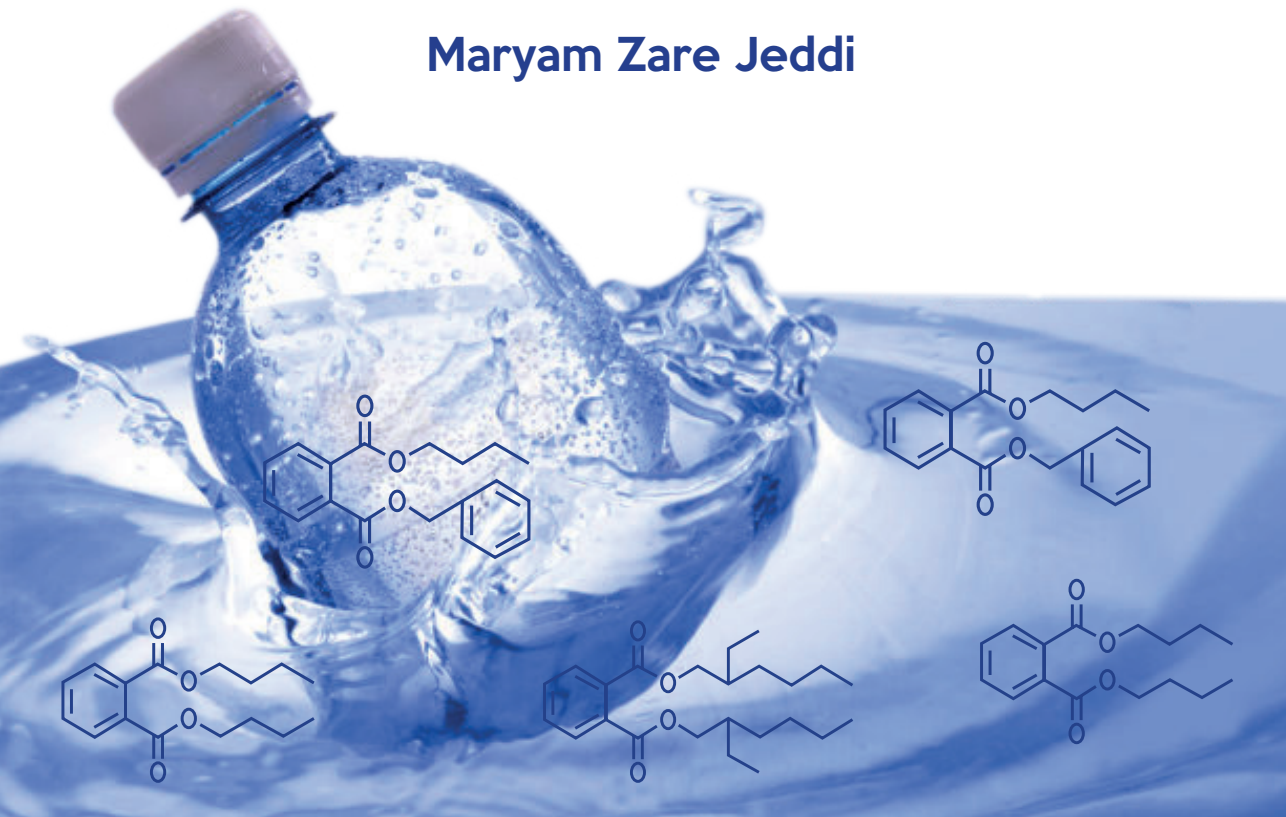
Phthalates mixtures in bottled water in Iran: human health risk assessment using direct and indirect exposure assessment

Maryam Zare Jeddi



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Thesis

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CHAPTER 1

General Introduction

Background

Packaging is an indispensable part of our modern food chain. According to the European legislation [Regulation European Commission (EC) No 1935/2004] food contact materials are all materials and articles intended to come directly or indirectly into contact with food (EC 2004; EFSA 2009). Food contact materials include a wide variety of materials such as plastics, paper, ceramics, glass, rubber, metals and their coatings, silicones, wood, printing inks, and many more. In turn, these materials are made of many different substances, for example, monomers converted into polymers, or additives such as plasticisers and stabilizers, and others. Contact of such materials with food may occur during all food stages comprising food production, processing, storage, preparation and serving. The safety of food contact materials requires evaluation, as chemical constituents can transfer (migrate) from the contact materials into food (Bolognesi, Castoldi et al. 2017). Plastic is one of the most common food contact materials (Marsh and Bugusu 2007). For example polyethylene terephthalate (PET) is a semi-crystalline plastic polymer belonging to the family of polyesters and universally used as packaging material for water and other drinks due to its strength, lightweight, flexibility, clarity, resistance to high temperature, and its negligible permeability to carbon dioxide (Cincotta et al. 2018). In the 1970s, a production process for PET bottles was developed. PET bottles were initially used for soft drinks, but gradually their use with bottled water became more popular. Nowadays, PET is the most widespread material for water packaging and the worldwide bottled water markets are increasingly naturalized (Andrady and Neal 2009; Brei 2018). Asia-Pacific was in 2016 the largest region in terms of volume with around 41% share of overall global bottled water consumption. The Americas were the second largest region at 33% of the total and European countries were in the third place with the top five biggest consumers of bottled water being Italy, Germany, Belgium, Hungary and Spain (Marcussen et al. 2013, EFBW 2016; Market Research 2018). Recently, other countries, including Iran, increasingly struggle with water scarcity leading to rapid development of the bottled water market (Hawkins 2017). Bottled water is considered a safe, healthy, and convenient packaged food product, and nowadays bottled water is available practically everywhere (Hawkins 2017). Water is counted as ‘food’ under Regulation (EC) No 178/2002 of the European Parliament and of the Council, and therefore it must comply with general principles and requirements of food safety and food hygiene (EC, 2002; EU, 2016). Not only in the EU but also in other parts of the world such regulations are in place including requirements defined by for example the U.S. Food and Drug Administration (U.S. FDA) and Codex Alimentarius

and the World Health Organization (WHO) (FDA 2018, 2012 CODEX 2001; WHO 2003). The Institute of standards and industrial research of Iran (ISIRI) is the sole organization in the country that can lawfully develop and designate official standards for products. Regarding bottled water safety, this institute has used the mixed regulatory guidelines for bottled water released by the U.S. FDA, the WHO and the Codex Alimentarius (CODEX 2001; WHO 2003; FDA 2012, 2018). However, ISIRI has not (yet) set any limits for phthalate levels in bottled water, such as done by the U.S. FDA for bottled water and by the WHO for drinking water.

In case of plastic materials in contact with foodstuffs, a declaration of conformity according to the EU Regulation No. 10/2011 is required (EC 2011). The regulation states that the risk assessment of a substance should cover the substance itself, relevant impurities, and foreseeable reaction and degradation products resulting from the intended use. EU Regulation No. 10/2011 also reports the list of the authorized monomers, other starting substances, additives, and polymers allowed in the production. Some substances are listed as allowed in food packaging materials with accompanying restrictions and/or specifications, which are indicated by their toxicological data. Regarding the identification and evaluation of migrating substances, experience has shown that more focus is needed on the finished materials and articles (EFSA 2016). The main impurities, reaction and degradation products that may unintentionally be present in food packaging materials have become known as non-intentionally added substances (NIAS). These substances should be considered in the safety evaluation in accordance with the current European legislation that also non-authorized substances that are present in food contact materials but non-intentionally added, should, if necessary, be included in restrictions and specifications of authorized substances (EC 10/2011). This is done to ensure that none of the NIAS migrates into foods in amounts that could endanger human health according to the Framework Regulation EC 1935/2004, Art. 3 and the Plastics Regulation EC 10/2011, Art. 19 (EC 2016; Bolognesi, et al. 2017, Cincotta et al. 2018). Consequently, the safety of NIAS has to be assessed. NIAS could arise from starting substances, such as monomers and catalysts, used for the initial polymerization step or from additives and plasticisers added during manufacturing to achieve special material properties. These substances can undergo degradation and decomposition reactions during polymer manufacture and use, resulting in compounds non-intentionally present in the plastic material that can leach to packaged food over time (Bach et al. 2013, Silano et al., 2017). Furthermore, starting substances or additives can contain impurities, which also might leach

from the packaging (Yang et al. 2011). However, it is not possible to list and consider all impurities and all reaction and degradation products in the authorization. Therefore, some NIAS may be present in the material or article while not included in the Union regulation list (EU 2011).

Phthalates are high-production volume synthetic chemicals produced and used worldwide since the 1920s as plasticisers and additives in many products, especially in polyvinyl chloride (PVC) household and textile products, toys, personal-care products, furniture upholstery, blood storage bags, and medical devices (Koch and Calafat 2009). Plasticisers are additives employed in various kinds of plastic to alter their properties and make them softer and more flexible. In the case of polyvinyl chloride (PVC), for example, the use of plasticisers is essential.

However, regarding the use of PET for water bottles, the material needs to be strong and a little more rigid so that the plastic can be thinner and lighter to facilitate the stacking of packs on pallets. Therefore, plasticisers do not serve in PET for plastic water bottles, indicating that PET bottles are expected to be free from any kind of plasticiser. The name of one of the chemicals used to make PET, terephthalic acid, sounds very much akin to phthalic acid, the material used as a starter for plasticisers, and this often leads to the mistaken belief that PET bottles contain plasticisers. Although phthalates are not thought to be used in the manufacture of PET bottles (ILSI, 2000), they have been found in PET material and in PET bottled water (Cao 2008, Montuori, Jover et al. 2008, Amiridou and Voutsa 2011). Therefore, the presence of phthalates in bottled waters is a clear example of the presence of NIAS in food packaging material. Whatever the origin of these migrants, the determination of NIAS in water is a priority due to the growing popularity of bottled water consumption.

To the best of our knowledge, the occurrence of phthalates in PET bottled water produced in Iran was not previously investigated. The aim of this thesis was to evaluate and quantify the levels of common phthalates (DMP, DEP, DBP, BBzP, and DEHP) in bottled water locally produced in the Iranian market and to investigate the effects of various storage conditions on the levels of these contaminants. In addition, human exposure to phthalates via consumption of bottled water and its possible consequences with respect to human health for children and adults are estimated. Furthermore, because phthalates are ubiquitous in daily life, and exposure is not only via consumption of bottled water, total exposure to phthalates was estimated by measuring urinary concentrations of phthalate metabolites in Iranian children.

Phthalate Esters; occurrence and chemistry

Among the numerous (approximately 30,000) substances that have been evaluated for their plasticizing properties, the economic viability, and broad spectrum physical properties of phthalates (dialkyl or alkyl/aryl esters of 1,2-benzenedicarboxylic acid) or phthalic acid esters (PAEs) make them an important class of plasticisers. The use of phthalates as plasticisers in plastics and as additives in innumerable consumer products is widespread due to their low costs, attractive properties, and the lack of suitable alternatives (Benjamin et al. 2017).

Phthalates are a group of substances with the same general chemical structure (*o*-phthalic acid), esterified with two aliphatic carbon chains (Figure 1). The toxicity and application of the different phthalates generally depends on the length of the carbon chains (Scholz, 2003).

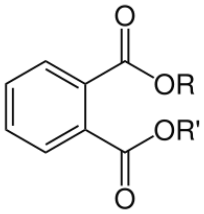


Figure 1. General chemical structure of phthalates (R_1 and R_2 = C_nH_{2n+1})

Phthalate esters (PEs) are used as plasticiser or solvent in various polymer and non-polymer products. Phthalates can be divided into high- and low-molecular-weight phthalates according to the length of their carbon chains (EC 2017). High molecular weight (HMW) phthalates include those with 7–13 carbon atoms in their carbon chains including diisononyl phthalate (hereafter referred to as DINP), diisodecyl phthalate (DIDP), and di-n-octyl phthalate (DnOP). Low molecular weight (LMW) phthalates contain 3–6 carbon atoms in their carbon chains and include compounds like di-2-ethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), and butyl benzyl phthalate (BBzP) (NRC 2009, DanishEPA 2013). The HMW phthalates are also called long chain phthalates and the LMW phthalates are called short chain phthalates (Koch and Angerer, 2011). HMW phthalates are widely used in industry as plasticisers to increase softness, flexibility, elongation and durability of rigid polymers such as polyvinyl chloride (PVC). The plasticized products include wire and cables, flooring, truck tarpaulins, wall coverings, self-adhesive films or labels, synthetic leather, coated fabrics, roofing membranes and automotive applications (AgPU 2006, Cao 2010, ECPI 2014). LMW phthalates also are used in PVC products, as well as in medical devices, adhesives, paints, inks, and enteric-coated tablets. However, LMW phthalates, like DBP, may

be too volatile for PVC applications and they are more likely to be used as a gelling aid in combination with other HMW plasticisers (Lassen et al., 2009a). Dimethyl phthalate (DMP) and diethyl phthalate (DEP), with one and two carbon atoms in their hydrocarbon chain respectively, are not used as plasticisers, so they are not related to PVC. These two phthalates belong to very LMW phthalates. Indeed, they can be categorized in a separate group (EC 2017). They are used as solvents and fixatives in fragrances, additives in cosmetics, medical devices, household and personal care products (EAG 2011).

Globally, phthalates are still the dominant plasticisers (Figure 2) and will continue to dominate the market in upcoming years (Research and Markets 2018). Phthalates like DINP (~25%) and DEHP (~50%) together claim over 75% of the global market share of phthalates, which is expected to rise to 6.76 million tons of production in 2019 from 5.35 million tons in 2014 (Micromarket Monitor, 2015). According to CMR (carcinogenic, mutagenic or toxic to reproduction) classification of substances under either Regulation (EC) No 1907/2006 on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH registration) or Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP Regulation), DEHP, DBP, BBP are classified as CMR plasticisers (ECHA 2012).

The EU demand for plasticisers has been steadily shifting away from CMR classified phthalates towards non-CMR classified phthalates and other non-CMR classified plasticisers. A similar movement from CMR classified phthalates has occurred in North America, but in the rest of the world (China, India, Middle East, Iran Africa and Latin America) CMR classified phthalates including DEHP and DBP are still dominant (KEMI 2015).

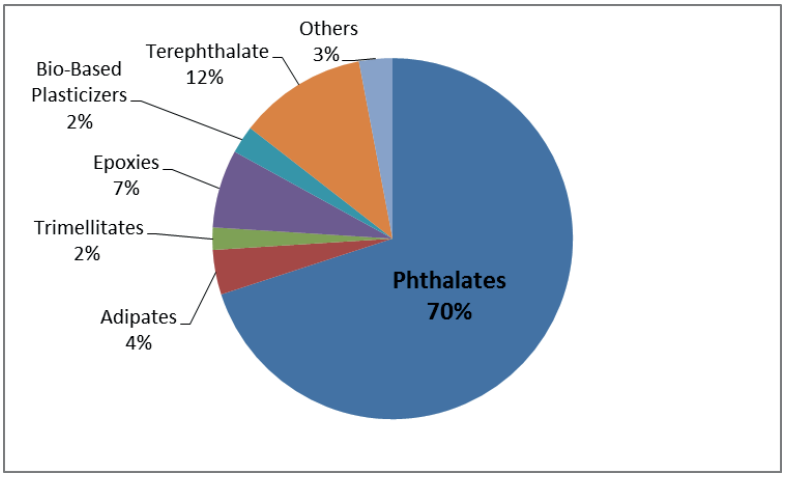


Figure 2. Global plasticiser application in 2014 by plasticiser types (CEH 2015).

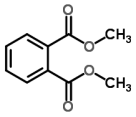
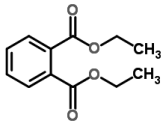
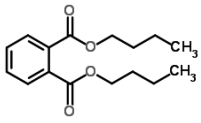
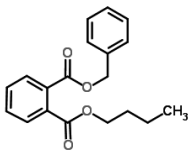
In recent years, the production of phthalates has changed in Europe due to their toxicity classifications, mandatory labelling, as well as because of restrictions and bans on several members of this group (CEH 2015). As a consequence, the EU production of three phthalates (DBP, DEHP and BBP) has reduced by more than 50% during the period 2010-2015 after their use has been banned in toys, childcare articles and food contact materials (FCM) for fatty foods and for repeated use, as well as infant feeding (Danish EPA 2013, EU 2018). In addition, under the EU RoHS 2 (Restriction of Hazardous Substances in electrical and electronic equipment), DEHP, BBP, DBP and DIBP will be restricted from 22 July 2019 for all electrical and electronic equipment apart from medical devices and monitoring and control equipment that will have an additional two years to comply by 22 July 2021 (EC 2017). Although these phthalates are strictly regulated within the EU today, they are abundant in products and materials still in use, and will be so for a long time to come (Research and Markets 2018). The estimated worldwide production of plasticizers in 2014 was about 14 billion pounds with the majority of the plasticizer consumption taking place in Asia Pacific, predominately China. About 75% of this volume consists of phthalate plasticisers (Godwin 2017). This indicates that the use and/or occurrence of these phthalates can be expected to continue throughout the world in the current century (Research and Markets 2018).

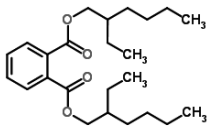
Iran is one of the main producers and consumers of phthalate plasticisers in the Middle East (Shokrolahi 2016). DEHP is still being used in Iran, because new phthalate plasticiser

alternatives are not produced, although, in some cases they are imported, which is costly (Shokrolahi 2016).

The physical and chemical characteristics related to the investigated phthalates in the present thesis and an overview of their major uses are given in Table 1.

Table 1. List of common phthalates, their chemical formula and structure, CAS No and examples of their use

Phthalate name	**Molecular formula**	**Chemical structure**	**CAS No.**	**Straight carbon backbones in the alkyl side chains**	**Total Carbon**	**Molecular weight type**	**Example of Use (s)**
DMP (Dimethyl phthalate)	$C_{10}H_{10}O_4$		131-11-3	1	1	very low	Insect repellent, plastic, additives in cosmetics, household products
DEP (Diethyl phthalate)	$C_{12}H_{14}O_4$		84-66-2	2	2	very low	Shampoo, scents, soap, lotion, cosmetics, industrial solvent, pharmaceutical coatings, additives in cosmetics, fragranced products
DBP (Dibutyl phthalate)	$C_{16}H_{22}O_4$		84-74-2	3/4	4	low	Adhesives, caulk, cosmetics, industrial solvent, pharmaceutical coatings, plasticiser in polymers, such as PVC, fillers, putties, plasters, modelling clay, inks and dyes, electronics (e.g. sewing machine, lamps)
BBP (Butyl benzyl phthalate)	$C_{19}H_{20}O_4$		85-68-7	4/6	4/6	low	Vinyl flooring, adhesives, sealants, industrial solvent, automotive trim, food conveyor belts, and artificial leather

Phthalate name	Molecular formula	Chemical structure	CAS No.	Straight carbon backbones in the alkyl side chains	Total Carbon	Molecular weight type	Example of Use (s)
DEHP (Di(2-ethylhexyl) phthalate)	$C_{24}H_{38}O_4$		117-81-7	6	8	Low/high*	Plasticiser in polymers, such as PVC, soft plastic, tubing, toys, home products, electronics, lamps, food containers, food packaging, medical devices, such as plastic tubing used for catheters and intravenous drug and fluid delivery, personal protective equipment - goggles

*There are different classifications of DEHP as high or low molecular wight phthalate considering the total carbon or straight carbon backbones in the alkyl side chains in the stracture.

References: Schierow 2012; ECHA 2016; European Plasticisers (ECPI) 2017

Exposure to Phthalates

When used as plasticiser, phthalates have no covalent linkage with and are reversibly attached to the polymer (Figure 3). As a result, slight changes in the environment [*e.g.*, high or low pH, temperature and pressure, irradiation (UV, sunlight, microwaving, *etc.*) or contact with lipid, solvents, *etc.*] may accelerate the leaching or migrating or vaporizing out of phthalates from the plastic into the surrounding environment (Bach, Dauchy et al. 2013; Benjamin, Masai et al. 2017). As a result, phthalates are omnipresent, for example in food, water, breathing air, soil, dust, dress materials, dwelling house, hospital. They have been the subject of numerous investigations and concerns because of their widespread applications, and their extensive use categorizes them as a ubiquitous group of environmental contaminants relevant for human exposure (Wormuth, Scheringer et al. 2006). The general population is exposed to phthalates via different routes and from different sources including:

- Oral route: uptake via the mouth and gastrointestinal tract via food, nutritional formulas, pharmaceuticals, nutritional supplements, pharmaceutical coatings (capsules and pills), toys placed into mouth, as well as other mouthing objects.
- Inhalation route: uptake via the lungs via inhalation of house dust, indoor air, or medical devices (e.g. for respiratory therapy).
- Dermal route: uptake via the skin via clothing, cosmetics, personal care products, sunscreens, modelling clay, toys, cleaning products, soil or dust.
- Intravenous route: uptake via medical devices (Schettler 2006).

In general, recent reports suggest food and beverages to be the predominant source of human exposure to phthalates and indoor environment and direct contact with articles can be considered as other main sources of exposure to phthalates, as well (ECHA 2016; Heinemeyer et al., 2013; Fierens et al., 2012).

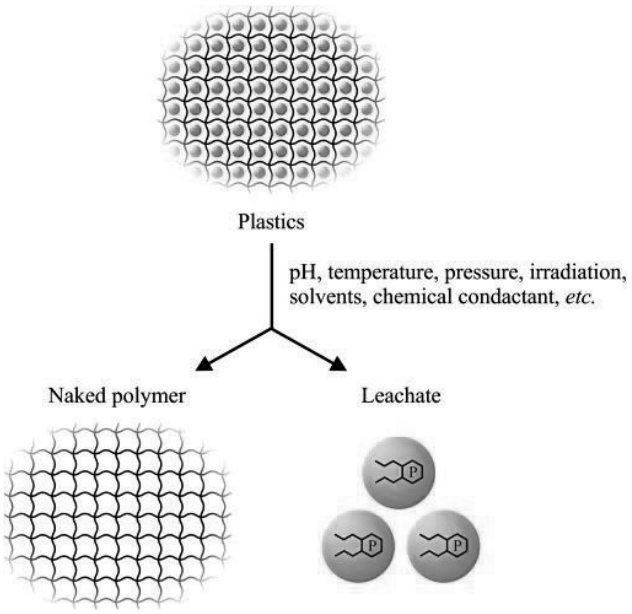


Figure 3. Migration of phthalates into the environment from the embodiment of plastics (Benjamin, Masai et al. 2017)

Both animal and human studies demonstrate that exposure may occur throughout the life span, from the developing fetus through early infancy, childhood, and beyond. In neonates, infants, and toddlers, exposure may come through vertical transmission or external sources. The most likely neonatal exposure pathway is vertical transmission through the placenta or breast-feeding (Zhou et al., 2000; Zhang et al., 2108). Phthalates pass the placenta and go into fetal blood, where they are found to have an extended half-life up to 6.2 hours in fetal serum and 64 hours in amniotic fluid as compared to 4.3 hours in maternal serum (Johns et al., 2015; Kessler et al., 2013; Genuis et al. 2012). Infant formula, baby food, and children's toys are additional sources of exposure, a realization that has prompted Europe to enact legislation limiting the use of these compounds in order to prevent adverse effects on development (Wormuth, Scheringer et al. 2006). Additionally, neonates or children who spent time in an intensive care unit and patients who are critically ill are exposed to high levels of phthalates through medical equipment including intravenous bags and tubing (Green et al. 2005).

Other common sources of exposure in the general population include ingestion of contaminated food and dust. Absorption of phthalates can also occur via dermal contact (Schettler 2006). This is a concern for products such as deodorant, perfumes, aftershave, hair

styling products, shampoo, skin and nail care products, as well as cosmetic products that have been found to contain varying amounts of phthalates, ranging from 1–15,000 mg/kg (Wormuth et al. 2006).

Different approaches to assess human exposure to phthalates

As humans are exposed to chemicals during their everyday lives, the best way to calculate or estimate the magnitude, frequency, and duration of exposure, along with the number and characteristics of the population exposed is to perform a human exposure assessment. Such an exposure assessment is needed to evaluate the potential health impacts of chemical exposures (Joas et al. 2017).

There are three main general approaches to assess human exposure: history/questionnaire information, environmental monitoring (i.e., measuring concentrations of the chemicals in environmental medias such as water and food), and biomonitoring (i.e., measuring concentrations of the chemicals or their metabolites in human specimens) (Calafat, 2018).

Different approaches to human exposure assessment are presented in Figure 4.

Questionnaire based exposure assessment methods are used in the exposure assessment of occupational and environmental epidemiological studies. Questionnaires may be the method of choice for assessing exposure when no other sources of information are available, or because they provide the most efficient data collection method, allowing a larger study size and greater statistical power than would be possible with other more accurate measurement techniques. They may be used in combination with other methods (Nieuwenhuijsen 2005). In order to consider phthalate exposure, questionnaires were usually used for scenario-based risk assessment approaches (SceBRA), such as food consumption questionnaire based exposure evaluations (English et al., 2015; Wormuth et al. 2006), or in combination with other methods like human biomonitoring approaches. Such combinations may facilitate a better understanding of contributions of different exposure routes (e.g. foodstuff) or exposures caused by individual life style (i.e. medical history, demographic data, socioeconomic status, product use) pivotal in identifying causes of disease particularly in cohort studies (Soomro et al., 2018; Wittassek, Koch and Angerer, 2011; Swan et al., 2008).

Environmental monitoring, also called a ‘forward’ and/or indirect approach, is the second method in exposure assessment. In this method, exposure to phthalates in humans can be assessed by measuring the concentration of these chemicals in external sources (e.g., air, dust,

food, personal care products, and etc.), and in combination with data from life style and daily intake of food and/or usage patterns of personal care products, daily exposure can be estimated (Zaki et al., 2018; Cincotta et al., 2018; Wittassek, Koch et al. 2011). This approach is also known as indirect exposure assessment. In the present study, the environmental monitoring approach was used for assessing exposure to phthalates through bottled water stored in various conditions. Exposure modelling was carried out by combining information on: (1) the concentrations of phthalates in bottled water, and (2) human behaviors, e.g., the daily intake of water. Based on this information, the amount of exposure through this specific route is calculated (Kamrin, 2009). In fact, the main aim of the environmental monitoring approach is to estimate possible contributions of different exposure sources and routes to total exposure (Koch and Angerer, 2011). Ideally, it describes the sources, pathways, routes, and the uncertainty in the assessment.

The third approach to estimating phthalate intake is through human biomonitoring (HBM), which has been defined as the 'backward' and/or direct approach (NRC 2006, Christensen, Makris et al. 2014). HBM is a commonly used technique to determine the internal exposure (i.e. body burden) by assessing whether and to what extent chemicals enter the human body (Joas et al. 2017). Indeed, HBM is an important tool to map exposure patterns to environmental chemicals throughout the population over time, inform policy decisions and evaluate the success of risk reduction strategies (Kolossa-Gehring, Fiddicke et al. 2017). HBM measures chemicals in body tissues and fluids using biological specimens such as blood, urine, and/or hair. HBM considers all routes of uptake and all relevant sources.

In this thesis, an HBM approach is used for investigation of phthalate exposure of Iranian children.

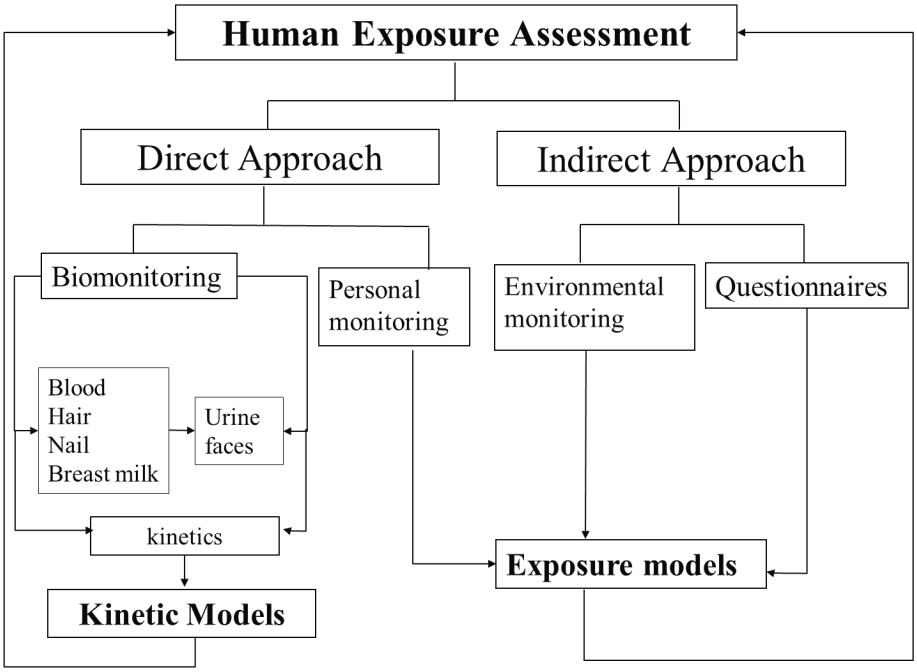


Figure 4. Different approaches to human exposure assessment.

Metabolism and elimination of phthalates

Phthalates are non-persistent chemicals; hence, once they enter the body, they are rapidly metabolized in three steps which lead to several metabolites as break-down products of phthalates (Koch and Angerer 2011). Metabolism of phthalates in humans is schematically illustrated in Figure 5. In the first step they are hydrolysed by lipases and esterases in the intestine or in other organs to their respective more active monoesters (Phase I = de-esterification). In the second step of phase I, the alkyl chain of the resulting monoesters can be modified by various oxidation reactions (Koch and Angerer, 2011). In the third step, both hydrolytic and oxidized secondary metabolites (OH-, oxo and carboxy (cx-)) can undergo phase II biotransformation to produce glucuronide conjugates (glucuronidation). This step is catalysed by uridine 5'-diphospho-glucuronosyltransferases (UDP-glucuronosyltransferases, UGTs) leading to formation of glucuronidated phthalates with higher water solubility (Calafat, Ye et al. 2006). The metabolites of phthalates from phase I (hydrolysis and oxidation) and phase II (conjugation) reactions are excreted as free (unconjugated) as well as glucuronyl conjugated forms in urine or feces (Braun 2017; Kumar et al., 2016; Wittassek et al., 2008). Phthalate metabolism is qualitatively similar among

species, beginning with formation of the monoester (NRC 2009). The ratio between free monoester and glucuronide conjugate excretion varies among different phthalates (Hauser and Calafat 2005). In fact, the extent of phase I and phase II metabolism depends on the alcohol part as well as the physiological characteristics of the individual subject (Silva et al., 2003; Meeker et al., 2012).

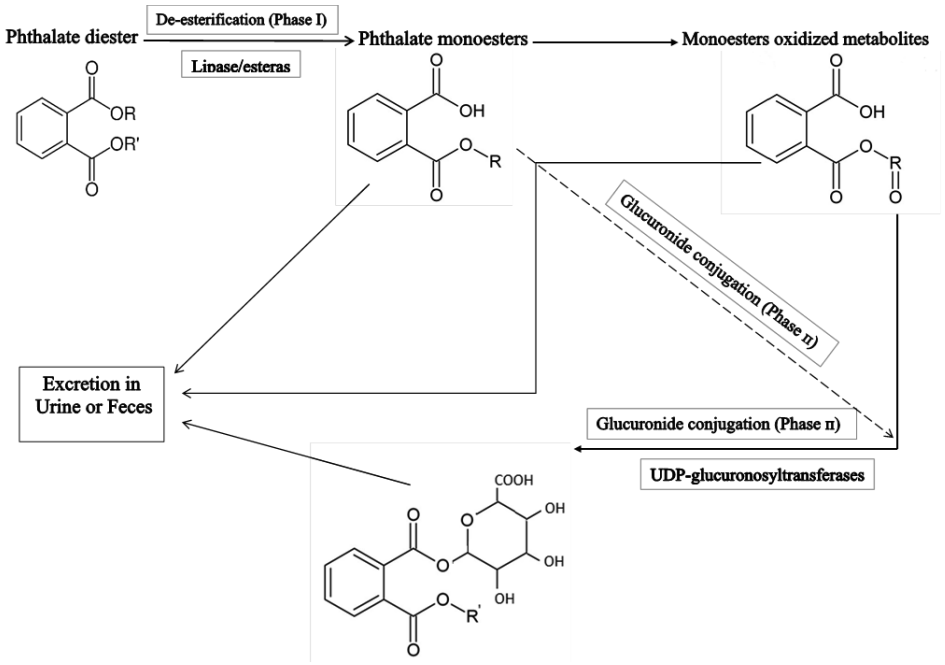


Figure 5. Metabolism of phthalate esters in human.

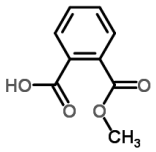
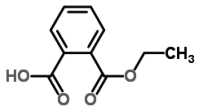
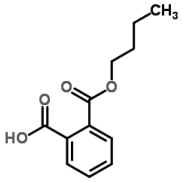
It is notable that, typically, for most xenobiotics hydrolysis results in detoxification, while in the case of phthalates in vitro and in vivo studies have shown that it leads to more bioactive monoesters (Ventrice et al., 2013; Frederiksen, Skakkebaek et al. 2007). Conclusive evidence on levels of phthalate bioaccumulation within specific organs and tissues of the body has not been reported (Genuis, Beesoon et al. 2012).

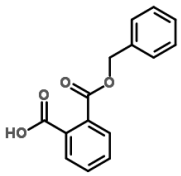
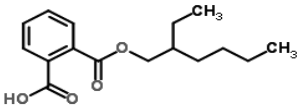
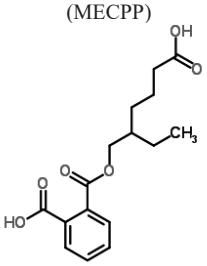
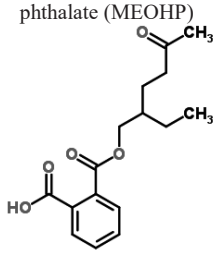
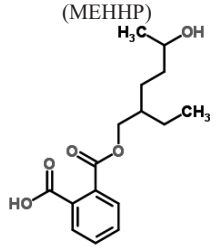
Biomarkers of exposure to phthalates

Biomarkers, measured as concentrations of chemical substances and/or their metabolites, display a time dependent concentration profile that is associated with patterns of exposure and elimination kinetics (WHO, 2015). Phthalate metabolites have been used as biomarkers to monitor exposure to phthalates from the environment, occupation, and lifestyle in several HBM surveys (Yoshida 2017, Choi et al., 2015).

Major metabolites of phthalates, which are used in epidemiological studies as biomarkers of exposure and also in the present thesis, are listed in Table 2 (Frederiksen et al., 2007). Monoesters are the main detected metabolites of the LMW phthalates including DBP, BBP, DEP, and DMP. For instance, for DBP about 90% of the urinary metabolites are MBP, whereas for DEHP, with more carbon atomes per alkyl chain, less than 10% of its primary metabolites consist of the monoester (Wittassek and Angerer 2008). Indeed, hydrolytic monoesters of DEHP (e.g., MEHP) usually are oxidized in a subsequent metabolic step to secondary products representing a variety of metabolites (Koch and Angerer, 2011; Praveena et al., 2018).

Table 2. Parent phthalates and their metabolites used as biomarkers in human biomonitoring surveys.

Phthalate		**Molecular Weight**	**Selected Metabolites**	
			Hydrolytic monoester (primary metabolite)	**Oxidized monoester(s) (secondary metabolite(s))**
DMP	Dimethyl phthalate	194	Monomethyl phthalate (MMP) 	-
DEP	Diethyl phthalate	222	Monoethyl phthalate (MEP) 	-
DBP	Di-*n*-butyl phthalate	278	Monobutyl phthalate (MBP) 	-

<table>
<tr><th colspan="2" rowspan="2">Phthalate</th><th rowspan="2">Molecular Weight</th><th colspan="2">Selected Metabolites</th></tr>
<tr><th>Hydrolytic monoester (primary metabolite)</th><th>Oxidized monoester(s) (secondary metabolite(s))</th></tr>
<tr><td>BBP</td><td>Butyl benzyl phthalate</td><td>312</td><td>Monobenzyl phthalate (MBzP)
  </td><td>-</td></tr>
<tr><td rowspan="3">DEHP</td><td rowspan="3">Di(2-ethylhexyl) phthalate</td><td rowspan="3">390</td><td rowspan="2">Mono(2-ethylhexyl) phthalate (MEHP)
  </td><td>Mono(2-ethyl-5-carboxypentyl) phthalate (MECPP)
  </td></tr>
<tr><td>Mono(2-ethyl-5-oxohexyl) phthalate (MEOHP)
  </td></tr>
<tr><td></td><td>Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)
  </td></tr>
</table>

Although, both primary and secondary metabolites of phthalates are considered as indicators of human exposure to phthalates, secondary oxidized metabolites are preferred whenever possible given that the secondary oxidized metabolites are unique, independent of external

contamination, and can be formed only by oxidation of monoester metabolites (Koch, Bolt et al. 2005).

Selection of biological matrix

The physicochemical properties of the chemical determine its metabolism and excretion routes, which will influence the selection of an appropriate matrix such as blood, urine, nail, hair and/or saliva in biomonitoring studies (Calafat and Needham, 2008). Therefore, it is important to consider the kinetics of a chemical when selecting the matrix for the biomonitoring studies (WHO, 2015). Although phthalate metabolites can be detected in several body fluids such as amniotic fluid, breast milk, saliva and seminal plasma (Anand-Ivell et al., 2018; Hines, Calafat et al. 2009; Main et al. 2006; Silva et al., 2005; Calafat et al. 2004), the presence of enzymes such as esterases in these matrices can cleave phthalates contaminating the samples from external sources into their monoesters (Kumar et al., 2016). As a matter of fact, one of the main issues for measuring phthalates is the risk of laboratory contamination, as phthalates can be present in water, organic solvents, ambient air, glassware and plastic material used for the analysis. Thus, a primary concern related to phthalate quantification is the risk of contamination during the analytical procedure, which can often lead to false positive or overestimated results (Net et al., 2015).

In general, in epidemiological studies urine has been considered the matrix of choice for non-persistent chemicals, such as phthalates, because urinary concentrations of these compounds or their metabolites are usually considerably higher than blood concentrations (NCR 2009). Phthalates have short biological half-lives ranging from 2 to 12h, with DEHP metabolites having a half-life of 10–15h; hence their blood levels are relatively low compared to urinary levels (Kumar et al., 2016; Genuis et al., 2012; Jeong et al., 2011; Frederiksen et al., 2010). This is in contrast to persistent compounds where blood is the preferred matrix for biomonitoring (Koch and Angerer, 2011). In addition, urine is a relatively abundant matrix, and its collection is simple and non-invasive, and a wide range of sensitive analytical methods are available to measure phthalate metabolites in urine (Kumar et al., 2016; Johns et al., 2015). The measurement of concentrations of phthalate metabolites in urine is currently a valuable and accepted approach for assessing exposure in environmental epidemiology studies (Benjamin, et al., 2017; Johns et al., 2015: WHO 2015). In environmental epidemiology studies, the body burden (levels) of phthalates and their metabolites is measured and assessed to determine health impacts or potential risks in humans (Table 3).

Table 3. Human biomonitoring studies for assessing exposure and health impacts.

Purpose	Subjects and country	Phthalates metabolites	Matrix examined	Inference	Reference
Exposure assessment					
Indicate the association between personal care products use and urinary concentrations of phthalate metabolites	N= 400 men the environment and reproductive health (EARTH) study	MEP MEHP MEHHP MnBP MBzP	Urine	A significant association was observed only for MEP with use of cologne/perfume (83%, p-value<0.01) and deodorant (74%, p-value<0.01). It seems personal care product use is an important source of exposure to DEP.	Nassan et al., 2017
Comparison of phthalate exposure among the Austrian population aged 6–15 and 18–81 years	N= 387 children and adolescents, 419 adults. 196 senior citizens	MiBP MnBP MBzP MEHP MEOHP and MEHHP	Urine	Phthalate metabolites detected in majority of samples. Children exhibited higher levels of exposure to the majority of investigated phthalates, except to MEP, which was found in higher concentrations in adults and senior citizens at a maximum concentration of 2,105 µg/l.	Hartmann et al., 2015
Determine the average exposure of Italian population to common phthalates	N= 157 (74 males and 83 females); Italy	MEP MEHP MEHHP MnBP MBzP	Urine	In females, MEP was highest (72.94 µg/g creatinine) followed by MnBP (20.26), MBzP (16.44) and MEHHP (10.77).	Tranfo et al., 2013
Determine the average exposure of Korean population to common phthalates	N= 111 adults: 45 male and 66 females (19-77 yrs); Korea	MiBP MnBP MBzP MEHP MEOHP and MEHHP	Urine	DEHP metabolites highest (75.92 µg/g creatinine) among population. In general, detected concentration of $\sum_8$ phthalate metabolites in female urine (200.76 µg/g) was 1.09 fold higher than of male. Rural regions had higher levels (211.96 µg/g) than samples from urban regions.	Kim et al., 2014
Measure the concentration levels of common phthalates in samples of amniotic fluid and maternal urine collected in the same day to investigate the mechanisms involved in fetal exposure	N= 70 women, in 16 - 17 weeks of gestation	MiBP MnBP MBzP MEHP MEOHP and MEHHP	Urine, Amniotic fluid	The concentrations of phthalate monoesters in amniotic fluid are lower than those found in maternal urine and the metabolites having a higher concentration in amniotic fluid are MnBP and MEHP.	Tranfo et al., 2014

Table 3. Continue.

Health impacts					
Purpose	**Subjects and country**	**Phthalates/ metabolites analyzed**	**Matrix examined**	**Inference**	**Reference**
Association between prenatal exposure and childhood allergies and infectious diseases	N= 127 pregnant women; N=654 Children up to 7 years of age	MEHP	Maternal blood	Maternal MEHP levels were negatively associated with cord blood IgE levels and increased risks of allergies and infectious diseases up to 7 years of age	Bamai et al., 2017
Association between phthalate exposure and autism spectrum disorder (ASD)	N= 48 with ASD (36 male, 12 female; ~ 12 years), Italy	MEHP MEHHP MEHOP	Urine	Significant association between DEHP metabolites and ASDs, especially 5-oxo-MEHP showed 91.1% specificity in identifying patients with ASDs	Testa et al., 2012
Relation between phthalates exposure and insulin resistance	N= 766 fasting (12-19-yrs) NHANES (2003–2008); USA	MEP MBP DEHP MEHP MEHHP MEHOP	Urine, blood	DEHP metabolites associated with increased insulin resistance (21.6% prevalence)	Trasande et al., 2013a
Association of urinary phthalate concentrations with childhood obesity	N = 2,884 (6-19 years) NHANES (2003-2008); USA	MMP MEP MEPP MEHP MEHOP MiBP MnBP MBzP	Urine	Metabolites of low molecular weight phthalates associated with overweight and obesity (21% and 22%, respectively)	Trasande et al., 2013c

One major limitation to measuring biomarkers in spot urine samples compared to blood is that the concentration of metabolite concentrations in urine is dependent on the degree of urine dilution (Aylward et al., 2014).

In this regard, several approaches for correcting for urine dilution have been defined including creatinine correction, specific gravity correction, calculation of urinary excretion rate, using creatinine or specific gravity as an independent variable in a model evaluating toxicant exposure and outcome (Barr, et al., 2005). The most common approach to compensate for urine dilution is to measure the concentration of creatinine, a breakdown product of muscle metabolism, in the urine sample, and correct the mass of the analyte by the mass of creatinine from the same sample resulting in what is called a creatinine-adjusted level (Johns et al., 2015; Cocker et al., 2011). This is also the way the correction for urine dilution was performed in the studies of the present thesis.

Health effects of phthalates and possible mechanism of action

Phthalates as industrial chemicals have received considerable attention over recent years and both animal and human studies have identified various possible adverse health effects (Koch and Angerer, 2011). The adverse health effects of phthalates relate to a large extent to their potential to act as endocrine active compounds, however, effects on other endpoints have been described as well, including oxidative stress, neurodevelopmental disorders, asthma and allergies, obesity or insulin resistance, diabetes, and respiratory effects (Kim et al., 2018; Dales et al., 2018; Braun JM, 2017; Harley et al., 2017; Bamai et al. 2017; Bai et al., 2017; Kataria et al., 2017; Duan et al., 2017; Franken et al., 2017; Ipapo et al. 2017).

Endocrine disrupting properties and related disorders

The ubiquitous exposure and associations with reproductive and developmental toxicity both in animal and human studies have made phthalates chemicals of concern (NRC 2009). Several phthalates are endocrine disrupting chemicals (EDCs) which are able to act as anti-androgens, estrogens, anti-estrogens or inhibitors of steroidogenic enzymes and/or are able to interact with thyroid hormones and their receptors (Fisher 2004). Compounds with an endocrine disrupting mode of action can seriously affect human reproduction (Sifakis et al., 2017). Phthalates do not possess any intrinsic hormonal activity in contrast to many other environmental endocrine disruptors, which means that phthalates do not seem to act via direct hormonal mimicking (Koch and Angerer, 2011). For DEHP and other phthalates the reduced activation of the androgen receptor is caused by interference with steroid hormone synthesis (ECHA 2014). Targeted studies on the mode of action of DEHP showed changes in steroidogenesis, including reduced testosterone production and down-regulation of genes involved in steroid synthesis. Thus, DEHP exerts its anti-androgenic action by suppressing human testicular steroidogenesis (Desdoits-Lethimonier et al., 2012; ECHA 2014; Toor et al., 2017).

The spectrum of effects in animals (male rats) is known as the phthalate syndrome which covers different reproductive abnormalities in male offspring of rats exposed during pregnancy including inhibition of fetal testosterone production, reduced male anogenital distance, decreased gene expression related to steroid biosynthesis, increased permanent nipple retention in male offspring, increased incidence of genital malformations (hypospadias and cryptorchidism), delayed puberty onset, reduced semen quality and testicular changes including decreased testes and epididymes weight, tubular atrophy and Leydig cell hyperplasia (Swan, 2008; Zhou et al., 2017). It is well understood that the cause for the

phthalate syndrome is suppression of fetal androgen action (Kortenkamp, Evans et al. 2012). The current scientific evidence from epidemiological studies shows that these effects as observed in male animals and are also observed in and are relevant for male humans (ECHA 2016; NRC 2009). It is hypothesized that the outcomes of the phthalate syndrome may comprise the "testicular dysgenesis syndrome" (TDS) in humans with a common origin in fetal life. Testicular cancer may also be part of TDS in humans (Harris et al. 2016). Therefore, phthalates may play a role in the development of these adverse health effects on reproduction and development in humans (NRC 2009).

Human biomonitoring studies support the notion that exposure to phthalates impairs semen quality and decreases sex hormone levels causing fertility problems in reproductive-age men (Bloom et al., 2015; Liu et al., 2012; Wang et al., 2016, 2018). Several studies found statistically significantly lower sperm concentration and sperm counts when exposure to phthalates increased (Pant et al., 2008, 2014; Kranvogl et al., 2014; Specht et al., 2014; Bloom et al., 2015; Chang et al., 2017). Some others observed that sperm motility was statistically significantly lower when DEHP exposure increased (Pant et al., 2008; Huang et al., 2011; Jurewicz et al., 2013; Kranvogl et al., 2014; Axelsson et al., 2015).

When it comes to the correlation between DEHP exposure and sperm DNA integrity, this still remains controversial. One study reported that DEHP levels are strongly associated with morphologic abnormality and the DNA fragmentation index in the semen of the general Indian population (Pant et al., 2008). Some others showed that there were no significant relationships between urinary sperm DNA damage and MEHP exposure (Duty et al., 2003; Jönsson et al., 2005). A recent study reported that neither low-level in vitro nor high-level in vivo DEHP exposure would result in significant sperm DNA damage (Huang et al., 2012).

Huang et al. reported that direct DEHP exposure would diminish fertilization capacity and embryonic development, indicating the reproductive hazard of DEHP (Huang et al., 2012). Urinary levels of MEP, MBP and MnBP are positively associated with sperm DNA damage (Hauser et al., 2006; Jurewicz et al., 2013).

Health impacts in women who have been exposed to phthalates include endometriosis, leiomyomata (Upson et al., 2013; Weuve et al., 2010), breast cancer (López-Carrillo et al., 2010, Chen et al., 2014) and type-2 diabetes (Sun et al., 2014). However, results of epidemiological studies are debated. There is no significant association between total urinary phthalate metabolites (MBP, MEP, and DEHP metabolites) and risk of breast cancer or uterine leiomyoma (Morgan et al. 2017; Fu et al., 2017). However, a significant positive correlation was found between the exposure to MEHP, DEHP's primary metabolite, and

breast cancer in the study conducted by Holmes et al. in 170 women (75 cases and 95 controls) (Holmes et al., 2014). Fu et al., reported that total urinary DEHP metabolites were significantly associated with both risk of breast cancer and uterine leiomyoma (Fu et al., 2017), which suggested that DEHP metabolites might play a more important role than other phthalate metabolites measured in urine in the development of breast cancer and uterine leiomyoma (Morgan et al. 2017; Fu et al., 2017; Kim et al., 2018). However, the exact roles of DEHP metabolites and other phthalates in breast carcinogenesis are still unclear (Zuccarello et al., 2018). Anti-androgenic activity of phthalates, especially of DBP, BBP and DEHP seems to influence women as well since these phthalates have been associated with lower testosterone levels in pregnant women (Sathyanarayana et al., 2014). Females exposed to these phthalates are more likely to get pregnant (Vélez et al., 2015) and have a decreased likelihood of polycystic ovary syndrome (PCOS), a condition characterized as hyperandrogenemia (Vagi et al., 2014). Finally, there may be an increased risk of pregnancy-induced hypertensive diseases for pregnant women exposed to BBP (Werner et al., 2015).

Although information on the effects of a mixture of phthalates on reproduction and prenatal development is limited it is important to note that a mixture of phthalates such as DEHP, DBP and BBP tested for anti-androgenic properties in vivo, acted together in line with the dose addition approach which is the mixture effect prediction approach for chemicals acting by the same mechanism of action (NRC 2009; Kortenkamp et al., 2011; Zhou et al., 2017; Conley et al., 2018).

Neurodevelopmental disorders

Some epidemiological studies have reported that phthalate exposure may contribute to the onset of numerous neurodevelopmental disorders including impaired concentration in girls, diminished motor function and masculine behavior in boys, autism spectrum disorders (ASD), attention-deficit hyperactivity disorder (ADHD), learning disabilities, and altered play behavior (Engel et al., 2009; Swan et al., 2010; Bellinger 2013, Braun et al. 2013, Ejaredar et al. 2015, Jeddi, Janani et al. 2016). However, there is considerable heterogeneity in the neurodevelopmental outcomes reported to be associated with phthalate exposure due to differences in study design including diverse diagnosis methods, varied times at which prenatal maternal exposure was assessed and different age groups evaluated (Ejaredar et al. 2015; Stroustrup et al., 2018).

In a study performed among newborns enrolled in a multiethnic birth cohort at the Mount Sinai School of Medicine in New York City, maternal urinary concentrations of phthalate

metabolites and behavior were assessed within 5 days of birth. There were strong, inverse associations between increasing concentrations of HMW phthalate metabolites and attention, orientation, and alertness among girls. Among boys, there was a slight positive association between increasing LMW metabolites and motor performance (Engel et al., 2009). Two other studies from the same cohort examined the associations between phthalate exposure and ADHD and autistic behaviors. Engel et al. reported more ADHD-like behaviors among 4- to 7-year-old children whose mothers had higher urinary levels of LMW phthalate metabolites during pregnancy (Engel et al., 2009), and Miodovnik et al. found autistic-like behaviors among 7- to 9-year-old children born to women with higher urinary LMW phthalate concentrations (Miodovnik et al., 2011). Finally, in the Study for Future Families cohort, Swan et al. reported that prenatal phthalate exposure was associated with decreased masculine behavior in preschool boys (Swan et al., 2010). Male and female newborns appear to be affected by different phthalates and in a different way, indicating that phthalates may have sex specific effects (Engel et al., 2009; Huang et al., 2014, 2009; Wolff et al., 2008). For example, exposure to HMW phthalates was reported to be inversely associated with orientation and quality of alertness in infant girls and LMW phthalates were positively associated with motor performance in boys (Engel et al., 2009).

Phthalates also may have an effect on the intelligence of school-aged children. Although IQ is dependent on familial and social factors, there is evidence that prenatal exposure to DEHP, DBP and DiBP is inversely associated with child verbal comprehension, processing speed, perceptual reasoning and working memory (Cho et al., 2010; Factor-Litvak et al., 2014).

Extensive research in rodent models has shown that phthalates primarily act as anti-androgens and impair testosterone production in Leydig cells (Foster et al., 2005); androgens have an extensive influence on brain development in regions of the brain, yet their etiological involvement remains unclear (Rotem et al., 2018). However, it has also been suggested that phthalates disrupt the endocrine system by interfering with thyroid homeostasis through various mechanisms including alteration of transcriptional activity of the sodium/iodine symporter, inhibition of the binding of T3 to purified thyroid receptors, and inhibition of T3-induced cell proliferation (Ghisari et al., 2009). Exposure to DBP still has unclear effects on thyroid activity in pregnant women. Altered maternal thyroid hormone levels during gestation induce mental retardation and have adverse effects on fetal neurodevelopment which may be visible later in childhood (Cohen, 2014; Morreale de Escobar et al., 2004; Morreale de Escobar, 2001; Oerbeck et al., 2003). Other possible mechanisms that have been proposed to explain neurodevelopmental effects of phthalate exposure include interference with

intracellular calcium signaling, disruption of peroxisome proliferator–activated receptor (PPAR) activation, and alteration of lipid metabolism (Miodovnik et al., 2014). Moreover, phthalates may influence sex hormone regulations like that of estradiol, and eventually affect normal fetal brain development (Kim et al., 2018). DEHP can affect brain-derived neurotrophic factor (BDNF) expression which is a protein that plays a critical role in the survival of existing neurons and promotes the enhancement and differentiation of new neurons and their synapses. Low-dose DEHP exposure (10 mg/kg body weight) has been shown to reduce BDNF levels and to down-regulate hippocampal BDNF mRNA expression (Smith and Holahan, 2014). A study conducted by Wójtowicz et al. assessed the neurotoxic and apoptotic effects of DBP in mouse neocortical neurons in primary cultures (Wójtowicz et al., 2017). Based on the results of this study, DBP stimulated caspase-3 activity, lactate dehydrogenase (LDH) release, and reactive oxygen species (ROS) formation in a concentration (10 nM to 100 μM) and time-dependent (6, 24, 48 h) manner. The study suggested that the Aryl hydrocarbon receptor (AhR) is involved in DBP-induced apoptosis and neurotoxicity by inducing AhR mRNA and protein expression (Wójtowicz et al., 2017). Similar to the effects of DBP observed in the study by Wójtowicz et al., the results of the in vitro studies conducted by Lim et al. (2009) and Lin et al. (2011) revealed that DEHP and its metabolite, MEHP, inhibit cell proliferation, increase DNA fragmentation, activate caspase-3, induce apoptosis in a concentration- and time-dependent manner, and activate expression of the PPARγ and Trim17 protein in a neuroblastoma cell line, Neuro-2a cells and embryonic stem (ES) cells (Lim et al. 2009; Lin et al., 2011).

The neurotoxicity of DEHP, DBP, and DIBP has also been observed in *Caenorhabditis elegans*, a nematode. DEHP exposure can lead to an accumulation of ROS intracellularly, pointing at oxidative damage as a critical factor in the mode of action by which phthalates may cause neurotoxicity (Tseng et al., 2013).

The complexity of neurodevelopment, and the importance of early neurogenesis, drew attention to identifying environmental influences in addition to genetic factors in the development of autism spectrum disorders (Picciotto et al., 2018). In this thesis, a systematic review was performed to summarise existing scientific evidence on phthalate exposure as an environmental risk factor for autism spectrum disorders.

Genotoxicity of phthalates

The possible genotoxic effect of phthalates has been thoroughly investigated in several different short-term tests. The European Union risk assessment report on DEHP in 2008 stated that DEHP and its metabolite, MEHP, are considered to be non-genotoxic substances. This conclusion was based on several in vitro studies performed in the period from 1980 to 1992 on bacteria, fungi and mammalian cells that evaluated gene mutation, DNA damage, and chromosomal effects (EU 2008). However, nowadays there are several other studies that assessed the genotoxic potential of phthalates, especially DEHP and MEHP, pointing at a possible indirect genotoxic effect. For example, the overall results of the study conducted by Erkekoglu et al., (2011) indicated that the induction of oxidative stress is one of the important mechanisms underlying the indirect genotoxic potential of DEHP and this is mainly through the effects of the metabolite MEHP. Free radicals comprise both ROS and reactive nitrogen species (RNS) able to cause structural damage to major macromolecules like DNA, RNA, proteins, and lipids thereby leading to cellular toxicity and genotoxicity (Franco et al., 2008). Erkekoglu et al. (2011) investigated the increased ROS production and activation of the tumor suppressor gene p53 (a transcription factor controlling cell cycle progression, cell survival, and DNA repair in cells exposed to genotoxic as well as non-genotoxic stresses) and p21 (representing a major target for the activity of the tumor suppressor gene p53, and thus being associated with linking DNA damage to cell cycle arrest) upon exposure of LNCaP cells (human prostatic cell line) to DEHP or MEHP. The LNCaP cell line is a good in vitro model for assessing the oxidative stress potential of phthalates as they express prostate specific antigen (PSA) and p53 protein (Chung et al., 1992). Erkekoglu et al. (2011) demonstrated that 24 h exposure of the cells to 3 mM DEHP or its main metabolite, (MEHP, 3 μM) caused strongly amplified production of ROS (Erkekoglu et al., 2011). In another study Erkekoglu et al. (2010) also reported the genotoxic potential of DEHP and MEHP in MA-10 Leydig cells (mouse Leydig cells) and that DEHP caused cytotoxicity and an increase in oxidative stress in MA-10 Leydig cells (Erkekoglu et al., 2010). In an in vitro study effects of phthalate exposure on DNA damage were investigated using the alkaline Comet assay and cells from the human hepatocyte HepG2 cell line exposed to various concentrations of DEHP (0, 2.5, 5, 10, 25, 50, 100, and 250 μM) for 24 or 48 h. After exposure to DEHP for 24 and 48 h, DEHP caused increases in DNA damage. Therefore, DEHP was considered to be genotoxic in HepG2 cells in a dose-dependent manner (from 10 to 250 μM) (Choi et al., 2010). Another study showed that MEHP induced oxidative DNA damage and apoptosis in HepG2 cells and induction of p53-mediated mitochondria-dependent signalling pathways

after 24 h treatment with MEHP (⩾25 μM) (Yang.et al., 2012). Likewise, Chen et al., suggest that MEHP (ranging 6.25–50 μM) could induce apoptosis of HepG2 cells through mitochondria- and caspase3-dependent pathways (Chen et al., 2012).

Recently, Chang et al. (2017) reported that also MEHP induces intracellular ROS production leading to DNA damage, with the amount of ROS generated being dependent on the MEHP concentration. Mammalian Chinese hamster ovary (AS52) cells were used in the Comet assay to evaluate whether MEHP causes DNA single-strand breaks at concentrations of 0, 10, 25 and 50 mM. The results showed that at 50 mM MEHP all cells died during collection. MEHP indeed caused significant single-strand breaks in the cells. Therefore, Chang et al., concluded that MEHP induces oxidative stress and causes DNA damage (Chang et al., 2017). It is important to note that in a Comet assay cytotoxicity resulting in apoptosis may also be the cause of DNA damage, given that apoptosis induces DNA fragmentation. This implies that results obtained at cytotoxic concentrations should be interpreted with caution.

Taken all data together at the current state of the art regulatory bodies have classified phthalates as non-genotoxic substances (EU 2008). Indirect genotoxicity potential of DEHP or MEHP via production of ROS may proceed by a threshold mode of action still enabling identification of a safe level of exposure.

Carcinogenicity and hepatocarcinogenicity of phthalates

Evidence from multiple reports demonstrates that DEHP induces hepatotoxicity and hepatic tumorigenesis in rats and mice (Lee et al., 2018; Ventrice et al., 2013). Data on carcinogenicity for other phthalates than DEHP are missing. The biological action of DEHP is very similar to that reported for chemicals that are collectively known as peroxisome proliferators (Rusyn et al., 2006). Peroxisome proliferators induce an increase in the number and size of peroxisomes in hepatocytes, a process called ‘peroxisome proliferation’ that result in elevation of fatty acid metabolism (Rusyn et al., 2006). Peroxisome proliferators are characterised as non-genotoxic rodent carcinogens (Roberts et al., 2000). MEHP and DEHP are peroxisome proliferators and act as an exogenous ligand of PPARs including PPAR alpha (PPARα) and PPARγ mediated gene expression resulting in hepatocarcinogenic cell proliferation (Nepelska et al., 2017; Oral et al., 2016; Hurst et al., 2003;). According to in vitro and in vivo studies, DEHP stimulated activation of PPARγ leading to the production of oxidative stress and downregulated expression of insulin receptor and GLUT4 proteins, disrupting the insulin-signalling pathway in the liver of SD rats and L02 cells (Zhang et al.,

2017). Activation of PPARα by phthalates can modify protein and gene expression that causes increased induction of cell proliferation, suppression of apoptosis and/or oxidative stress (Hurst et al., 2003).

In addition, Bility et al. (2004) reported that in in vitro assays, some other phthalate monoesters, including MBP, MBZP, MOP, and MIDP, can activate PPARα (Bility et al., 2004) with the ability of phthalate monoesters to activate PPARα increasing with increasing chain length (Lampen et al., 2003; Bility, et al. 2004).

In general, PPARα activation occurs in both rodents and humans, but due to differences in PPARα density and the signaling pathways, the PPARα mode of action is generally assumed unlikely to be relevant in humans (Felter et al., 2018). Thus, there are marked species differences in response to peroxisome proliferators (Hasmall et al., 2000). The difference in responsiveness between rodents and humans has been attributed to the different levels of PPARα expression and differences in the ability of PPARα to activate target gene expression (Rusyn et al., 2006). Indeed, PPARα expression in humans is about 10-fold lower than that in rats or mice (Ito et al., 2008). On the other hand, inactive forms and polymorphic forms of PPARα have been found in human liver, suggesting that the expression of full-length functional PPARα is very low (Ito et al., 2008). In addition, the function of the PPARα signalling in liver cell proliferation induced by chemical exposure is not always similar in mice and humans. In the rodent liver, peroxisome proliferators cause peroxisome proliferation, induce cell proliferation and suppress both spontaneous and transforming growth factor b1 (TGFb1)-induced apoptosis (Hasmall and Roberts 1999). In contrast, humans appear to be refractory or only weakly responsive to the adverse effects of peroxisome proliferators (Hasmall et al., 2000).

To understand if human PPARα can be affected by phthalates a study was conducted in which the human PPARα gene was inserted in mice lacking this gene. These mice produce human PPARα, but not mouse PPARα. Treatment of these mice with a PPAR agonist, such as fenofibrate, did not cause significant hepatomegaly or hepatocyte proliferation (Yang et al., 2007a). In conclusion, in rats and mice, induction of PPARα leads to increases in hepatocyte proliferation and liver weight, and, under chronic exposure conditions, liver tumour formation, while PPARα activation in humans does not lead to increases in liver to body weight ratios (Felter et al., 2018).

With respect to the carcinogenicity of DEHP, animal studies concluded that DEHP is carcinogenic to rats and mice inducing liver tumors at doses of 50 to 1000 mg/kg/day by a non-DNA-reactive mechanism involving peroxisome proliferation (IARC 2000, Praveena et

al., 2018). Therefore, a Working Group of the "International Agency for Research on Cancer" (IARC) has concluded that the hepatocarcinogenic effects of DEHP are unlikely to occur in humans and DEHP has been classified by the IARC as belonging in group 3, meaning that this chemical is not classified as carcinogenic to humans (Klaunig, Babich et al. 2003, IARC 2000).

Nevertheless, further studies have suggested that phthalates may promote and induce carcinogenesis in a variety of tissues by a mechanism independent of PPARα activation through AhR-mediated genomic and non-genomic pathways or other mechanisms such as mitochondria- and caspase3-mediated pathways (Ito et al., 2007; Wang et al., 2012; Chen et al., 2012; Mankidy et al., 2013). The AhR is a protein that controls the expression of a diverse set of genes and is best known for its role in mediating dioxin-like toxicity (Krüger et al., 2008). Based on a summary by Schlezinger et al. (2006) and Wang et al. (2012) experimental evidence suggests that AhR plays an important role in cell proliferation and differentiation as well as in tumour development and tumorigenesis in the mammary gland (Schlezinger et al., 2006, Wang et al., 2012). Phthalates exhibit weak potency as agonists of the AhR (Mankidy et al., 2013; Kruger et al., 2008).

In addition, it has been postulated that induction of pancreatic acinar-cell tumours and Leydig cell tumours (testicular tumours) in male rats may also be caused by a mode of action including activation of PPARα (Voss et al. 2005; Klaunig, Babich et al. 2003; David et al. 2000). However, the exact mode of action underlying the induction of Leydig cell tumors is unclear and the EU risk assessment report (EU, 2008) considered the induction of Leydig cell tumors in the rat relevant for humans, and Voss et al. reported that for these tumors the PPAR mediated mode of action might be relevant for humans (Voss et al., 2005).

Following oral exposure, four long-term carcinogenicity studies (Moore 1996, 1997; NTP studies, 1982a, b) performed in rats and mice are of good quality and are considered adequate for evaluation of the carcinogenicity of DEHP in experimental animals (EU 2008). DEHP shows clear evidence of hepatocarcinogenicity in both sexes of rats and mice in the four studies and an increase in the incidence of Leydig cell tumours was observed in Sprague-Dawley rats exposed for DEHP, at dietary dose levels of 30, 95 and 300 mg/kg, in a lifelong study (published as an abstract) (Berger 1995; EU 2008). As indicated above, in the EU risk assessment report the induction of Leydig cell tumours in rats reported by Berger is considered relevant for human risk assessment. However, although other reports support the results of Berger with respect to phthalate-induced Leydig cell tumour formation in rats (Mylchreest and Foster, 1998; Schilling et al., 1999), further comprehensive investigations

are necessary before concluding on the possible carcinogenic risk of DEHP, and no classification for carcinogenicity is currently proposed (EU 2008).

The epidemiological evidence for an association between exposure to DEHP and cancer incidences is considered very weak and inconsistent (ECHA 2017, IARC 2013). The carcinogenicity of DEHP was reported for the first time in the nineties. A case–control study (Selenskas et al., 1995), conducted among workers in a plastic industry (vinyl and polyethylene production) in the United States, showed a markedly increased risk of pancreatic cancer. This study had small sample size (only 9 cases and 40 controls). Among these workers, an elevated risk for pancreatic cancer was observed among those exposed for more than 18 years (Selenskas et al., 1995). A recent epidemiological survey in Mexico evaluated the association between urinary levels of nine phthalate metabolites and breast cancer. Lopez-Carrillo et al. (2010) reported increased odds ratios for breast cancer that were associated with urinary concentrations of the DEHP metabolite MEHP and for the risk of developing breast cancer associated with phthalate metabolites detected in about 82% of all women (Lopez-Carrillo et al., 2010). However, another nested case-control study within the Women's Health Initiative (WHI) prospective cohort study on the association of phthalate exposure and breast cancer in 2018 reported that urinary phthalate metabolite levels are not related to increased breast cancer risk. In this study, they measured the level of phthalate metabolites among 419 invasive cases and 838 matched controls over 1 to 3 years. The results did not show statistically significant associations between individual phthalate metabolites and breast cancer risk in analyses adjusted for matching factors, creatinine, body mass index, smoking status, and race/ethnicity (Reeves et al., 2018).

Prospective data on whether phthalates affect human cancer risk are lacking. Nevertheless, IARC again reviewed the classification of DEHP in 2013 and changed their conclusion to ‘possibly carcinogenic to humans (Group 2B)’ as it was before the last change in 2000, based in part on the Ito et al. (2007) and Yang et al. (2007b) data (IARC 2013). The results of Ito et al. (2007) displayed the existence of pathways for DEHP-induced hepatic tumorigenesis that are independent of PPARα. These results suggested that increases in oxidative stress induced by DEHP exposure may lead to the induction of inflammation and/or the expression of protooncogenes through the increased levels of 8-OHdG and NFκB, resulting in a high incidence of tumorigenesis in PPARα-null mice. In its re-evaluation of DEHP carcinogenicity, IARC stated, “multiple molecular signals and pathways in several cell types in the liver, rather than a single molecular event, contribute to the induction of cancer in rats and mice. Thus, the relevance to human cancer of the molecular events that lead to cancer

elicited by DEHP in several target tissues (e.g. the liver and testis) in rats and mice cannot be ruled out" (IARC 2013).

Similar to what was reported for DEHP, among other phthalates BBP has also been shown to activate PPARα and PPARγ. BBP has been reported to induce DNA and chromosomal damage in rats as well (Hsieh et al., 2013). BBP tested negative for carcinogenicity in mice (Hsieh et al, 2013); in rats findings of mononuclear cell leukaemia, benign pancreas tumours and urinary bladder tumours were of doubtful significance (Hsieh et al., 2013).

The U.S. Environmental Protection Agency (U.S. EPA) classified BBP as a Class C "possible human carcinogen" in 1993 (U.S. EPA, 1993b). In 2000, IARC determined that the evidence of the carcinogenicity of BBP in humans was inadequate, and the evidence in experimental animals was limited, and classified BBP in Group 3 "Not classifiable as to its carcinogenicity to humans" (IARC, 2000). For DBP, DEP and DMP, no carcinogenicity studies are available (Danish EPA 2013).

Health based guidance values for exposure to phthalates

Health based guidance values defining acceptable levels of exposure for several phthalates have been derived by different regulatory agencies such as the U.S. EPA and European Food Safety Authority (EFSA), and can be identified in authoritative national and international guidance documents. In addition, the German Human Biomonitoring Commission (HBM Commission) derived health-related guidance values (Human Biomonitoring assessment values, HBM values) to achieve a harmonized assessment of human's internal exposure to pollutants. In the present thesis, Tolerable Daily Intake (TDI) values, and Reference Dose (RfD) values are used for the risk assessment, both reflecting the level to which a person can be exposed every day during a life time without experiencing an adverse health effect. The fact that TDI and RfD values have been defined, also reflects that the potential carcinogenicity, whenever relevant, is considered to be due to a threshold mode of action and the TDI/RfD is protective also for these endpoints. Table 4 and the next sections summarize information on the TDI and RfD values currently available for phthalates included in the studies of this thesis.

Table 4. Health based guidance values for phthalates including Tolerable Daily Intake (TDI), Reference Dose (RfD) and Human Biomonitoring assessment values (HBM values), established by the EFSA, U.S. EPA and the German Human Biomonitoring Commission.

Phthalates	**TDI (EFSA)[1] [µg/kg body weight/day]**	**RfD (U.S. EPA)[2] [µg/kg body weight/day]**	**HBM (µg/l)[3]**		
DEP	-	800	Children (6–13)	Women	Men ≥14
DMP	-	-	-	-	-
DBP	10	100	-	-	-
BBP	500	200	-	-	-
DEHP	50	20	500	300	750

Abbreviations: EFSA, European Food Safety Authority; TDI, Tolerable Daily Intake; RfD, Reference Dose; HBM, Human Biomonitoring assessment values; U.S. EPA, United States Environmental Protection Agency
[1] Sources: EFSA (2005a, 2005b, 2005c)
[2] Sources: IRIS EPA (1987a,b; 1988)
[3] Sources: HBM Commission, 2007

Tolerable Daily Intake (TDI)

The TDI is the estimated dose of a substance which can be ingested daily over a lifetime without significant risks to human health (Sand, Parham et al. 2017). TDIs for several phthalates have been defined by the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) of the EFSA based on No Observed Adverse Effect Levels (NOAELs) or Lowest Observed Adverse Effect Levels (LOAELs) and the default uncertainty factors (EFSA 2005a, EFSA 2005b, EFSA 2005c).

Previously, temporary Tolerable Daily Intake (t-TDI) values of 0.05, 0.1 and 0.05 mg/kg bw were set by the Scientific Committee for Food (SCF) for DBP, BBP and DEHP respectively, based on the endpoint of peroxisome proliferation in rodent liver. Given that there is now a scientific consensus that liver peroxisome proliferation in rodents is not relevant for human risk assessment, in the recent European Union Risk Assessment Report (RAR) it is announced that the critical effects of DBP, BBP and DEHP relate to reproduction. Therefore, EFSA changed the critical endpoint from peroxisome proliferation in rodent liver to reproduction and development endpoints on which to base the risk assessment for DBP, BBP and DEHP (Lhuguenot, 2009). In this regard, the EFSA AFC panel allocated a TDI of 0.05 mg/kg body weight/day for DEHP, based on a NOAEL of 5 mg/kg body weight/day for testicular toxicity and an uncertainty factor of 100. The TDI for DBP is determined at 0.01 mg/kg bw/day, based on a LOAEL of 2 mg/kg body weight/day and making use of an uncertainty factor of 200.

The EFSA AFC Panel allocated a TDI of 0.5 mg/kg body weight/day for BBP, derived from a NOAEL of 50 mg/kg body weight/day based on developmental effects (reduced anogenital distance (AGD)) with an uncertainty factor of 100.

The effects are considered to have an anti-androgenic mode of action for all abovementioned phthalates (DBP, BBP and DEHP). The critical observations for these phthalates were as follows.

For DEHP, a multigenerational reproductive assessment study by Wolfe and Layton (2003) (Wolfe and Layton 2003), was the pivotal study for the selection of the starting point for the definition of the TDI. The AFC Panel considered that the study conducted by Wolfe and Layton (2003) was more robust than those underpinning the previous NOAELs based on reproductive toxicity. Wolfe and Layton reported a NOAEL of 5 mg/kg body weight/day based on testicular effects (germ cell depletion, reduced testis weight) in male offspring (Wolfe and Layton 2003). From this value the AFC Panel derived the TDI of 0.05 mg/kg body weight/day (EFSA 2005a).

For DBP, a developmental toxicity study in rats (Lee et al. 2004), with dietary exposure to DBP during the period from late gestation (gestational day 15) to the end of lactation (postnatal day 21), showed effects on the development of male and female offspring at lower doses than those found previously. Lee et al. (2004) observed delayed germ cell development and a persistent male mammary gland change at 2 mg/kg body weight/day. A LOAEL of 2 mg/kg body weight/day was selected from this study. A NOAEL could not be established for DBP due to the fact that in several reproductive toxicity studies NOAEL values were established that varied approximately 30-fold (Mylchreest et al., 2000). Therefore, the TDI for DBP was allocated based on the LOAEL level using an extra uncertainty factor of 2 for extrapolation of the LOAEL to NOAEL, in addition to the default uncertainty factor of 100 for inter- and intraspecies differences (EFSA 2005b).

For BBP, a multi-generation study carried out by Tyl et al. (2004) was used as a reference for establishing the TDI based on reproductive and developmental toxicity (Lhuguenot 2009). In this study BBP was administered in the diet at 0, 50, 250, and 750 mg/kg body weight/day. At ≥250 mg/kg bw/day, there were reduced F1 and F2 male anogenital distance and male reproductive system malformations. There were no effects on parents or offspring at 50 mg/kg body weight/day. Therefore, Tyl et al. (2004) reported a NOAEL of 50 mg/kg body weight/day based on the presence of reduced anogenital distance in F1 and F2 males (Tyl et al., 2004). From this value the AFC Panel derived the TDI of 0.5 mg/kg body weight/day (EFSA 2005c).

The EFSA AFC Panel did not issue TDIs for DMP and DEP. The EFSA AFC Panel reviewed relevant studies (Fujii et al., 2005; Pereira et al., 2007a,b) reported since the 2003 review by the WHO and concluded that they did not suggest a need to modify the TDI proposed by the WHO (TOX/2011/04). EFSA categorised DMP and DCHP as List 7 'Substances for which some toxicological data exist, but for which a TDI could not be established. Required additional information should be furnished' (SCCP/1016/06).

The WHO evaluated the health effects and environmental effects of DEP in 2003 (WHO 2003). A TDI of 5 mg/kg body weight/day was established based on a NOAEL of 1600 mg/kg body weight/day based on no malformations but skeletal (rib) number variations in mice at higher dose levels, to which an uncertainty factor of 300 was applied. This uncertainty factor consisted of a factor 3 for incompleteness of the database and the values of 10 each for interspecies and intraspecies variation. The existing information on other investigated phthalates in this study including DEP was considered by regulatory bodies sufficient to conclude that DEP does not exhibit endocrine disrupting effects in terms of human health similar to DBP, BBP and DEHP and an endocrine disrupting mechanism cannot be attributed to DEP's effects on the male reproductive system (ECHA 2015). Effects of DEP on general reproductive performance were limited since a dose of 4400 mg/kg caused only decreased body weight gain in the F1 generation (ECHA 2015; SCCNFP 2002).

Toxicity data associated with DMP exposure are limited. Overall, a lack of comprehensive studies pertaining to particular organ systems or exposure durations (i.e. acute, subchronic, or chronic) hampers the calculation of a TDI for systemic, reproductive, or developmental effects (U.S. CPSC , 2011). Even though NOAEL or LOAEL values could be described for a particular study (i.e. bodyweight decrements, changes in hemoglobin, increases in liver weight), the lack of other supporting studies was taken to conclude that there was "inadequate evidence" for the designation of a TDI for DMP (U.S. CPSC , 2011).

Reference Dose (RfD)

The RfD set out by the U.S. EPA Integrated Risk Information System (IRIS) is defined as "an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime." For the RfD derivation, a NOAEL, a LOAEL or a benchmark dose can be used, and uncertainty factors are applied for reflection of limited data and inter- and intraspecies differences (Sand, Parham et al. 2017).

Based on a study on guinea pigs an RfD of 2×10^{-2} mg/kg body weight/day was determined for DEHP using the LOAEL of 19 mg/kg/day for increased relative liver weight and an uncertainty factor of 1000 (IRIS EPA 1987a). Uncertainty factors of 10 each were used for interspecies and intraspecies variation, and an additional factor of 10 was used since the guinea pig exposure was longer than sub chronic but less than lifetime, and because, the RfD is set based on a LOAEL, the latter eventhough the effect observed was considered to be minimally adverse (IRIS EPA 1987a).

The RfD of 1×10^{-1} mg/kg body weight/day was issued for DBP based on a study in male Sprague-Dawley rats with a NOAEL of 125 mg/kg body weight/day for increased mortality and considering an uncertainty factor of 1000 (IRIS EPA 1987b). A factor of 100 was applied to account for inter- and intraspecies variation. An additional factor of 10 was used to account for both the less-than-chronic duration of the study and deficiencies in the study, such as the use of only male animals (IRIS EPA 1987b).

A 6-month rat study conducted by NTP (National Toxicology Program) in 1985 was considered the pivotal study in the process of defining an RfD for BBP. In this study 15 males/group were administered concentrations of 0, 0.03, 0.09, 0.28, 0.83, or 2.5% BBP in the diet for 26 weeks. All the rats given 2.5% had small testes upon gross necropsy; some had soft testes and one group had a small prostate and seminal vesicles. At the 0.83% treatment group the absolute liver weight, liver-to-body weight and liver-to-brain weight ratios were significantly ($p<0.05$) increased. Therefore, a LOAEL of 0.83% (equivalent to 470 mg/kg body weight/day) for increased liver weight was identified in the NTP (1985) study. The NOAEL of 0.28% (equivalent to 159 mg/kg body weight/day) was used to derive the RfD because the NTP (1985) study had longer duration compared to other related studies, and this study was well conducted providing a complete description of methods including study design and clinical analysis. The RfD of 2×10^{-1} mg/kg body weight/day is allocated for BBP making use of an uncertainty factor of 1000. Uncertainty factors of 10 each were used for intra- and interspecies sensitivity, and an additional factor of 10 was used for extrapolating from a sub chronic to a chronic NOAEL (NTP 1985: IRIS EPA 1988).

The RfD for DEP was based on the results of a 16-week feeding study using CD rats (Brown et al., 1978). Rats were fed 0, 0.2, 1.0, or 5.0% DEP in the diet. The authors reported significantly decreased growth rate, decreased food consumption and altered organ weights in male and female rats given 5% DEP. According to the results of this study, the RfD of 8×10^{-1} mg/kg body weight/day was established based on a NOAEL of 5% (equivalent to 750 mg/kg body weight/day) with an uncertainty factor of 1000. This factor of 1000 consisted of the

default uncertainty factor of 100 for inter- and intra- species differences while an additional factor of 10 was used for extrapolation from sub-chronic to chronic exposure (Brown et al., 1978; IRIS EPA 1987).

DMP was not assessed under the U.S. EPA IRIS Program.

Comparing the RfD values set out by the U.S. EPA and the TDI values defined by the EFSA, it appears that values for the same phthalates may vary up to 10-fold. The reason for these discrepancies might be due to the choice of the critical effect, the key studies used to derive the point of departure (POD) and exposure scenarios, as well as different NOAEL or LOAEL values and uncertainty factors used to define the health based guidance values (Gurusankar, et al., 2017; Søeborg, Frederiksen and Andersson, 2012).

Human Biomonitoring assessment values (*HBM values)*

HBM values represent another category of health based guidance values. These values define concentrations of biomarkers (metabolites) in urine, which reflect an acceptable chronic exposure, since the basic assumption is a direct relation between external exposure and internal burden (Angerer, Aylward et al. 2011; Angerer et al., 2007).

The HBM Commission applies three methods for deriving HBM values as follows: deriving HBM values based on epidemiological data providing evidence of a relationship between concentrations of a substance and/or its metabolites in human biological samples and the occurrence of adverse effects. Human data are the best fundament for the value's derivation. Secondly, HBM values can be established on the basis of internationally agreed TDI/RfD values. This concept was used for the first time to derive the HBM value for the sum of the DEHP metabolites. Thirdly HBM values can be established on the basis of critical effects observed in animal experiments for which sufficient human data or generally accepted tolerable intake values are missing (Apel et al., 2017). In this approach two type of HBM values exist as follows:

- HBM-I values: HBM-I values are control values. According to current knowledge, no need for action exists when the internal exposure (the measured HBM level) remains below or at the HBM-I value, because adverse health effects are not expected.
- HBM-II values: HBM-II values are intervention values. Concentrations of a substance in a body fluid/tissue, which exceed this value at the current state of evaluation by the HBM-commission, may lead to adverse health effects. Acute need for action to reduce exposure exists, and care by experts of environmental medicine is necessary (Schulz, Wilhelm et al. 2011).

However, due to a lack of human studies on biological effects, only a few health-related HBM values could be determined for phthalates. In the case of phthalates, the commission derived HBM values for DEHP metabolites in urine of children, women and males based on the TDI (HBM 2007). HBM-I values for DEHP metabolites in urine are estimated based on the TDI values and the composite sum of the DEHP metabolites, MEOHP and MEHHP (Table 4) on the basis of the following equation (HBM commission 2007):

Σ(MEOHP+MEHHP) in 24-h urine= TDI× (Molecular weights of the metabolites/ Molecular weight of DEHP)× 0.4

In this formula the factor 0.4 reflects that 40 percent of the applied oral dose is excreted into urine as the two metabolites MEOHP and MEHHP so that 1 mol DEHP corresponds to ~ 0.4 mol Σ(MEOHP+MEHHP) in 24-h urine.

Filling out the respective values this equals: Children and the remaining general population:

Σ(MEOHP + MEHHP) in 24-h urine = 50 µg/kg body weight/day × 293/390 × 0.4 = 15 µg/kg body weight/day

Thus, this HBM-I value it is based on the assumption that the maximum adult's daily intake of DEHP that would be of no safety concern equals the TDI (50 µg/kg body weight/day) (EFSA 2005a), 40 percent of the applied oral dose is excreted renally as the two metabolites 5 MEOHP and MEHHP (1 mol DEHP corresponds to ~ 0.4 mol Σ(MEOHP + MEHHP). Thus, the HBM-I value for DEHP metabolites was derived based on a TDI of 50 µg/mg/kg body weight/day for children and the remaining general population (HBM commission 2007). For women of childbearing age a specific HBM was derived in the same way but based on a TDI of 20 µg/mg/kg body weight/day (HBM commission 2007).

For women of childbearing age:

Σ(MEOHP + MEHHP) in 24-h urine = 20 µg/kg body weight/day × 293/390 × 0.4 = 6 µg/kg body weight/day

The HBM values refer to the amount of a substance (µg/day) in a complete 24-hour urine sample. Therefore, the calculated metabolites' excretion can be referred to a body weight-related urine volume of 30 mL/kg body weight/day for children and 20 mL/kg body weight/day for all other population subgroups.

This results in the following HBM values for DEHP metabolites in urine:

HBM-I value for the adult population (Table 4):

15 µg/kg body weight/day÷ 0.020 L/kg body weight/day=750 µg/L

HBM-I value for children (Table 4):

*15 µg/kg body weight/day÷ 0.030 L/kg body weight/day=500 µg/*L

HBM-I value for women of child bearing age (Table 4):

6 µg/kg body weight/day÷ 0.020 L/kg body weight/day= 300 µg/l

Quantitative risk assessment for carcinogenicity as the critical effect

Given that some phthalates may cause carcinogenicity, albeit by a threshold mode of action, some regulatory bodies have also defined parameters that enable a quantitative cancer risk estimation. Such a quantitative cancer risk estimation is not adopted by EFSA but has been frequently used by other regulatory bodies. The U.S. EPA IRIS has classified DEHP as B2 (probable human carcinogen) in 1988, and has developed a slope factor and unit risk, based on animal studies in rats and mice, to enable a quantitative cancer risk estimation (EPA1993). The slope factor is the result of application of a low-dose extrapolation procedure and is defined as the upper-bound estimate of the probability that an individual will develop cancer if exposed to a chemical for a lifetime of 70 years and is expressed in (mg/kg body weight /day)$^{-1}$.

In addition, the European Commission's Scientific Committee on Occupational Exposure Limits (SCOEL) and the European Chemicals Agency (ECHA)'s Risk Assessment Committee (RAC) have agreed on the approach of quantitative cancer risk estimation for the risk assessment of non-genotoxic carcinogens for which a threshold exists (e.g., peroxisome proliferators, hormones and local irritants) and of genotoxic carcinogens with an indirect genotoxicity mechanism (indirect mechanisms that cause damage to DNA or chromosomes such as production of ROS) (ECHA 2017). In their approaches, the unit risk is the quantitative estimate in terms of either risk per µg/L drinking water or risk per µg/m^3 air breathed, and risk numbers are presented as the drinking water or air concentrations providing cancer risks of 1 in 10,000, 1 in 100,000 or 1 in 1,000,000.

Based on the approach defined by the U.S. EPA, the quantitative estimate of the carcinogenic risk of DEHP upon oral exposure is calculated as follows:

A cancer slope factor of 1.4×10^{-2} (mg/kg body weight /day)$^{-1}$ for oral exposure was calculated based on the combined incidence of hepatocellular carcinomas and adenomas in male mice (Kluwe, et al., 1982, IRIS EPA, 1987a). Orally administered DEHP produced significant dose-related increases in liver tumor responses in rats and mice of both sexes. The probability of an individual developing cancer over a lifetime is estimated by multiplying the cancer slope factor (proportion of the population affected per mg/kg body weight/day) for the substance by the chronic (70-year average) daily intake (mg/kg body weight/day) (CHEST, 2003).

The Drinking Water Unit Risk = 4.0×10^{-7} per (µg/L) was calculated based on the oral slope factor. The median carcinogen risk estimate is calculated by dividing the cancer slope factors by 70 kg (average weight of man) and multiplying it by 2 L/day (average water consumption rate of an adult) (CHEST, 2003).

Specified Risk Levels defined by the U.S. EPA and related DEHP concentrations in drinking water are shown in Table 5. In the present thesis these risk factors were used to perform a quantitative risk assessment for exposure to DEHP via bottled water.

Table 5. Drinking Water Concentrations of DEHP at Specified Risk Levels (EPA1993).

Risk Level (proportion of the population affected)	**Concentration in drinking water causing this effect**
E-4 (1 in 10,000)	300 µg/L
E-5 (1 in 100,000)	30 µg/L
E-6 (1 in 1,000,000)	3 µg/L

Phthalate exposure estimation for risk assessment

For application of direct or indirect exposure assessment data in risk assessment, based on the concentration of phthalates in respectively environmental/food media or human biomonitoring samples, the estimated daily intake (EDI) should be determined (Kamrin, 2009).

The EDI of phthalates can be calculated based on indirect exposure assessment methods using the detected chemical concentrations of the phthalates in relevant sources (food, air, soil, etc.) to develop estimates of intake from these sources. There is uncertainty associated with these estimates including variations in reported concentrations and intake of various media, reliance on surveys of product use and the potential for sample contamination. As a

consequence, there is a tendency to default to worst case inputs to conservatively address the uncertainty (Kransler et al., 2012).

With the knowledge on human metabolism and elimination characteristics of the phthalates as a precondition, a translation from the urinary metabolite levels to the doses of the parent phthalates taken up, and thus a so-called direct exposure assessment, becomes feasible. The estimation of the EDI values of the phthalates based on human biomonitoring data is carried out based on the urinary concentrations of phthalate metabolites adjusted for daily creatinine excretion, body weight and/or other related parameters.

To do so, some approximations must be made such as a steady state regarding exposure and metabolic clearance. When extrapolating intake doses from urinary metabolite levels, the molar fraction of the urinary monoester metabolite excretion (F_{UE}) plays a crucial role. These factors describe the molar ratio of the external oral phthalate dose to the amount excreted in urine as one or several specific metabolites. For example, a value of 84 for DEHP and its metabolite means that within 24 h after oral intake of DBP, 84% of the dose is excreted as its metabolite in urine (Koch et al., 2005).

For several phthalates, F_{UEs} have been determined in human toxicokinetic studies by the administration of isotope-labeled phthalate diesters (Table 6) (Koch and Angerer, 2011).

Table 6. Molar excretion fractions (F_{UE}) of phthalate metabolites in urine related to the ingested oral dose of the parent phthalate determined in human metabolism studies after oral application.

Phthalates	Metabolites	F_{UE} (%)	Reference
DBP	MBP	84	Koch et al., 2012
DEP	MEP	69	Koch and Angerer, 2011
DMP	MMP	69	Koch and Angerer, 2011
BBP	MBzP	73	Anderson et al., 2001
DEHP	MEHP	6.2	Anderson et al., 2011
	MEHHP	14.9	
	MEHOP	10.9	

Ultimately, for risk assessment, the EDI values calculated for phthalates from both approaches can be compared to one another and also to health based guidance values such as TDI and RfD values established by authorities such as EFSA and U.S. EPA to estimate whether an adverse effect of a particular chemical can be expected under particular circumstances. In this process of risk assessment, the level of concern can be quantified by calculating a Hazard Quotient (HQ) or Hazard Index (HI). The HQ is the ratio between the

EDI and the TDI or RfD of a given chemical. The HI is the sum of the HQs for several chemicals that have a similar toxicological endpoint. If the HQ or HI equals or exceeds one, the chemical exposure under consideration is regarded as more likely to lead to adverse health effects because it exceeds the relevant TDI or RfD (Koch and Angerer, 2011). In addition, with HBM values it is possible to compare the HBM concentrations actually detected in a biomonitoring study to HBM reference values to quantify the risk (Gurusankar et al. 2017). When the toxicological data base is considered inadequate for definition of a TDI, the risk can be evaluated considering the margin of exposure (MOE). With this approach the daily intake is not compared to the TDI or RfD but to the NOAEL (or the Benchmark dose lower confidence limit (BMDL) from an experimental animal study. Uncertainty factors are used to determine the acceptable MOE. For a non-genotoxic chemical the ratio between the NOAEL (or BMDL) and daily intake should be higher than 100 to conclude the exposure is not of concern, with the values of 100 being based on the UF of 10 for interspecies differences and the UF of 10 for interspecies differences.

Since chronic co-exposure to various phthalates may constitute a risk of anti-androgenic effects during the stages of puberty and development of reproductive organs, the National Research Council in the recent recommendations has reported that phthalates met the conditions necessary to warrant a cumulative risk approach (NCR 2008). This conclusion provides the basis for assessment of risks from combined exposure to the three phthalates (DBP, BBP, DEHP) owing to their structural and metabolic similarities and similar endpoint (anti-androgenic effects). Combined toxicity of these three phthalates has been best predicted with dose addition models by using HI values (e.g., NCR 2008; CHAP 2014; Health Canada 2015).

In the present thesis, excess lifetime cancer risk was used to calculate the potential cancer risk via drinking bottled water by multiplying the cancer slope factor (proportion of the population affected per mg/kg body weight/day) for the substance with the estimated daily intake (in mg/kg body weight/day). This approach for quantification of cancer risk levels can be used for both genotoxic and non-genotoxic carcinogens. Excess cancer risk should be compared to a cancer risk that is considered acceptable which is usually expressed as a population risk, such as 1×10^{-6}, which means that no more than one in 1 million exposed people, is expected to develop cancer upon life time exposure. The dose levels causing this 1×10^{-6} additional cancer risk upon life time exposure is considered the Virtual Safe Dose

(VSD). The U.S. Environmental Protection Agency (U.S. EPA) typically uses a target reference risk range of 10^{-4} to 10^{-6} for carcinogens in drinking water (Cotruvo 1988) (Table 5), which is in line with the World Health organization (WHO) guidelines for drinking water quality which, where practical, base guideline values for carcinogens on the upper bound estimate of an excess lifetime cancer risk of 10^{-5} (WHO, 2001). It is important to note that risk estimates obtained in this way are not scientific estimates of actual cancer risk; they are upper bounds on actual cancer incidences that are useful to regulators and risk managers for setting priorities and for setting exposure limits.

Classification labelling and regulation of phthalates

Depending on use, phthalates are potentially regulated and classified by various regulatory agencies, including the U.S. EPA, the Occupational Safety and Health Administration (OSHA), the U.S.FDA, the Consumer Product Safety Commission (CPSC) and in the European Union by the European Chemicals Agency (ECHA) and EFSA.

U.S. EPA regulates various phthalates released into the environment under most of its statutes. For example, DEHP is regulated as a hazardous air pollutant, a drinking water contaminant, a water pollutant, and a hazardous waste. REACH as a regulation of the European Union included four phthalates from the Union list, the ESCO list and/or the FACET database, that are subject to authorization as food contact material (FCM) substances under REACH (Table 7) (Geueke et al., 2017). Table 7 provides an overview of regulations for the phthalates included in the present thesis as FCM.

Table 7. Food contact material (FCM) substances that are subject to authorization under the European Chemicals Regulation (REACH).

CASRN	Name	Union list	ESCO list	FACET list	Annex XIV
84-74-2	Dibutyl phthalate (DBP)	+	+	+	+[a]
85-68-7	Butyl benzyl phthalate (BBzP)	+	+	+	+[a]
117-81-7	Bis(2-ethylhexyl) phthalate (DEHP)	+	+	+	+[a]

Abbreviations: ESCO list initiated by the European Food Safety Authority (EFSA) and established by EFSA's Scientific Cooperation Working Group, http://www.efsa.europa.eu/en/supporting/pub/139e.htm.
FACET list is developed as modelling tool to estimate exposure to chemicals in food and which includes 6475 FCM substances, https://www.foodpackagingforum.org/food-packaging-health/phthalates.
Union list: Substances on this list underwent risk assessment and have been authorized by the European Commission (EC) for use in plastic FCMs.
Amendments to Annex XIV:
Commission Regulation (EU) No 143/2011

The EU has confirmed that DBP, BBP and DEHP have endocrine disrupting properties and classified them as Category 1B reproductive agents that have been identified as Substances of Very High Concern (SVHC) and placed them on the REACH Authorisation List. This means that these phthalates can be placed on the EU market only for those uses for which an authorisation has been granted to specific applicants (EC 2014).
An overview of the globally harmonised classification and labelling of the phthalates according to legislation (EC) no. 1272/2008 is displayed in Table 8.

Table 8. Harmonised classification and labelling of the phthalates (ECHA 2016).

Substance	**CAS no.**	**Classification and labelling according to Regulation 1272/2008**	
		Hazard class and category codes	Hazard statement codes
DEHP	117-81-7	Repr.1B	H360-FD
BBP	85-68-7	Repr.1B; Aquatic Acute1; Aquatic Chronic1	H360-Df; H400; H410
DBP	84-74-2	Repr.1B; Aquatic Acute1	H360-Df; H400

Repr.1B: Toxic to reproduction category 1B
H360-FD: May damage fertility. May damage unborn child
H360-Df: May damage unborn child. Suspected of damaging fertility
H400: very toxic to aquatic life
H410: very toxic to aquatic life with long lasting effects

Together this information supports that phthalates can be of concern, and confirms that evaluation of exposure and associated risks is of use, especially in countries like Iran where such strict regulations are not (yet) in place. Therefore, identifying exposure sources and routes for phthalates and estimating total intake of phthalates in the general population based on human biomonitoring needs to be well understood.

Objectives and Outline of thesis

The aim of the present thesis was to evaluate human exposure to phthalates via consumption of bottled water and its possible consequences with respect to human health for children and adults in Iran.

This work is presented in seven chapters:

Chapter 1, the current chapter, presents an overview of the basic principles that are of importance for this thesis, including an introduction to the occurrence and chemistry, metabolism, toxicity and biomarkers of exposure of phthalate esters as well as a short introduction to risk assessment and regulatory status for phthalates. The aim and overall objective of the thesis are presented.

In **chapter 2** an analytical method for measuring the phthalates in water is defined. A new magnetic PDMS/MWCNTs-OH composite, which has good dispersibility in aqueous solutions, was used as magnetic solid-phase extraction (MSPE) adsorbent for the extraction of phthalates from water samples.

In order to investigate to what extent the presence of phthalates in PET-bottled water available on the Iranian market would be of concern for the health of its consumers, **chapter 3** describes: (1) the levels of DBP, BBP and DEHP in bottled water following different storage conditions, (2) the estimated daily intake of phthalates via drinking this bottled water (µg/kg bw/day) for children and (3) a risk characterization of this estimated intake using the hazard quotient (HQ) approach and, for DEHP, also a cancer risk estimate approach, and (4) a comparison of the EDI of the phthalates via bottled water in relation to the TDI value of each phthalate. **Chapter 4** presents concentrations of DEP in bottled water stored under various conditions, the estimated daily intake of DEP via consumption of this bottled water and an assessment of the potential health risk posed by daily intake of DEP via consumption of this bottled water by children, adult pregnant and lactating women by using the margin of exposure (MOE) approach. Also, the contribution of the daily intake of DEP via bottled water to the TDI of DEP was calculated. In **chapter 5**, based on the concentrations of DBP, BBP and DEHP in bottled water, the estimated daily intake of the respective phthalates was assessed for adult, pregnant and lactating women. Further, the potential non-cancer risk for these individual phthalates and also the cumulative risk for anti-androgenicity were determined using HQ and HI approaches. Excess cancer risks were estimated for DEHP in adult, pregnant and lactating women.

Chapter 6 provides a review of the current literature on association between prenatal and/or childhood exposure to phthalate and autism spectrum disorder (ASD) in order to identify data gaps for future studies.

Considering that combined exposure assessment of phthalates is of paramount importance because of the similar anti-androgenic effects, **chapter 7** intends to estimate the total phthalate exposure of Iranian children for assessing potential risk posed by phthalate exposure via multiple routes and pathways, by direct exposure assessment via analysis of phthalate metabolites in urine samples. The combined risk of exposure to anti-androgenic phthalates was estimated by using the dose addition approach.

Finally, **Chapter 8** presents a summary of the results described in the previous chapters, the overall discussion and future perspectives to be addressed.

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CHAPTER 2

Magnetic solid-phase extraction based on modified Magnetic nano-particles for the determination of phthalate diesters in water samples

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Abstract

In the present study, it is sought to extract Phthalate Acid Esters (PAEs) from bottled water by applying surface-functionalized magnetic particles (MPs) as the adsorbent of Magnetic Solid-Phase Extraction (MSPE). In order to do so, MPs along with Polydimethylsiloxane and Multi-Walled carbon nano-tubes, due mainly to their excellent adsorption capability, were utilized. By amalgamating the MSPE with Gas Chromatography-Mass Spectrometry (GC-MS), a reliable, sensitive, and cost-effective method for the simultaneous determination of six main PAEs was developed. The factors which could influence the extraction were investigated in depth. The results indicated that under optimized conditions, the limits of detection (LODs) and limits of quantification (LOQs) were in the range of 0.01-0.025 and 0.025-0.05 Fg L-1, respectively. Also, the calibration curves were linear ($r2 \geq 0.992$) over the concentration ranges from 0.05 to 20 Fg L-1. In addition, a satisfying reproducibility was achieved by evaluating the intra- and inter-day precisions with relative standard deviations (RSDs) less than 11.71% and 12.40%, respectively. The recoveries of the five PAEs ranged from 91.5 % to 97.8 % with the RSDs less than 10.64 %. DMP, DEP, DBP and DEHP were detected in most of the samples. Based on the results, the MSPE-GC-MS method developed in the current study provides a new option for the determination of PAEs in water samples.

Introduction

Industrial development and urbanization have led to a high exposure to environmental pollutants especially through consumption of food and drinking (Martine et al., 2012). Over the last 50 years, increasing application of plasticizers such as phthalate acid esters (PAEs) for improvement of physicochemical properties of polymers has drawn public attention due to their effect on human health (Amiridou et al., 2011). Recent studies have shown that phthalates may act as endocrine disruptors and teratogens. They can also produce reproductive and developmental effects in rodents (Kondo et al., 2010). These and many other similar findings have caused considerable concern about phthalate exposure (Martine et al., 2012; Bang et al., 2012; Al-Saleh et al., 2011; Bach et al., 2011; Kovacic et al., 2010; Sathyanarayana et al., 2008; Liang et al., 2008; Casajuana et al., 2003). As a matter of fact, PAEs can be found in many raw materials, which are used for production of toys, food, drugs and cosmetic products (Martine et al., 2012; Al-Saleh et al., 2011; Casajuana et al., 2003; Caldwell et al., 2012). It is also noteworthy that PAE compounds can be released into the environment through different phases of polymer production, including synthesis, molding, deformation, lamination, storage, incineration of the polymeric materials and even when they are being used by costumers. The lack of a strong chemical bond between phthalate, when used as a plasticiser, and polymers is one of the reasons why such hazardous compounds can be easily released into the environment (Amiridou et al., 2011; Muncke et al., 2009). Application of plastic and polymers in production of bottles for packaging drinking water is routine (Andra et al., 2012). Some of the facts such as, 25% increase in the consumption of bottled water per capita from 2004 to 2009 in the world, and that people in several developed countries drink bottled water more than tap water, should make us more concerned (Reimann et al., 2010; Andra et al., 2011). Based on the 2001–2008 National Health and Nutrition Examination Survey, phthalate metabolites had been detected in >97% of the studied cases (Ceretti et al., 2010). A reliable and highly sensitive method for quantification of PAEs in samples is necessary to have an estimate of the degree of pollution. Also, performing sample cleanup and enrichment process before instrumental analysis is vital due mostly to the phthalates low concentration in the samples and the presence of inevitable interferences. In this regard, different sample preparation methods such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE) have been used widely in the previous studies (Caldwell et al., 2012; Saeed et al., 2010). However, these methods have some disadvantages. LLE is a time-consuming method and usually needs large volumes of organic solvents, which have been proven to be harmful to environment. The other separation method, SPE, is usually tedious, relatively expensive and is always accompanied with complexity (Cao et al., 2010). Recently, a new method, called magnetic solid-phase extraction (MSPE), has been established upon the old SPE method by

which sample pretreatment can be conducted effectively (Sharpe et al., 2000). In this method, magnetic particles (MPs) are adopted as SPE adsorbents without need for packing into the SPE cartridges. MPs disperse in sample solutions completely and absorb the analytes quickly. These features are beneficial for achieving high-extraction efficiency. After the extraction, the MPs can be collected and separated from liquid phase by applying an external magnet. This technique simplifies the SPE procedure greatly. Comparing to the traditional SPE procedure, MSPE is a time effective and less laborious approach, which makes it a promising technique for preparing samples. Many different adsorbents have been used for MSPE. Among them, magnetic carbon nanotubes (MCNTs) have attracted great attention due to their unique properties (Liang et al., 2010; Kataoka et al., 2000). The hydrophobic and π–π electron donor–acceptor interactions between MCNTs and aromatic compounds such as phthalates make it a promising MSPE adsorbent (Bocchini et al., 2009). In our previous study, we used MCNTs as MSPE adsorbent for the determination of phthalate monoesters in urine samples (Xu et al., 2012). The data analysis of the results indicated that the prepared MCNT is a useful tool for biological monitoring of exposure to phthalates through the determination of urinary phthalate metabolites. In the other study, the same adsorbent was used successfully for the determination of polycyclic aromatic hydrocarbons in grilled meat samples (Fankhauser-Noti et al., 2007). The aim of the present study was to find a magnetic sorbent with higher performance for separation of phthalates from environmental samples. To achieve this goal, we used a different nanocomposite of MSPE adsorbent. Due to its excellent adsorptive properties, polydimethylsiloxane (PDMS) has been employed frequently as SPE and SPME adsorbent for the extraction of phthalates (Wegelin et al., 2001; Alzaga et al., 2003; Jara et al., 2000). It could be anticipated that a composite of MCNTs and PDMS will show superior performance compared with each one alone. However, the low solubility of PDMS in aqueous matrices prevents its wide applications as MSPE adsorbent (Cao et al., 2008). Even when the PDMS is modified as MPs, it accumulates at the top of aqueous phase, a situation that is unfavorable for the extraction. Therefore, a surface modification of PDMS to the PDMS-functionalized MPs is necessary to enhance the dispersibility of the magnetic sorbent in aqueous matrices. Recently, Xu *et al*. (Cao et al., 2008) have adopted MWCNTs-OH and hydroxyl-terminated PDMS (OH-PDMS) as coating materials for the preparation of functionalized MPs. The developed MPs were used as MSPE adsorbents for the extraction of four fluoroquinolones, from water samples followed by capillary liquid chromatography analysis. In the present study, the capabilities of PDMS/MWCNTs-OH nanocomposite as MSPE adsorbent for the extraction of PAEs from water samples were investigated. In addition, to find the optimum condition, the variables that can influence the extraction efficiency were identified and studied in detail. Under optimized conditions, a rapid,

sensitive and cost-effective method for the determination of PAEs was established by coupling the developed MSPE technique with GC–MS instrumental analysis.

Experimental

Chemicals and reagents

A PAEs standard solution, containing six compounds [dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), di-*n*-octyl phthalate (DNOP) and bis(2-ethylhexyl) phthalate (DEHP) at 2.0 mg/mL in *n*-hexane], was obtained from Sigma–Aldrich (St. Louis, MO, USA). The phthalates stock solution, at a nominal 100 µg/mL concentration, was prepared in methanol. The phthalates working standard solutions were prepared by sequential dilution of the stock solution in methanol–water (50: 50, v/v). Benzyl benzoate (internal standard, IS) was added to each sample at a final concentration of 1 µg/L. The quality control (QC) samples were prepared from diluted stock standard solutions on the same day of the analysis. In order to safely keep and protect the solutions, all of them were kept at 4°C in the dark until analysis. All the other chemicals and solvents were of analytical reagent grade or better. Considering the fact that PAE compounds are existed in many of the laboratory products (e.g., chemicals and glassware), they may interfere with the analysis of PAEs in real samples. In order to minimize such interferences, all the laboratory glassware used in this study was immersed in acetone for at least 30 min, rinsed with *n*-hexane and then dried at 120°C for at least 4.0 h before use. The organic solvents were treated with aluminum oxide according to the previous reports (Prapatpong et al., 2010); 300 mg aluminum oxide was mixed with 10 mL solvents, and shaken for 30 s.

Samples

Bottled water samples were purchased from markets located in different regions of Tehran in October 2012. All the samples were stored at 4°C until analysis and subjected to analysis <4 months after their production date.

Instrumentation

Agilent gas chromatograph 6890 plus (Agilent Technologies, Palo Alto, CA, USA) equipped with a 5973 quadrupole mass spectrometer was applied for GC–MS analysis. The gas chromatograph was fitted with an HP-5 (MS) capillary column (30 m, 0.25 mm i.d., 0.25-µm film thickness). The instrumental temperatures were as following: injector temperature, 290°C and initial oven temperature, 50°C which was held for 1 min and then increased to 280°C at a rate of 30°C/min, and

finally increased to 310°C at a rate of 15°C/min held for 4 min. The inlet was operated in splitless mode. The temperature of the transfer line was maintained at 310°C. As carrier gas, helium (99.9999%) was used at 1 mL/min (constant flow). The source and quadrupole temperatures were kept at 230 and 150°C, respectively. The electronic beam energy of the mass spectrometer was set at 70 eV. The mass selective detector was operated in electron impact mode, using selected ion monitoring (SIM). The dwell time of each ion was set at 100 ms. The GC conditions were selected to minimize the analysis time while allowing all the analytes to elute in acquisition groups containing suitable number of ions for monitoring (Table 1).

Table 1. Selected Ions Used for the Quantification and Qualification of the Target Analytes by GC–MS (SIM Mode).

Ion group	Analyte	Time window (min)	Quantification ion (abundance) (*m*/*z*)	Confirmation ions (abundance) (*m*/*z*)
1	DMP	6.3–6.8	163 (100)	194 (15), 135 (15)
2	DEP	7.0–7.4	149 (100)	222 (15), 177 (28)
3	Benzyl benzoate (IS)	7.6–8.2	105 (100)	212 (40), 194 (35)
4	DBP	8.2–8.8	149 (100)	223 (6), 205 (6)
5	BBP	9.5–10.0	149 (100)	206 (21), 91 (72)
6	DEHP	10.0–10.6	149 (100)	279 (36), 167 (50)
7	DNOP	10.6–11.6	149 (100)	279 (18), 261 (10)

MSPE procedure

The magnetic PDMS/MWCNTs-OH particles were prepared based on a two-step reaction as described previously (Cao et al., 2008). The Fourier transform infrared spectroscopy (FTIR) spectrum of magnetic PDMS/MWCNTs-OH particles was illustrated in Figure 1. The typical absorption peaks of Fe_3O_4, OH-PDMS and MWCNTs-OH in the FTIR spectra of magnetic PDMS/MWCNTs-OH particles indicate that both OH-PDMS and MWCNTs-OH have bonded to the surface of Fe_3O_4 particles successfully. The similarity between acquired spectrum and the one reported for this composite makes it evident that the composite was synthesized successfully (Cao et al., 2008). The extraction of PAEs from water samples was conducted in consecutive steps. First, 10 mg magnetic PDMS/MWCNTs-OH particles were accurately weighed and activated with methanol and water separately in sequence. Then, the activated MPs and 1 g NaCl were added to the 10 mL of water sample.

The mixture was shaken vigorously for 4.0 min to extract the analytes. The magnetic adsorbent was gathered to the side of the vial (within ~90 s) with the aid of an external magnet. The supernatant was then discarded followed by addition of 2 mL acetone along with 2.0 min of vigorous vortex to elute PAEs from the adsorbent. Afterwards, the magnetic adsorbent was gathered to the side of the vial again. The desorption solvent was collected and evaporated to dryness at 40°C under gentle stream of nitrogen followed by reconstituting in 0.1 mL methanol for the subsequent GC–MS analysis.

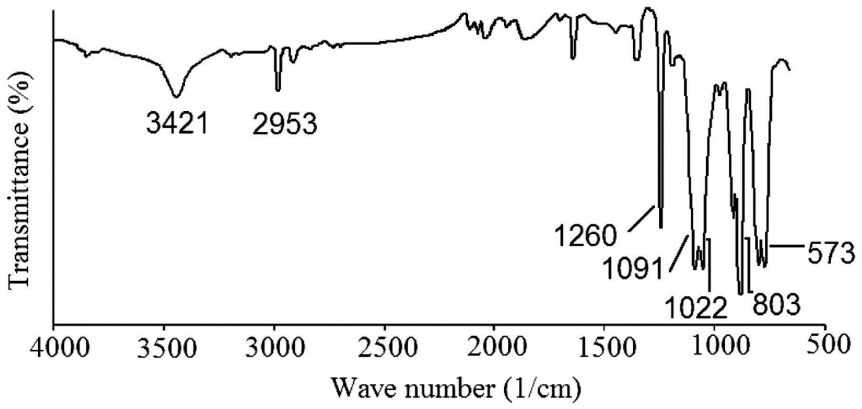

Figure 1. The FTIR spectrum of synthesized magnetic PDMS/MWCNTs-OH composite.

Method optimization

The method optimization was carried out based on "one factor at a time" protocol. In this procedure, the optimum conditions are determined by consecutive experiments in which all the influencing factors are kept constant except one and the remaining one is gradually modified to find the optimum value. After optimizing each factor, the experiment is repeated to find the optimum value for another factor, whereas studied factors are adjusted to their determined optimum values. Finally, the overall procedure is repeated while all the factors are adjusted to their determined optimum values. The method conditions will be optimal or near optimal if the value of determinant parameter (in this case "extraction yield") in the last experiment is the best between acquired values (24).

Method validation

Method validation was conducted in accordance with the currently accepted US Food and Drug Administration Guideline for Industry (Xu et al., 2010).

Results

Optimization of the extraction procedure

A certain amount of magnetic PDMS/MWCNTs-OH sorbents is required so as to have satisfactory recoveries of target compounds. Regarding this matter, some experiments were carried out by adding 0.5–2 mg/mL magnetic nanoparticles to each sample and as the results indicated (Figure 2A), an amount equal to 10 mg of nanoparticles was sufficient to extract the analytes from 10 mL of water sample. Based on the results, at this level the recoveries of target analytes were all over 90%. In addition, salting out effect on PAEs extraction efficiencies was assessed. Salting out effect has been well established in the previous works through adding different salts (mostly NaCl and Na_2SO_4) to the samples. Most authors are in agreement that salt addition positively affects the extraction efficiency in SPE. Therefore, a series of experiments [i.e., adding different amounts of NaCl and Na_2SO_4 (from 0 to 2 g) to the samples] were conducted to evaluate the effects of salt addition on the extraction efficiencies (Figure 2B). Based on the results, there were no significant differences between the salts and the highest extraction efficiencies were obtained by adding 1 g of NaCl to the sample. Therefore, the subsequent experiments were conducted using 1 g of NaCl per 10 mL of sample. Extraction time, which is the time that the magnetic PDMS/MWCNTs-OH particles were exposed to the water samples, had a significant effect on the recovery values of the extracted compounds. The extraction efficiency for all the analytes versus different exposure times of the sorbent to the sample, which was in the range of 0.5–6 min, is shown in Figure 2C. As it is evident, the optimum values for extraction times were obtained at 4 min. For the desorption process, two parameters need to be optimized including desorption solvent and desorption time. Therefore, four solvents, including acetone, methanol, ethyl acetate and *n*-hexane, were used as desorption solvent to evaluate their effects on the extraction efficiencies. The results indicated that the best extraction efficiencies were obtained by using acetone as desorption solvent (Figure 2D). In addition, the other experiments indicated that the highest extraction yield will be achievable if the volume of desorption solvent exceeds 1.5 mL. Therefore, 2 mL acetone was selected as desorption solvent for the following experiments. Also, the experiments with different desorption times from 0.5 to 6 min were conducted. The adequate extraction yields were obtained in 2 min as it is shown in Figure 2E.

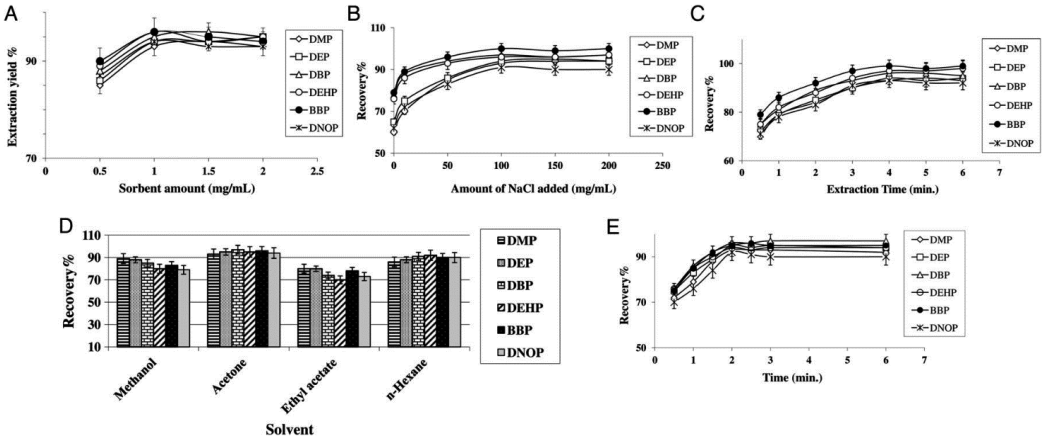

Figure 2. The effect of different influencing factors on extraction efficiencies: effect of sorbent amount in the sample solution (A), effect of salt concentration in the sample solution (B), investigation of the extraction time (C), optimization of desorption solvent (D) and investigation of the desorption time (E).

Method validation

The results of method validation are shown in Table 2. In addition, representative chromatograms of fortified and real samples are demonstrated in Figure 3.

Table 2. Estimated Recoveries, Accuracies and Precisions for Determination of the Analytes at Different Concentrations (n = 6).

Target compound	Samples	Nominal concentration (μg/L)	Mean of calculated concentration (μg/L)	RSD(%) of calculated concentration (interday)	RSD(%) of calculated concentration (intraday)	RE(%) of calculated concentration	Estimated recoveries (%)	RSD(%) of calculated recovery
DMP	QC_1	0.075	0.067	11.51	9.09	−10.67	91.5	10.21
	QC_2	0.250	0.229	10.43	7.53	−8.39	93.6	8.16
	QC_3	7.500	7.997	7.22	5.40	6.63	92.2	7.22
	QC_4	15.000	15.827	4.03	3.76	5.50	94.3	6.43
DEP	QC_1	0.075	0.068	12.40	11.18	−9.32	92.1	9.32
	QC_2	0.250	0.269	10.02	9.91	7.59	93.8	7.86
	QC_3	7.500	7.217	6.04	6.42	−3.76	93.7	6.72
	QC_4	15.000	15.757	4.44	5.09	5.05	95.6	6.13
DBP	QC_1	0.075	0.082	12.36	11.23	9.32	91.7	10.25
	QC_2	0.250	0.230	9.52	7.94	−8.00	94.7	9.74
	QC_3	7.500	7.181	6.22	6.42	−4.24	95.8	7.12
	QC_4	15.000	14.457	5.01	4.27	−3.61	97.8	6.63
BBP	QC_1	0.075	0.067	12.15	11.71	−10.67	92.3	9.87
	QC_2	0.250	0.230	8.85	7.85	−8.00	94.8	8.21
	QC_3	7.500	7.136	7.35	7.36	−4.84	95.2	8.14
	QC_4	15.000	14.417	6.22	6.29	−3.89	94.7	7.25
DEHP	QC_1	0.075	0.065	11.75	11.10	−13.32	91.8	10.64
	QC_2	0.250	0.225	9.87	8.82	−10.00	96.4	9.64
	QC_3	7.500	7.997	7.45	4.46	6.63	95.4	8.31
	QC_4	15.000	14.507	5.27	5.71	−3.29	95.8	6.67
DNOP	QC_1	0.075	0.084	11.92	9.09	12.00	93.1	9.54
	QC_2	0.250	0.277	9.43	7.53	10.79	94.6	8.95
	QC_3	7.500	8.057	6.66	6.40	7.43	93.9	7.94
	QC_4	15.00	15.907	4.56	5.46	6.05	93.4	6.51

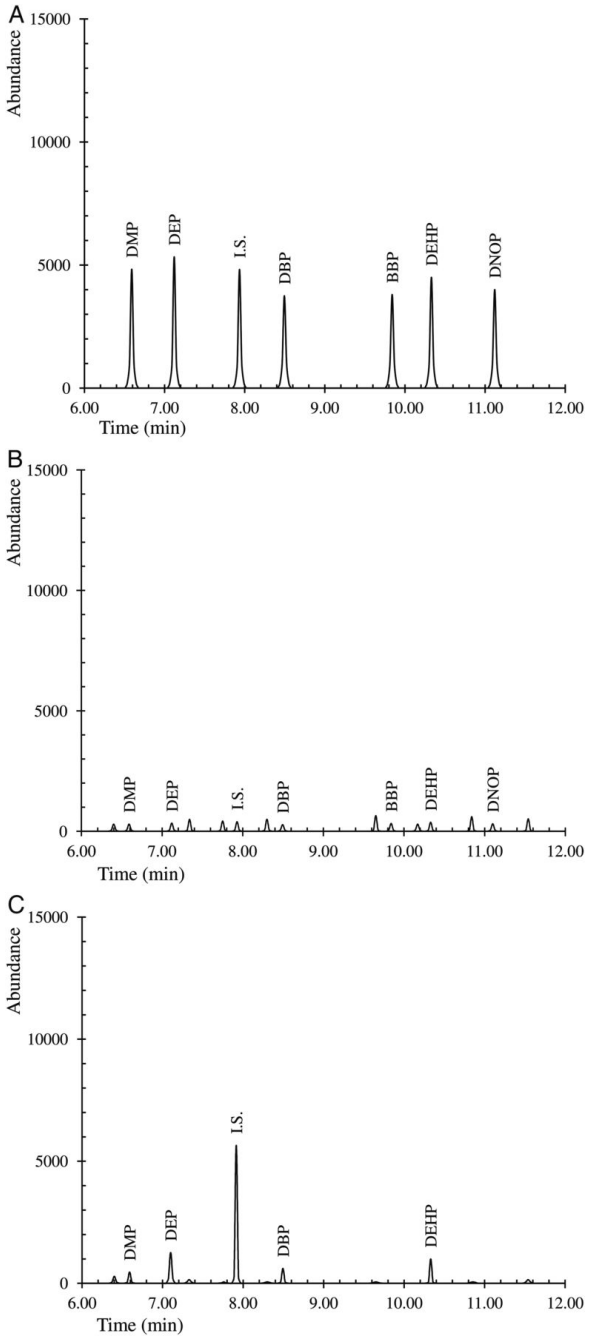

Figure 3. Representative MSPE–GC–MS chromatogram (SIM mode) of a QC sample, spiked with the analytes at 3 µg/L (A); a blank water sample spiked with the analytes at their LOQ levels (B) and one of the analyzed sample containing DMP (0.12 µg/L), DEP (0.66 µg/L), DBP (0.16 µg/L) and DEHP (0.43 µg/L) (C).

Discussion

Quantitative analysis

The linearity of the calibration curves was determined in the range of 0.05–20 μg/L. Coefficient of correlation ranged from 0.992 to 0.996. The LODs were defined as three times of the standard deviation of the baseline noise (n = 6) and determined by spiking serially diluted analyte standards into a blank water sample. According to the International Conference on Harmonization of Technical Requirements for Bioanalytical Methods (ICH) guideline for analytical method validation, for each analyte the limit of quantification (LOQ) was determined as the lowest concentration on the calibration curve with a precision of <20% coefficient of variation and an accuracy of 80–120% (Xu et al., 2010). The results showed that the LODs and LOQs for the target analytes ranged from 0.01 to 0.025 and 0.025 to 0.05 μg/L, respectively. The precision of the method was determined in terms of intermediate precision through calculating the analyte concentrations in QC samples, which were prepared at four levels (each six replicates) on 3 consecutive days. As it is shown in Table 2, interday precision values for the analytes were always <12.40%. The RSDs% of determined responses of six replicates of QC samples are taken for the expression of the repeatability (or intraday precision). The estimated recoveries at four different concentration levels are also listed in Table 2. To determine the recovery, mean peak area of each analyte at each concentration level was determined for a blank water sample spiked with the analyte (n = 6). The determined value was compared with the mean value obtained from spiking the same amount of the analyte in 100 μL methanol. All these results indicate the feasibility and reliability of the developed method for determining PAEs in water samples. The selectivity of the method was confirmed by analyzing 50 different water samples from different sources. There was no interfering peak in the region of the analytes and IS. A brief comparison of different analytical methods for the determination of PAEs was demonstrated in Table 3. As indicated by the LOQ and LOD values, the method developed in the current study is more sensitive than previous methods. It is also noticeable that the method is significantly more sensitive than our previous MSPE method for the determination of phthalate monoesters in aqueous sample (Xu et al., 2012). In that study, we used magnetic multiwall carbon nanotubes as MSPE adsorbent. The results of the present study indicate superior performance of developed magnetic PDMS/MWCNTs-OH composite over magnetic multiwall carbon nanotube for the extraction of phthalates from aqueous samples. Furthermore, the total analysis time (including sample preparation and instrumental analysis) of the developed method is ~0.5 h, which is considerably shorter than previous methods.

Table 3. Comparison of Different Analytical Methods for the Determination of PAEs in Water Samples.

Method	Matrix	Adsorbent	Estimated time of analysis (min)[a]	LOQ[b] or LOD[c] (μg/L)	Ref.
LLE–GC–MS	Water	LLE with dichloromethane	<120	LOD: range of LODs (0.02–0.05)	Amiridou et al., 2011
MSPE–GC–MS	Human urine	Magnetic multiwall carbon nanotubes	≤200	LOQ: range of LOQs (0.125–0.250) for phthalate monoesters	Xu et al., 2012
SPME–GC–MS	Water and urine	PDMS/divinylbenzene	≤60	LOQ: range of LOQs (0.3–8.6) for phthalate monoesters	Alzaga et al., 2003
SPE–HPLC–UV	Water	Poly(styrene–divinylbenzene)	<60	LOD: DEHP (0.1), BBP (0.05)	Jara et al., 2000
HS–SPME–GC–MS	Water	PDMS /divinylbenzene, PDMS, divinylbenzene/carboxen/PDMS	≤180	LOD: DNOP (0.003), BBP (0.085)	Cao et al., 2008
SPE–GC–FID	Water	C18, Florisil	<120	LOD: range of LODs (25–50)	Prapatpong et al., 2010
SPE–HPLC–UV	Water	Nylon6 nanofibers	≤60	LOD: DMP (3), DEP (2), DBP (6), DEHP (10) and DNOP (33)	Xu et al., 2010
MSPE–GC–MS	Water	Magnetic PDMS/MWCNTs-OH composite	≤30	LOD: range of LODs (0.01–0.025)	This study

[a]Total time required for sample preparation and instrumental analysis.
[b]Limit of quantification.
[c]Limit of detection.

Application to real samples

As mentioned in Introduction, PAEs represent an important class of endocrine disruptors and their presence in food and water has been intensively studied. However, due to insufficient method sensitivity, some of the PAEs, which are expected to be found in analyzed samples, were not determined in most of the previous studies and there are few reports about the simultaneous determination of all these six PAEs in a single analytical run. Therefore, to show the application of the developed method, some real bottled water samples were collected and analyzed. Determined concentrations of target PAEs in these samples were listed in Table 4, and one of the acquired chromatograms was shown in Figure 3C. As the results indicate, DMP, DEP, DBP and DEHP were detected in most of the samples. This could be due to the fact that the PAEs with a low-molecular weight (DMP, DEP and DBP) have larger water solubility and they are not bonded to the polymer chemically. Hence, these kinds of PAEs can easily migrate from the PET bottle into water (Cao et al., 2010). Also, DEHP was found in all bottled water. The reason could be that DEHP was used frequently as plasticizer and therefore its presence is expected (Cao et al., 2010). BBP and DNOP were found in fewer samples. The reason could be that these compounds are usually used as

plasticizer in flooring materials such as carpets, notebook covers and explosive materials. However, they are suspected of causing cancer and hence the manufacturers are under pressure to reduce using these compounds (Kondo et al., 2010, Liang et al., 2008). Although there are some differences, the overall results are in accordance with the previous results reported by other researchers (Amiridou et al., 2011, Liang et al., 2008; Cao et al., 2010). The results of real sample analysis confirm the applicability of the developed method for analysis of PAEs in water samples.

Table 4. Estimated Concentrations (μg/L) of Phthalate Esters in Analyzed Samples.

Samples	Container type	Water type	DMP	DEP	DBP	BBP	DEHP	DNOP
1	PET	Bottled	0.11	0.12	0.08	n.d.	0.23	n.d.
2	PET	Bottled	0.12	0.66	0.16	0.07	0.43	n.d.
3	PET	Bottled	0.05	0.17	n.d.	n.d.	0.15	n.d.
4	PET	Mineral	0.07	0.23	n.d.	n.d.	0.21	0.04
5	PET	Mineral	0.09	0.04	0.05	n.d.	0.52	n.d.
6	PET	Mineral	0.21	0.35	0.09	0.04	0.19	n.d.

n.d., not determined.

Conclusion

In this study, a new magnetic PDMS/MWCNTs-OH composite, which have good dispersibility in aqueous solutions, were used as MSPE adsorbents for the extraction of PAEs from water. The strong adsorption property makes magnetic PDMS/MWCNTs-OH composite an excellent candidate for serving as MSPE adsorbent. The developed method simplifies the sample preparation procedure since the adsorption and desorption processes are fast and the magnetic adsorbents can be rapidly separated from the sample solutions by applying an external magnet. This method could be a good alternative to conventional techniques for routine analysis due to its sensitivity, simplicity and reliability. The results showed that the established method possesses good performance in terms of limits of detection and LOQs, linearity, accuracy and reproducibility. Based on these features, the developed method is a useful tool for environmental monitoring of phthalate pollution as well as biological monitoring of exposure to phthalates through the determination of urinary phthalate concentrations.

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CHAPTER 3

Concentrations of phthalates in bottled water under common storage conditions: Do they pose a health risk to children?

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Abstract

Of recent concern is the migration of phthalates from plastic products such as Polyethylene Terephthalate (PET) bottles into the water contain. These concerns should be addressed, especially considering the steady growth of the consumption of bottled water and the toxicological effects of phthalates. In this regard, special attention should be paid to children's consumption because of their particular susceptibility to the effects of phthalates.

The aim of this study was to determine the concentrations of phthalates, including dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and bis (2-ethylhexyl) phthalate (DEHP), in bottled water and to estimate the health risk of endocrine disrupting chemicals due to water intake in children for the first time.

Migration of phthalates was investigated in PET-bottled water under various storage conditions using gas chromatography–mass spectroscopy. A phthalate exposure assessment was performed to characterize their risk to the children's health via a calculated hazard quotient (HQ).

It seems that increase in the temperature and the duration of storage affect phthalate migration, but the level of DEHP in bottled water was always very low and not exceed than

26.83% of the U.S. EPA maximum concentration limit (MCL). In particular, phthalate migration was not substantial at low temperatures (<25 ºC) and freezing conditions and for the most aboundant phthalate (DEHP) was not more than 10.6% MCL.

The estimated child intake ranged from 0.01 μg/kg/day for BBP to 0.24 μg/kg/day for DEHP.

Estimated phthalate intakes are generally in the safe range and exposure decreased with increasing age. Toxicological risk assessment of the maximum concentrations measured revealed a maximum HQ of 0.012 in the worst condition. Furthermore, a negligible carcinogenic risk of 6.5×10^{-7} for DEHP was observed. Consequently, risk evaluation showed that bottled water is safe for consumption by children.

Introduction

Food packaging is an integral part of today's lifestyle and food contact materials are a major source of chemical food contaminants (Muncke, 2009). Its applications cover a wide and variegated range, which is still growing. It has become routine for drinking water to be bottled and sold for human consumption, especially in areas where there is a lack of potable public water (Guart, Bono-Blay, Borrell, & Lacorte, 2014). Plastic packaging plays a very important role in this regard (Fasano, Bono-Blay, Cirillo, Montuori, & Lacorte, 2012).

Today, the most widespread water bottling material is Polyethylene Terephthalate (PET) (Bach et al., 2013). Over the last few decades, due to the low cost of PET production (Petrelli F, L, & M, 2006) ease of transport, size and strength, the consumption of PETbottled water has increased substantially worldwide and has effectively replaced tap water in several developed countries (Andra, Makris, & Shine; IBWA, 2009). Lately, due to the increasing popularity of bottled water consumption, questions have been raised about possible migration of chemical compounds from the bottles into the water and whether this poses a health risk to consumers? The diesters of 1,2-benzenedicarboxylic acid (phthalic acid), commonly known as phthalates, are a large group of man-made chemicals with versatile applications (Serrano, Braun, Trasande, Dills, & Sathyanarayana, 2014). These compounds are mainly employed as softening additives and, plasticizers in the production of plastic products (Singh & Li, 2011). With respect to health effects, phthalates include agents such as dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and bis (2-ethylhexyl) phthalate (DEHP), which are classified as endocrine disrupting chemicals (EDCs) or hormonallyactive agents (HAAs) because of their ability to interfere with the endocrine system and anti-androgenic or pro-estrogenic effects in the body (U.S. National Toxicology Program, 2007).

The impact of phthalate exposure on human health had been extensively reviewed and reported about by the National Toxicology Program — Center for the Evaluation of Risks to Human Reproduction (U.S. National Toxicology Program, 2007). There is strong evidence in rodents that exposure to phthalates causes developmental and reproductive toxicity (ATSDR, 2002; Martino Andrade & Chahoud, 2010; Meeker, Sathyanarayana, & Swan, 2009). In rodents, some phthalates, namely BBP, DBP and DEHP (Borch, Axelstad, Vinggaard, & Dalgaard, 2006; Foster, 2006; Gray et al., 2000; Howdeshell et al., 2007), have been identified as anti-androgens and reduction of testosterone in a dose-additive fashion in rats (Hannas et al., 2011; Howdeshell et al., 2008). They can modulate the endogenous production of fetal testicular testosterone and influence insulin-like factor 3 and folliclestimulating hormone production (Sharpe & Irvine, 2004), resulting in functional and structural impairment of male reproduction and development (Foster, 2006; Koch & Calafat,

2009; Tyl et al., 2004). Phthalates have been linked to adverse health effects particularly in relation to early life exposures and studies have shown that the most sensitive life stages are fetal > peri-pubertal > adult(Hauser & Calafat, 2005; Serrano et al., 2014).

A study by Singh et al. (2011) recently reported analyses of the toxicogenomics and possible adverse effects on human health of phthalate exposure. They reported that the top three phthalate (DBP, BBP and DEHP) toxicity categories were cardiotoxicity, hepatotoxicity and nephrotoxicity, and the top 20 diseases included cardiovascular, liver, urologic, endocrine and genital diseases (Singh & Li, 2011).

Existing exposure pathway assessments for phthalates have included consideration of food and water ingestion, soil and dust ingestion, dermal contact and inhalation (Schecter et al., 2013; Swan, 2008). Due to the complex chemistry of polymers several unknown substances can be incorporated in the final plastics material and potentially migrate into the food. These substances are the so-called NIAS ("non-intentionally added substances"). A declaration of conformity according to European regulation No. 10/2011 is required to ensure the safety of plastic materials in contact with foodstuffs (EU, 2011). Some compounds are subject to restrictions and/or specifications according to their toxicological data. However, over 50% of compounds migrating from food contact materials are NIAS (Bradley, Driffield, Harmer, Oldring, & Castle, 2008; Grob, Biedermann, Scherbaum, Roth, & Rieger, 2006). The European Commission and Chinese authorities have limited phthalates in food contact materials made of plastic since 2008–2009 (CFR, 2014a, 2014b; Petersen & Jensen, 2010) and they are not thought to be used in the manufacture of PET-bottles (ILSI, 2000). Despite this, the analysis of PET reveals some NIAS produced by authorized initial reactants and additives. Therefore, owing to the ubiquitous use of phthalates, phthalates as a NIAS (impurities) in PET-bottle materials can also migrate into bottled drinking water (Grob et al., 2006; Muncke, 2009; Skjevrak et al., 2005). Due to the lack of covalent bonding between the phthalate chemicals and their parent materials, they can be easily removed from plastic materials (Heudorf, Mersch-Sundermann, & Angerer, 2007; Kim et al., 2011; Liu H, 2008; Serôdio & Nogueira, 2006; Serrano et al., 2014). Consequently, the migration of phthalates into PET- bottled drinking water occurs and presents a current public health concern. Furthermore, several studies have demonstrated that the possibility of the presence of DBP, DEHP and BBP in bottled water is augmented by factors including storage duration, temperature change and sunlight (Table 1). Nevertheless, convincing explanations have never been offered because the origin of these compounds has not been clearly established (possible origins include the PET container, cap-sealing resins, background contamination, water processing steps, NIAS, recycled PET, etc.) (Bach, Dauchy, Chagnon, & Etienne, 2012).

Table 1. Bibliographic data on the content of phthalates in PET-bottled water.

Compound name	Type of water	Exposure temperature	Exposure conditions	Concentration range (µg/L)	Concentration mean (µg/L)	References
	Still water	-	-	0.08-0.32	0.357 ± 0.606	Cao et al., 2008
	Water	Up to 30°C	10 weeks	0.020-0.070	0.046	Casajuana and Lacorte 2003
DBP	Bottled water	4°C	1 month	<0.856	-	Al-Saleh, et al. 2011
	Bottled water	Outdoors	30 days	-	0.044	Amiridou and Voutsa 2011
	Still water	-	-	<0.085	-	Cao et al., 2008
	Water	Up to 30°C	10 weeks	<0.004 to 0.010	<0.004	Casajuana and Lacorte 2003
	Military packaged water	23.0 °C to 60°C	120 days	-	0.43	Greifenstein, et al. 2013
BBP	non-carbonate mineral water	22, 40, 50 and 60 °C	24, 48, 72 h	0.006-0.1	-	Keresztes Szilvia et al. 2013
	Bottled water	r.t.	2 month	0.581-2.69	-	Al-Saleh, et al. 2011
	Bottled water	4°C	1 month	1.94-21.128	4.592±3.081	Al-Saleh, et al. 2011
	Bottled water	40-45°C	3 month	0.315-3.520	-	Al-Saleh, et al. 2011
	Bottled water	Outdoors	30 days	-	ND	Amiridou and Voutsa 2011
	Dionised water	r.t.	17 h, darkness	0.14-0.24	0.19 ±0.05	Schmid, et al. 2008
	Dionised water	r.t.	17 h, sunlight	0.10-0.38	0.26 ±0.10	Schmid, et al. 2008
	Dionised water	60 °C	17 h, sunlight	0.15-0.71	0.36 ±0.21	Schmid, et al. 2008
	Still water	-	-	0.05-0.093	0.102 ±0.055	Cao et al., 2008
DEHP	Water	Up to 30 °C	10 weeks	<0.002 to 0.188	0.134	Casajuana and Lacorte 2003
	non-carbonate mineral water	22, 40, 50 and 60 °C	24, 48, 72 h	0.016-2.9	-	Keresztes Szilvia, et al. 2013
	Mineral water	>45 °C	3 months	<0.696-1.03	0.793±0.009	Al-Saleh, et al. 2011
	Mineral water	r.t.	2 months	<0.696-0.788	0.716±0.012	Al-Saleh, et al. 2011
	Mineral water	4 °C	1 month	<0.696-1.254	0.663±0.209	Al-Saleh, et al. 2011
	Military packaged water	23.0 °C to 60°C	120 days	0.47–0.60	-	Greifenstein, et al. 2013
	Bottled water	Outdoors	30 days	-	0.350	Amiridou and Voutsa 2011

DBP: dibutyl phthalate; BBP: benzylbutyl phthalate; DEHP: di (2- ethylbutyl phthalate; r.t.: room temperature; ND: Not Detected.

Up to now, most studies have been performed at temperatures above 25 ºC. However, there have been concerns in the general population regarding frozen bottles of drinking water or storage in outdoor conditions. In addition, some websites persuade the public not to use frozen bottles claiming the release of toxic compounds during freezing. Therefore, there is an increasing demand for more comprehensive studies to determine the role of possible factors in the migration of these toxicants into water in order to reduce concerns about the safety of PET-bottled water under common usage conditions. In fact, studies have demonstrated that children are more exposed to these environmental pollutants than adults because they consume more food and water per unit body weight (Dewalque, Charlier, & Pirard, 2014; U.S.EPA, 2002) which constitutes a matter of concern due to the potential vulnerability of this sub-population to the developmental problems and endocrine toxicity caused by phthalates (Gray et al., 2000).

Accordingly, to quantitatively assess the human risk of specific chemicals, the ratio between the level of exposure (e.g., estimate of daily intake) and an acceptable level of exposure for the same period (e.g., daily) is traditionally used. This ratio is sometimes referred to as the hazard quotient (HQ) (Kranich, Frederiksen, Andersson, & Jørgensen, 2013).

The purpose of this study was to describe for the first time the presence and concentration of DBP, DEHP and BBP in bottled water that has been frozen or kept in other common storage conditions in homes and retail stores. Moreover, for the first time, this study evaluated exposure to DBP, DEHP and BBP via consumption of bottled water under conditions of common use in infants and preschool children to determine their carcinogenic and non- carcinogenic effects, to resolve the concerns about consumption of PET-bottled water.

Material and methods

Chemicals and reagents

A phthalic acid esters (PAEs) standard solution, containing three compounds (dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), bis (2-ethylhexyl) phthalate (DEHP) at 2.0 mg/mLin n-hexane) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The phthalates stock solution, at a nominal 100 µg/mL concentration, was prepared in methanol. Benzyl benzoate (internal standard, I.S.) was added to each sample at a final concentration of 1 µg/L. In order to safely store and protect the solutions, all of them were kept at 4 °C in the dark until analysis. All the other chemicals and solvents were of analytical-reagent grade or better.

Since phthalates occur in many laboratory products (e. g., chemicals and glassware), they may interfere with the analysis of phthalates in real samples. In order to minimize such interference, all

the laboratory glassware used in this study was immersed in acetone for at least 30 min, rinsed with n-hexane, and then dried at 120 °C for at least 4.0 h before use. It was shown that phetalate contamination could be minimized adequately by this procedure (Jeddi, Rastkari, Ahmadkhaniha& yunesian, 2014; Rastkari &Ahmadkhaniha, 2013).

Samples collection and storage conditions

The focus of this study was to determine factors that influence the migration of three common phthalates including di-n-butyl phthalate (DBP), butyl benzyl phthalate (BBP) and bis (2-ethylhexyl) phthalate (DEHP) into bottled water. For this purpose, six brands of frequently consumed PET-bottled water (coded as A–G), were chosen. Twenty four bottles from each brands were purchased from factories immediately after production to confirm that the samples were stored in similar conditions before the experiment and to make sure that the source of phthalate esters in bottled water was the PET-bottle materials. Water samples were from identical batches. First, characteristics of the bottles and water were investigated. For this purpose, in one sample of each brand of bottled water, the amount of cations and anions were measured by Ion-Chromatograph. Other chemical properties of water including pH, total hardness and electrical conductivity were measured by the corresponding method. One sample of each brand was analyzed in terms of physical characteristics by evaluation of bottle weight and bottle wall thickness. Thereafter, each sample was individually analyzed for DBP, DEHP and BBP at various intervals, immediately after production and during storage. Samples were stored in six different conditions for forty five days: outdoors (directly exposed to sunlight during the period November-February 2012. Cloudy, rainy and snowy days were not considered. The minimum and maximum temperatures were 12-26.5°C) and at different temperatures [-18°C (freezing), 0°C, 4 to 8°C (refrigerator), 25°C (room temperature) and 40°C] in 0.5-L PET containers. Finally, the release of DBP, DEHP and BBP was measured in the first 24 hours and on day 10, day 30, and day 45 in each storage condition to compare the obtained residual levels with regulatory safe levels in order to evaluate the safety of PET bottled water use. Therefore, six bottles (one for each brand) were analysed in each storage condition. Replicate experiments for some of sample were employed. To ensure the results are representative, some of the samples were analyzed in triplicate.

Instrumental analysis

Agilent gas chromatograph 6890 plus (Agilent Technologies, Palo Alto, CA, USA) equipped with a 5973 quadruple mass spectrometer was applied for GC-MS analysis. The gas chromatograph was fitted with an HP-5 ms capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The

instrumental temperatures were as follows: injector temperature, 290 °C; initial oven temperature, 50 °C which was held for 1 min and then increased to 280 °C at a rate of 30 °C/min, and finally increased to 310 °C at a rate of 15 °C/min held for 4 min. The inlet was operated in splitless mode. The temperature of the transfer line was maintained at 310 °C. As carrier gas, Helium (99.9999%) was used at 1 mL/min (constant flow). The source and quadrupole temperatures were respectively kept at 230 and 150 °C. The electron beam energy of the mass spectrometer was set at 70 eV. The mass selective detector was operated in electron impact (EI) mode, using selected ion monitoring (SIM). The dwell time of each ion was set at 100 ms. The GC conditions were selected to minimize the analysis time while allowing all the analytes to elute in acquisition groups containing suitable number of ions for monitoring.

MSPE procedure

The magnetic PDMS/MWCNTs-OH particles were prepared based on a two-step reaction as described in (Xu, Jiang, Lin, & Jia, 2012). The typical absorption peaks of Fe3O4, OH-PDMS and MWCNTs-OH in the FTIR spectra of magnetic PDMS/MWCNTs-OH particles indicates that OH-PDMS, MWCNTs-OH have both been bonded successfully to the surface of Fe3O4 particles . The extraction of PAEs from water samples was conducted in consecutive steps. First, 10 mg magnetic PDMS/MWCNTs-OH particles were accurately weighed and activated with methanol and water separately in sequence. Then, the activated MPs and 1 g NaCl was added to the 10 mL of water sample. The mixture was shaken vigorously to extract the analytes for 4.0 min. The magnetic adsorbent was gathered to the side of the vial (within ~90 s) with the aid of an external magnet. The supernatant was then discarded followed by addition of 2 mL acetone along with 2.0 min of vigorous vortex to elute phthalates from the adsorbent. Afterwards, the magnetic adsorbent was gathered to the side of the vial again. The desorption solvent was collected and evaporated until dry at 40 °C an under gentle stream of nitrogen followed by reconstitution in 0.1 mL methanol for the subsequent GC-MS analysis.

Method validation

Method validation was conducted in accordance with the currently accepted U.S. Food and Drug Administration Guideline for Industry (USFDA, 2012). Under optimized conditions, the limits of detections (LODs) and limits of quantifications (LOQs) achieved were in the range of 0.01-0.025 and 0.025-0.05 µg/L respectively.

Daily phthalate intake and hazard index

Two methods are used for assessing overall human exposure to phthalates. One is risk assessment through the exposure assessment of specific environmental media, such as food and water, and behavioral assessments. The second is biomonitoring of phthalate or phthalate metabolite levels in human fluids and calculating exposures based on these analyses. In this study, the first method was used for assessing exposure to phthalates through drinking water. Exposure modelling was carried out by combining information on: (1) the levels of phthalates in the environment, e.g., food, water, toys, air, etc., and (2) human behaviors, e.g., the amount of food ingested, water intake, etc,. Based on this information, the amount of exposure through each route, as well as total exposure through all routes, may be calculated (Kamrin, 2009).

Daily dietary intakes of phthalates in the target population [infants (0-6 and 7 -12 months), toddlers (1-3 years old), and preschool children (4-6 years old)] were estimated from the water consumption rate and concentration of phthalates in water as in the following formula (De Fátima Poças & Hogg, 2007; Schecter et al., 2013).

$EDI = MC \times Water\ Consumption$

Where:

EDI = Estimated daily intake via drinking water (µg/kg body weight/day).

MC= the maximum concentration values of DBP, BBP and DEHP investigated in the bottled water samples in the present study (µg/L).

Water Consumption= daily drinking-water requirement in the target groups based on body weight. This estimate is provided in units of liters per kilogram of body weight per day (L/kg body weight /day) (Table 2). The daily drinking-water requirement for children were those recommended by the 2011 Exposure Factors Handbook (EFH) based on the U.S. EPA analyses of National Health and Nutrition Examination Survey (NHANES) 2003-2006 data and Panel on Dietary Reference Intakes for Electrolytes, & Water by Institute of Medicine (US) (Medicine, 2005; U.S.EPA, 2011). Also, in the present paper, body weights are default recommended by World Health Organization (Table 2) (WHO, 2007).

Table 2. The parameters use for calculation of HQ in different age groups.

Age	Mean body weight (kg)	Mean Water requirement (Liter/day)	Water Consumption (Liter/kg body weight /day)
Infants			
1-6 month	5.4	0.75	0.15
7-12 month	8.6	1	0.12
Children			
toddlers (1-3 years old)	11.8	1.5	0.124
preschool children (4-6 years old)	16.3	1.8	0.108

Afterward, daily intakes of non-carcinogenics were estimated, non-carcinogenic hazard was measured in terms of a Hazard Quotient (HQ) based on comparisons of oral exposure estimates to RfD values which are calculated for each target group along with toxicological reference values for each phthalate as follows:

$$HQ = \frac{EDI}{RfD}$$

Where:
HQ = Hazard Quotient associated with the exposure via the specified exposure route (unitless).
EDI = Estimated daily intake via drinking water (μg/kg body weight/day).
RfD = Reference dose (μg/ kg body weight /day).
For the acceptable level of exposure to phthalate, the Integrated Risk Information System (IRIS) of the U.S. EPA's proposed value of Reference Doses for chronic oral exposures (RfD) based on chronic health hazard assessment for non-carcinogenic effects was estimated in order to evaluate non-cancer risk from oral exposure.
An HQ of 1 or less in the event that only one contaminant and/or exposure route was assessed, indicates that the receptor's exposure is equal to or less than an "allowable" exposure level, and adverse health effects are considered unlikely to occur.
Additionally, there are several ways to be exposed to phthalates, tolerable daily intake [TDI; μg/kg body weight/day] was calculated for all routes of exposure. On the other hand, consumption of the chemical contaminant with drinking water should only contribute to less than 20% of the TDI (SCHER, 2010). Among the chemicals, guideline derivation allocation to DEHP in water was 1% of TDI (WHO, 2003). Therefore, the contribution of the daily intake of these compounds via consumption of drinking water was estimated as follows:

$$\text{Contribution via drinking water} = \frac{EDI}{TDI} \times 100$$

Furthermore, among the phthalates considered, DEHP is the only probable human carcinogen (IARC, 2013). The reference carcinogenic unit risk from drinking water is 4.0×10-7 per μg/L (EPA, 2009). Unit risk (UR) is the upper-bound excess lifetime cancer risk estimated to result from continuous exposure to an agent at a concentration of 1 μg/L in water. The excess lifetime cancer risk due to water consumption was calculated in all storage conditions using the current standard as follows:

ELCR = Drinking Water Unit Risk × MC

Where:

ELCR = Excess Lifetime Cancer Risk associated with exposure to the chemical via the specified route of exposure (unitless).
Drinking Water Unit Risk = The unit risk is the quantitative estimate in terms of either risk per μg/L drinking Water (4.0×10^{-7} per μg/L, 4 excess cancer cases (upper bound estimate) are expected to develop per 10,000,000 people if exposed daily for a lifetime to 1 μg of the chemical in 1 liter of drinking water).
MC= Maximum concentration of DEHP for each storage condition (μg/L).

Excess Lifetime Cancer Risk less than 1 in million is typically considered negligible or minimum ($ELCR<10^{-6}$).

Statistical Analysis

SPSS (version 20.0) was employed for statistical analysis of the results. Means and standard deviations for the concentrations of various phthalate esters (DBP, DEHP and BBP) in bottled waters are reported. Before performing this statistical analysis, normality of concentrations in each group was checked using the Kolmogorov-Smirnov test. Because the distribution of data was not normal, we used nonparametric the Kruskal-Wallis H test for comparing concentrations from different storage conditions. A *P*-value of less than 0.05 was considered to indicate statistical significance. The concentrations from the exposure periods were compared using the Friedman test.

Results

The results of the present study showed that storage conditions and time are factors that may, to some extent, explain the release of phthalates from PET-bottles into water. Information on bottles and water characteristics are presented in Table 3. The results of the analyses of bottled water before storage (immediately after production) showed that the initial levels of DEHP and DBP were very low while BBP was not found at detectable levels in PET-bottled water prior to storage (Table 4). The effect of various storage conditions on the migration of DBP, DEHP and BBP into PET-bottled water regardless of their exposure periods are summarized in Table 4. Also, the results of the Kruskal-Wallis test for comparing concentrations in the six conditions based groups are reported. The concentration of DBP, DEHP and BBP in all water samples categorized by their storage conditions indicated that BBP was not detected at low temperatures (under the 25°C).

Table 3. Characteristics of the examined bottled waters.

	Brand of bottled water*					
	A	B	C	D	E	G
Bottle characteristics						
Bottle type	PET	PET	PET	PET	PET	PET
Color[a]	CL	CL	LB	CL	LB	CL
Resin identification code	1	1	1	1	1	1
Bottled Thickness (mm)	0.19-0.24	0.18-0.25	0.18-0.23	0.18-0.25	0.18-0.25	0.17-0.24
Weight (gr)	15.67	16.54	16.58	18.38	16.45	17.16
Volume (ml)	500	500	500	500	500	500
Water characteristics						
Water type[b]	NMW	NMW	NMW	BDW	BDW	BDW
PH	7.71	6.35	7.78	6.31	7.97	7.11
EC (µS/cm)	610	96.8	562	210	247	118.8
Hardness (mg/L)	150.5	16.5	143	28.5	60	19.5
TDS (mg/L)	360	57	331	124	146	66
Cl^- (mg/L)	31.100	0.747	14.020	24.246	1.425	2.248
NH_4^- (mg/L)	-	-	-	-	0.028	-
F^- (mg/L)	2.441	0.235	0.257	-	0.220	0.086
Na^+ (mg/L)	11.716	4.494	14.062	15.936	1.303	8.278
K^+ (mg/L)	0.957	1.988	1.324	1.289	0.488	0.409
Ca^{2+} (mg/L)	101.990	14.301	83.665	1.782	46.948	19.445
Mg^{2+} (mg/L)	23.690	2.814	26.826	17.292	10.030	5.112
SO_4^{2-} (mg/L)	27.557	0.399	34.766	66.392	4.323	21.583
NO_3^- (mg/L)	12.126	3.830	15.509	0.254	3.835	4.750
NO_2^- (mg/L)	0.057	0.117	0.062	-	0.072	0.030

*Brands were named in this work by using A, B, …G)

[a] CL, clear; LB, light blue.

[b] NMW, natural mineral water; BDW, bottled drinking water.

Although the Kruskal-Wallis test showed significant differences between storage conditions, posthoc results only revealed a difference between 40°C and the other conditions. A pronounced increase in the concentration of phthalates was observed at 40°C after exposure periods (24 hr, 10, 30, 45 days). Total increases of 349.1% (DEHP), 935.8% (DBP) and 333.0% (BBP) were observed at 40°C over the initial level before storage (bottled water immediately after production) (Table 5). DEHP and DBP values in the freezing conditions of 0°C and -18°C beyond their initial amounts before storage increased 52.1%, 187.8%, 59.6%, and 251.4% respectively. BBP was not detected in bottled water before storage or after storage in freezing conditions (Table 5). In addition, the average increment in DEHP concentration at 40°C, the worst condition was two times higher than that measured at -18°C, and 1.19 times higher than that at refrigerator temperatures.

Measurements for DBP in the same conditions were 9.56 and 3 times higher, respectively. BBP concentrations increased by 275% and 40% at 40°C compared to room temperature (25°C) and outdoor, respectively. Besides temperature, duration of exposure affected phthalate migration into water.

Table 4. Result of phthalates migration (mean±SD) in µg/L from PET-bottles into water under different storage conditions.

Storage conditions	n	DBP	DEHP	BBP
Control*	12	0.135±0.078	0.217±0.092	<LOD**
Outdoor condition	24	0.114±0.088	0.418±0.196	0.043±0.018
40 °C	24	0.303±0.172	0.917±0.342	0.063±0.031
Room (25 °C)	24	0.116±0.095	0.411±0.161	0.020±0.004
Refrigerator (4-8 °C)	24	0.124±0.099	0.423±0.150	<LOD
Zero (0 °C)	24	0.088±0.080	0.331±0.147	<LOD
Freezing (-18 °C)	24	0.079±0.089	0.317±0.124	<LOD
P-value		<0.001	<0.001	<0.001

* Before storage immediately after production.
** Limit of Detection.

Table 5. Increasing the concentration of phthalates (%) in different storage conditions relative to the initial level before storage and after storage at 25 ° C in several time intervals.

Phthalates	Storage conditions	Before storage	After Storage at 25 °C			
			24 hr	10 days	30 days	45 days
DBP	Storage at -18°C	187.8	-3.2	-25.8	-49.2	-54.6
	Storage at 0°C	251.5	-8.5	-21.2	-41.7	-14.3
	Storage at 4-8°C	349.1	-5.5	2.0	14.9	2.4
	Storage at 40°C	935.8	207.5	415.2	339.5	428.5
	Outdoor	301.4	3.2	-15.9	61.6	29.3
DEHP	Storage at -18°C	52.1	4.3	-16.4	-32.8	-34.0
	Storage at 0°C	59.6	3.9	-18.0	-29.8	-28.7
	Storage at 4-8°C	105.9	4.8	17.5	2.4	-4.0
	Storage at 40°C	349.1	92.4	131.4	129.2	152.0
	Outdoor	100.9	-2.1	-17.12	9.2	8.3
BBP	Storage at -18°C	0.0	0.0	0.0	-9.5	-19.1
	Storage at 0°C	0.0	0.0	0.0	-9.5	-19.1
	Storage at 4-8°C	0.0	0.0	0.0	-9.5	-19.1
	Storage at 40°C	333.0	49.3	234.7	386.4	428.5
	Outdoor	108.5	0.0	0.0	126.2	218.1

The Friedman test showed a significant difference (p<0.001) between time periods of measurements (After 1 day, 10 days, 30 days and 45 days of storage), post-hoc results showed that DEHP and DBP concentrations had significant differences between all time intervals, except the first day with the tenth day. Results of BBP monitoring showed that significant differences observed only between the tenth day with the forty fifth day and the first day with the forty fifth day. According to our findings after 45 days of storage in different conditions, a significant increase was observed in the concentration of phthalates (Figure 1). In other words, as the storage time prolonged, regardless of the storage condition and brand of the bottled water, concentrations of phthalate esters increased (Figure 1). In addition, no significant correlation was found between the phthalate concentrations and the physicochemical properties of the different brands of water samples (Table 2).

This study collected information on target substances and the exposure scenarios of preschool children and infants and computed their HQs; these levels are shown in Table 6. Risk assessment for DBP, DEHP and BBP in PET-bottled water is based on an exposure scenario using the estimated water requirements in each age group, with the assumption that they meet their needs (Sylvia & L-Katleen, 2012) and that the maximum concentration of DBP, DEHP and BBP found in the various storage conditions is present.

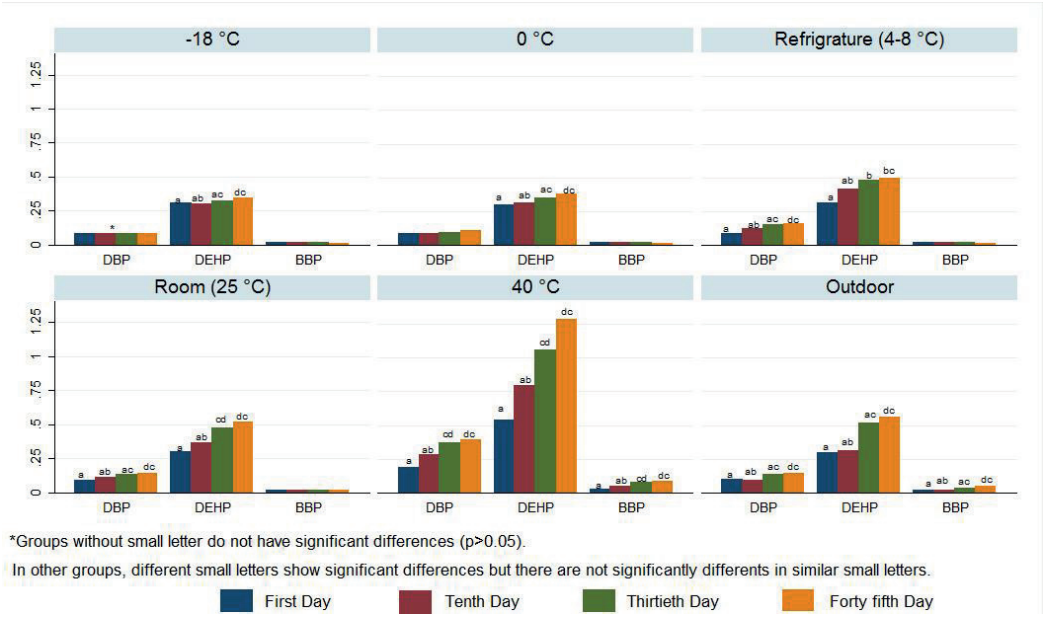

Figure 1. The effect of time duration (24 hr, 10 days, 30 days and 45 days) at different storage conditions on phthalate concentrations (µg/L) in PET-bottled water.

Based on the migration data from bottled water, the EDI of infants (0-6 and 7-12 months), toddlers (1-3 years old), and preschool children (4-6 years old) to DEHP from PET water bottles at 40 ºC was estimated to be 0.240, 0.218, 0.20 and 0.174 µg kg/body weight/ day, respectively. After considering the daily exposure of phthalates in the target group of children and calculating the Hazard quotients of non-carcinogenic toxic phthalates. HQs were calculated to be far less than one in all scenarios, and then no adverse health effects are expected as a result of exposure to phthalate via water intake (Table 6).

When looking at the contribution of water to exposure to phthalate according to daily intake, the infant and young children phthalate intake did not exceed 0.5% of the TDI for DEHP and thus satisfied the allocation level to drinking water (1% of the respective TDI).

The highest contribution of an individual phthalate to the TDI was for DBP (1.1% of the TDI), that it was far below the 20% of the TDI for consumption of the chemical contaminant with drinking water (SCHER, 2010).

According to the present results, the cancer risk due to DEHP exposure via water intake stored at 40 ºC was greater than for the other storage conditions. The extra risk of cancer associated with lifetime exposure to DEHP in drinking water at this worst condition was 6.5 in 10,000,000.The least carcinogenic risk (2.4×10^{-7}) from DEHP was observed for the bottled water stored under freezing conditions (-18 ºC).

Table 6. Estimation of exposure to phthalates via consumption of bottled water after 45 days storage under the various storage conditions in infant and children.

	Storage at 40°C			Storage at 25°C			Storage at 4-8°C			Storage at 0°C			Storage at -18°C			Storage at Outdoor		
	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP
Detected maximum concentration [µg/L]	1.610	0.702	0.130	0.711	0.328	0.250	0.687	0.375	-	0.678	0.248	-	0.589	0.274	-	0.802	0.302	0.071
EDI[a]																		
Infant																		
0-6 month	0.24	0.11	0.02	0.11	0.05	0.04	0.10	0.06	-	0.10	0.04	-	0.09	0.04	-	0.12	0.05	0.01
7-12 month	0.22	0.10	0.02	0.10	0.04	0.03	0.09	0.05	-	0.09	0.03	-	0.08	0.04	-	0.11	0.04	0.01
Children																		
Todler:1-3yr	0.20	0.09	0.02	0.09	0.04	0.03	0.09	0.05	-	0.08	0.03	-	0.07	0.03	-	0.10	0.04	0.01
Preschool:4-6yr	0.17	0.08	0.01	0.08	0.04	0.03	0.08	0.04	-	0.07	0.03	-	0.06	0.03	-	0.09	0.03	0.01
MCL[b] (WHO 2008, EPA 2009)	8/6	-	-	8/6	-	-	8/6	-	-	8/6	-	-	8/6	-	-	8/6	-	-
Calculated safety factor (WHO /EPA)	5/3.7	-	-	11.3/8.4	-	-	11.6/8.7	-	-	11.8/8.8	-	-	13.6/10.2	-	-	9.9/7.5	-	-
TDI[c] (EFSA 2013a, EFSA 2013c, EFSA 2013b)	50	10	500	50	10	500	50	10	500	50	10	500	50	10	500	50	10	500
Contribution via drinking water (%)																		
Infant																		
0-6month	0.50	1.10	0.004	0.22	0.50	0.008	0.20	0.60	-	0.20	0.40	-	0.18	0.40	-	0.24	0.50	0.002
7-12month	0.44	1.00	0.004	0.20	0.44	0.007	0.19	0.51	-	0.18	0.34	-	0.16	0.37	-	0.22	0.41	0.002
Children																		
Todler:1-3yrs	0.40	0.90	0.003	0.18	0.41	0.006	0.17	0.47	-	0.17	0.31	-	0.15	0.34	-	0.20	0.37	0.002
Preschool:4-6yrs	0.35	0.80	0.003	0.16	0.36	0.005	0.15	0.41	-	0.15	0.27	-	0.13	0.30	-	0.18	0.33	0.002

Table 6. Continue.

	Storage at 40°C			Storage at 25°C			Storage at 4-8°C			Storage at 0°C			Storage at -18°C			Storage at Outdoor		
	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP
Risk characterization																		
RfD[d] (EPA 2009)	20	100	200	20	100	200	20	100	200	20	100	200	20	100	200	20	100	200
HQ[e]																		
Infant																		
0-6months	0.012	0.001	0.0001	0.006	0.0005	0.0002	0.005	0.0006	-	0.005	0.0004	-	0.005	0.0004	-	0.006	0.0005	5×10^{-5}
7-12months	0.011	0.001	0.0001	0.005	0.0004	0.0002	0.005	0.0005	-	0.005	0.0003	-	0.004	0.0004	-	0.006	0.0004	5×10^{-5}
Children																		
Todler:1-3yrs	0.01	0.0009	8×10^{-5}	0.005	0.0004	0.0001	0.004	0.0005	-	0.004	0.0003	-	0.004	0.0003	-	0.005	0.0004	4.5×10^{-5}
Preschool:4-6yrs	0.009	0.0008	7×10^{-5}	0.004	0.0004	0.0001	0.004	0.0004	-	0.004	0.0003	-	0.003	0.0003	-	0.005	0.0003	4×10^{-5}
Drinking water unit risk[f]	4.0×10^{-7}	-	-	4.0×10−7	-	-	4.0×10−7	-	-	4.0×10−7	-	-	4.0×10−7	-	-	4.0×10−7	-	-
ELCR[g]	6.5×10^{-7}	-	-	2.8×10-7	-	-	2.7×10-7	-	-	2.7×10-7	-	-	2.4×10-7	-	-	3.2×10-7	-	-

[a] Daily intake via drinking water (EDI: µg/kg body weight/ day)

[b] Maximum concentration limit (MCL) basis of WHO drinking water Guidelines value (µg/L)/ U.S.EPA standard (µg/L)

[c] Tolerable daily intake (µg/kg bw)

[d] IRIS RfD chronic non-carcinogenic effect risk (RfD, µg/kg bw/day)

[e] Hazard Quotient (HQ)

[f] U.S.EPA Drinking water unit risk of carcinogenic (per µg/L)

[g] Excess Lifetime Cancer Risks (ELCR)

Discussion

The aims of this study were (1) to estimate the influence of common storage conditions of bottled water in homes and retail stores on phthalate esters' migration and (2) to assess the safety of exposure to phthalates from daily water intake in infants and children by comparing their intakes to well-recognized reference values. The presence of phthalates in bottled water immediately after production in the factory can be attributed to (a) water contamination in the bottling plant (Amiridou & Voutsa, 2011; Schmid, Kohler, Meierhofer, Luzi, & Wegelin, 2008), (b) contamination in water treatment facilities and, (c), the existence of phthalates in the source of the water (ground water or tab water) used to fill in the PET-bottles. Nevertheless, after 45 days of storage in different conditions an increase was observed in the amount of phthalates in the bottled water samples. Comparing the results of analyses of DBP, DEHP and BBP concentrations in bottled water before and after storage, the present study concluded that poor storage conditions cause an increase in the concentrations of DBP, BBP and DEHP in bottled water. Similarly, in the study conducted by Casajuana and Lacorte (2003), levels of phthalates in the initial water samples in PET, PE, and glass containers were below or close to the detection limits. Slightly higher levels of phthalates were detected in these samples after storage for 10 week outdoors, with the highest average concentrations being 0.003, 0.432, 0.046, 0.196 μg/L for DMP, DEP, DBP, DEHP, respectively (Casajuana & Lacorte, 2003).

In most previous studies, bottled waters was purchased from retail stores or initial levels of phthalates were not measured before storage (Al-Saleh, Shinwari, & Alsabbaheen, 2011; Amiridou & Voutsa, 2011; Keresztes Szilvia , Enikő, Czégény Zsuzsanna, & Victor, 2013); therefore, previous poor storage and exposure of these samples with environmental factors (e.g., high temperature, outdoors, etc.) from production up to purchase cannot be excluded. As a result, one could not see the net effect of each storage condition on phthalates migration. Due to the aforementioned points, it is unsure to demonstrate the reason behind the presence of phthalates in the bottled water; hence, in the present study, the increase observed in phthalate concentrations was due to migration from the PET materials in different storage conditions. Furthermore, the presence of phthalates in the bottles material confirms the results (Zare Jeddi, Yunesian, Ahmadkhaniha, Karimi, & Rastkari, unpublished results).

In accord with other studies (Table 1), our findings showed that high temperatures caused phthalates migration. Similarly, the presence of DEHP in bottled water was reported by other studies wich found that it was the most abundant phthalate in bottled water under all storage conditions (Amiridou & Voutsa, 2011; Greifenstein, White, Stubner, Hout, & Whelton, 2013; Keresztes Szilvia et al., 2013; Schmid et al., 2008). In contrast, Saleh et al. (2011) found that BBP

was the most abundant phthalate ester in three storage conditions (4 °C, room temperature, outdoor conditions) and the highest values (4.592±3.081 µg/L) were detected at 4 °C (Al-Saleh et al., 2011). The results of this investigation (Saleh et al. 2011) showed that the leaching pattern of phthalates among Saudi Arabian bottled water is somewhat different from that reported in other studies. The concentration of BBP in bottled water at low and high temperature is generally much higher than that reported in the present study in Iran and other countries (Amiridou & Voutsa, 2011; Cao, 2008; Keresztes Szilvia et al., 2013).

In the field of drinking water, the WHO and U.S.EPA only set a maximum permitted level for DEHP (EPA, 2009; WHO, 2008). The concentration of DEHP in all tested conditions was similar to that found by previous studies (Table1), which was always far below the maximum contaminant level (MCL) determined by U.S. EPA standards and WHO guidelines (6/8 µg/L respectively). It should be noted that the U.S. EPA, under the Safe Drinking Water Act, has defined the maximum contaminant level goal (MCLG) for di(2-ethylhexyl) phthalate (DEHP) in water as zero. The U.S. EPA has set this goal based on the best available science to prevent potential health problems. MCLs are set as close to the health goals as possible, considering cost, benefits and the ability of public water systems to detect and remove contaminants using suitable treatment technologies. This is consistent with previous studies described in Table 1, the amount of DEHP is within safe limits even in the worst case (Table 1). To the best of our knowledge, the present study is the first to conclude that phthalate migration under freezing conditions is not substantial and that this storage condition is not sub optimal storage conditions for consumption. Among the different storage conditions, storage at 40°C and storage at -18°C resulted the highest and lowest migrate respectively. However, phthalates migration in all storage conditions is negligible. Although the detected concentrations were far below toxic levels, considering the widespread consumption of bottled water in developed and developing countries, it was prudent to determine the risk to children and infants as susceptible groups. Data on the assessment of phthalate exposure via water intake for children is scarce; however, some researchers have investigated phthalate exposure through other routes. For instance, Wormuth et al. (2006) estimated total phthalate intake for Europeans using a multipathway approach. Infants and toddlers experienced higher daily exposure to all phthalates than teenagers or adults. Their maximum exposure to DEHP was higher than 100 µg/kg bodyweight/day, mainly by mouthing of soft plastic toys and from the ingestion of food and dust. The maximal exposure predicted in their scenarios was considerably higher than the tolerable daily intake (TDI) for DEHP (Wormuth, Scheringer, Vollenweider, & Hungerbuhler, 2006).

Our results show that children's phthalate exposure through water reduces with increasing age, since children's water intake is higher campared to their body weight.

However, phthalate exposure through water intake was extremely low (0.002 - 1.1 % TDI) and was considered safe even for the worst storage conditions. As shown in Table 6, the calculated HQ for DEHP, DBP and BBP in water at 40ºC was higher compared to the other storage conditions, but even in these conditions, the HQ is smaller than 1in vulnerable groups, therefore, there is no risk.

Clark et al. (2003), in an assessment of exposure pathways, reported that generally drinking water represented less than 0.2% of exposure to phthalates (i.e. DEP, DBP, and DEHP), except for formula-fed infants in whom ingestion of drinking water accounted for higher contributions (0.7% for DEHP, 2.9% for DBP and 21.4% for DEP) (Clark, 2003).

Our data demonstrated that the intake of individual phthalates via drinking water was much lower than published RfD benchmarks (Table 6); for example, the currently published RfD for DEHP on U.S. EPA's IRIS database is 20 µg/kg/day (U.S. EPA, 2012c), whereas our value for the intake of DEHP in infants (the most vulnerable group) by consumption of water kept in the worst conditions (40 ºC) was only 0.24µg/kg/day (1.2% RfD). However, the U.S. EPA also states that not all doses below the RfD are acceptable, but that all doses in excess of the RfD are unacceptable or will result in adverse effects. In addition, it is important to note that RfDs pertain to all pathways of exposure. Furthermore, regulatory values like EFSA TDIs and U.S. EPA RfDs are normally established in the perspective of the average adult. Children weigh were less than adults and thus experience higher relative intake of chemicals provided the absolute intake is the same. This leads to higher risks when EFSA TDI values, U.S. EPA RfDs, or similar regulatory values are used for assessing the risk for children (Søeborg, Frederiksen, & Andersson, 2012).

Furthermore, according to The International Agency for Research on Cancer (IARC) among all phthalate esters only DEHP is possibly carcinogenic to humans (Group 2B), (U.S. EPA, 2012c). Our results demonstrate that the carcinogenic risk posed by the more critical concentration of DEHP was extremely below the accepted risk level of 10^{-6} cancer risk, because the concentration of DEHP in drinking water corresponding to an excess estimated lifetime cancer risk of 1 in 1,000,000 is 3 µg/L (U.S. EPA, 2012c). Finally, the carcinogenic risk posed by the highest concentration of DEHP in bottled water is negligible.

It appears that, the contribution of bottled water in intake of phthalates compared to the extent permitted total daily intake is negligible. While the current experiment focused only on DBP, DEHP and BBP exposure through water consumption, the use of phthalates in many consumer products has been considered within the scientific and regulatory community as an important issue for human health. In this case, the food industry plays a key role in diminishing consumer exposure to phthalates (Wormuth, Scheringer, Vollenweider, & Hungerbuhler, 2006).

Conclusions

Because of the widespread use of PET plastic worldwide in containers for bottled water, PET-bottled water was studied under common usage conditions to obtain a clear answer to public questions about the safety of the presence of phthalate in bottled water. In this sense, the present survey on three common phthalate (DBP, BBP and DEHP) migrating from PET-bottles to drinking water confirmed that storage at low temperatures (refrigerator and freezing conditions) especially when compared to high temperatures (>25ºC) did not cause significant migration of these hazardous contaminants. Generally regarding to low level of DBP, BBP and DEHP concentrations in bottled waters, all common storage conditions of bottled waters in retail outlets, supermarkets and homes are considered safe for consumers.

Furthermore, the current study is the first to assess the exposure of infants and preschoolers to endocrine disruptors phthalates (DBP, DEHP and BBP) via consumption of bottled water. These groups are particularly sensitive to the toxicological effects of phthalates and often have more routes of exposure and greater prevalence of these compounds in their proximity.

Therefore, the main conclusion of this work is that bottled water does not represent a relevant ingestion source of phthalate esters for those who consume bottled water, and the levels of phthalates observed in bottled water are not a matter of concern from the standpoints of carcinogenic or non-carcinogenic effects.

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CHAPTER 4

A margin of exposure approach to assessment of non-cancerous risk of diethyl phthalate based on human exposure from bottled water consumption

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Abstract

Phthalates may be present in food due to their widespread presence as environmental contaminants or due to migration from food contact materials. Exposure to phthalates is considered to be potentially harmful to human health as well. Therefore, determining the main source of exposure is an important issue. The purpose of this study was (1) to measure the release of diethyl phthalate (DEP) in bottled water consumed in common storage conditions specially low temperature and freezing conditions; (2) to evaluate the intake of DEP from polyethylene terephthalate (PET) bottled water and health risk assessment; and (3) to assess the contribution of the bottled water to the DEP intake against the tolerable daily intake (TDI) values. DEP migration was investigated in six brands of PET-bottled water under different storage conditions room temperature, refrigerator temperature, freezing conditions (40 °C ,0 °C and −18 °C) and outdoor] at various time intervals by magnetic solid extraction (MSPE) using gas chromatography–mass spectroscopy (GC-MS). Eventually, a health risk assessment was conducted and the margin of exposure (MOE) was calculated. The results indicate that contact time with packaging and storage temperatures caused DEP to be released into water from PET bottles. But, when comprising the DEP concentration with initial level, the results demonstrated that the release of phthalates were not substantial in all storage conditions especially at low temperatures (<25 °C) and freezing conditions. The daily intake of DEP from bottled water was much lower than the reference value. However, the lowest MOE was estimated for high water consumers (pre-schooler> children > lactating women> teenagers> adults> pregnant women), but in all target groups, the MOE was much higher than 1000, thus, low risk is implied. Consequently, PET-bottled water is not a major source of human exposure to DEP and from this perspective is safe for consumption.

Introduction

Emerging contaminants (ECs) have been recognized as important issues in environmental chemistry and attracted increasing attention over the past decades (Yang et al. 2014). Meanwhile, the substances migrating from materials in contact with food are still being discussed. It is believed that many ECs such as phthalate acid esters (PAEs) are ubiquitous environmental pollutants because of their widespread manufacture, use, and disposal (Matsumoto et al. 2008; Serôdio and Nogueira 2006), and polyethylene terephthalate (PET) is also a controversial material in this sense. Throughout the world, PET is the most popular material for packaging, accounting for >99 % of all beverage bottles (Carneado et al. 2015; ILSIEurope 2000). Nevertheless, the problem of potential migrants from PET and adverse health effects is long standing. Chemically, PET is polyester of terephthalic acid and ethylene glycol. It is obtained by polycondensation of dimethyl terephthalate with ethylene glycol or terephthalic acid with ethylene glycol/ethylene oxide. According to the PET report made by the Fraunhofer Institute for Process Engineering and Packaging (EFBW 2013), PET does not contain plasticizers and is characterized by a limited range of additives and low diffusion of potential migrants in the polymer matrix. In addition, PET used for water bottles is not plasticized in order to obtain good mechanical and gas barrier properties (Dévier et al. 2013).

Hence, even if starting substances and additives are strictly regulated by EU regulation no. 10/2011, there might be even several non-polymer origins of non-intentionally added substances (NIAS) that can be exist in the final plastic material (EU 2011) and potentially leaching into food and beverages. The recent regulation on food contact materials (Regulation 10/2011/EU) recognizes that during the manufacture and use of plastic materials and articles, NIAS can be formed as a result of the interactions between different ingredients in the packaging materials, from degradation processes and mainly from the impurities may be introduced along with colorants master batches, catalysts, polymerization, and production aids present in the raw materials used for their production (Kassouf et al. 2013; Nerin et al. 2013), and assessments of health risks used for establishing packaging standards must also include the NIAS (Muncke 2009). Accordingly, any potential health risk caused by exposure to these contaminants should be considered.

Phthalates (dialkyl or alkyl aryl esters of o-phthalic acid) are a group of organic chemicals that have applications in many industrial and consumer sectors for more than 50 years in our daily lives (EPA 2007; Sioen et al. 2012). Among phthalate group of chemicals, the diethyl ester of phthalic acid (DEP), one of the low molecular weight phthalate esters, is the one which has many industrial uses especially as plasticizers (API 2001). Since phthalates are not covalent bound in plastic materials, they can be release slowly from products into the surrounding environment and into food items (EPA 2007; Leitz et al. 2009; Wittassek et al. 2011). As a result, the general population is widely

and continuously exposed to phthalates through dietary intake, inhalation, and dermal contact throughout their lifetimes (Heudorf et al. 2007; Sioen et al. 2012). Suspicions of harmful effects of phthalates on human health have recently been brought to the attention (Singh and Li 2011). Lately, the World Health Organization (WHO) had also published a Concise International Chemical Assessment Document (CICAD) on DEP, which claims that in spite of human exposure to DEP being significant with higher levels found in children and women of childbearing age (Kobrosly et al. 2012; Mapuskar et al. 2007; WHO 2003). Sufficient animal data supported the conclusion that oral exposure to DEP induced toxicity in a variety of organ systems such as the liver (hepatotoxic ant) and kidney. Maternal exposure to DEP also induced developmental effects (i.e., increased number of variations and categorized as a developmental toxicant (CPSC 2011). While human studies are limited, the adverse effects on fertility parameters and development are considered relevant to humans, where the exposure level of DEP is high and within a critical window of development. Data from animal studies indicate that DEP is rapidly and almost completely absorbed following oral or inhalation exposure, with 100 % bioavailability by these routes. Bioavailability via dermal (skin) absorption is not likely to exceed 10 % in humans. DEP is also rapidly metabolized to monoethyl phthalate (MEP) and excreted, predominantly via the urine (NICNAS 2013; U.S. EPA 2007). Tissue distribution of DEP is widespread including fetal tissues. It has also attracted international attention for its long-term health impact on pregnant, lactation women, and children. Thus, they are easily vulnerable to toxic chemicals due to the immaturity of their organs (Wang et al. 2014). In human studies of children, a 2010 study reported that among 171 children age 4–9, low molecular weight phthalates (including dibutyl phthalates (DBPs) and DEP metabolites were associated with higher scores for aggression, conduct problems, attention problems, and depression (Engel et al. 2010)). Other studies have reported associations between gestational exposures to phthalates, including di-n-butyl phthalates (DnBP), benzyl butyl phthalate (BBzP), di(2- ethylhexyl) phthalate (DEHP), and DEP and outcomes suggesting impaired behavioral development (Braun et al. 2013; Engel et al. 2009; Swan et al. 2010; Whyatt et al. 2012). In addition, early life DEP exposure, especially before 2 years of age, may increase the risk of allergic sensitization and atopic disorders (Wang et al. 2014). Besides, Pereira et al. (2007) showed a relation between chronic exposure to low doses of DEP from food, gestation and lactation, and toxic effects after three generations, indicating phthalates as critical for risk assessment (Pereira et al. 2007). There is, therefore, an urgent need to implement measures that lead to reductions in exposures, particularly for pregnant and lactating women and women of childbearing age (CHAP 2014). Therefore, daily oral exposure to DEP through food and beverage items is important, and this contaminated food event caused shock and panic among the general population.

In this regard, several studies have shown that PET bottles can release harmful chemicals such as diethyl phthalate (Table 1) but with a wide range of concentrations and storage times which makes data comparison difficult. Contradictory results have been published on the occurrence of phthalates in PET-bottled waters. As mentioned before, DEP and other phthalates not used directly in the PET production but DEP as NIAS during the manufacturing of PET may come from a wide variety of sources such as a cross-contamination in production line or impurities in raw materials. Furthermore, PET-bottled water can be contaminated in different phases of the production process, includes from supplying of the materials to handling, storing, and distribution (Amiridou and Voutsa 2011); therefore, its safety becomes a controversial issue. In recent years, concern about PET-bottled water is being raised because some websites and/or publications claimed that disposable plastic water bottles may cause harmful effects in human if they are stored in the freezing conditions or if they are left in the car or outdoor, due to the migration of hazardous chemicals such as phthalates (Al-Saleh et al. 2011; Andra et al. 2011; Culora 2009, Hosseini 2008). Public health risks from DEP exposure were assessed by using a margin of exposure (MOE) approach for two exposure scenarios include toys and childcare articles and cosmetic products containing. However, to the best of our knowledge, this is the first study to investigate the intake of DEP aimed to determine the health risks to children and adults from the use of DEP in bottled water consumption. Therefore, the major objectives of this study were (1) to analyze the leaching of DEP contained in PET-bottled water in order to investigate the influence of usual storage conditions, (2) to assess potential health risks based on maximum concentration of DEP in PET-bottled water, and (3) to assess the contribution of the bottled water to the DEP intake against tolerable daily intake (TDI) values.

Table 1. Bibliographic data on the content of diethyl phthalate in bottled water.

Compound name	Media	Exposure temperature	Exposure conditions	Concentration range (µg/L)	Concentration mean (µg/L)	References
Diethyl phthalate (DEP)	Still water	-	-	0.054-0.1	0.080± 0.016	Cao et al., 2008
	Water	30 °C	10 weeks	0.082-0.355	0.214	Casajuana and Lacorte 2003
	military packaged water	23 °C to 60°C	120 days	0.13–0.32	-	(reifenstein, et al. 2013
	Bottled water	Outdoors	30 days	-	0.033	Amiridou and Voutsa 2011
	Bottled water	4 °C	30 days	0.578-1.778	-	Al-Saleh, et al. 2011
	Bottled water	25 °C	60 days	<0.58	-	Al-Saleh, et al. 2011
	Bottled water	-	-	-	0.07	Montuori, et al. 2008
	Mineral bottled water	25 °C	One-year stored	0.857–4.30		Guart, et al. 2014

Material and method

Chemicals and reagents

A phthalate ester standard solution, containing DEP, at 2.0 mg/mL in n-hexane was obtained from Sigma-Aldrich (St. Louis, MO, USA). The phthalate stock solution, at a nominal 100 µg/mL concentration, was prepared in methanol. Benzyl benzoate (internal standard (I.S.)) was added to each sample at a final concentration of 1 µg/L. In order to safely keep and protect the solutions, all of them were kept at 4 °C in the dark until analysis. To prepare blank water for making calibration and quality control samples, we used doubledistilled deionized water which was originally collected by melting natural snow. After purification steps, the water was analyzed for any phthalate residues. The samples that contained DEP below the LOD of the applied method were used as blank matrices. These samples were collected in amber glass-stoppered bottle and kept at 4 °C until analysis. All the other chemicals and solvents were of analytical-reagent grade or better. Considering the fact that phthalate compounds are existed in many of the laboratory products (e.g., chemicals and glassware), they are usually interfering with the analysis of phthalates in real samples. In order to minimize such interferences, the entire laboratory used in this study was immersed in acetone for at least 30 min, rinsed with n-hexane, and then dried at 120 °C for at least 4.0h before use (Zare Jeddi et al. 2015a).

Samples collection and storage conditions

The focus of this survey is to determine the factors that may influence the migration of phthalate in bottled water. For this purpose, six different brands of PET-bottled water available in Iran purchased from factories immediately after production to confirm that the samples stored at the same condition before experiment. Later, each sample was analyzed to determine DEP at different time intervals including, at production date, and after 45 days of storage in different conditions: outdoor condition (exposed to sun) and different temperatures, −18 °C (freezing), 0 °C, 4 to 8 °C (refrigerator), 25 °C (room temperature), and 40 °C, in the form of 0.5 L PET bottles. Release of DEP measured in the first 24 h, day 10th, day 30th, and day 45th in each storage conditions to determine the effects of bottled water storage conditions on phthalate releasing.

MSPE procedure

The magnetic PDMS/MWCNTs-OH particles were prepared based on a two-step procedure as described previously (Xu et al. 2012). The typical absorption peaks of Fe3O4, OH-PDMS, and MWCNTs-OH in the FTIR spectra of magnetic PDMS/MWCNTs-OH particles indicates that OH-PDMS and MWCNTs-OH have been both bonded to the surface of Fe3O4 particles successfully.

The extraction of phthalate acid esters (PAEs) from water samples were conducted in consecutive steps. Firstly, 10 mg of magnetic PDMS/MWCNTs-OH particles were accurately weighed and activated with methanol and water separately in sequence. Then, the activated MPs and 1 g NaCl was added to the 10-mL water sample. The mixture was shaken vigorously to extract the analytes for 4.0 min. The magnetic adsorbent was gathered to the side of the vial (within ~90 s) with the aid of an external magnet. The supernatant was then discarded followed by addition 2 mL of acetone along with 2.0 min of vigorous vortex to elute DEP from the adsorbent. Afterwards, the magnetic adsorbent was gathered to the side of the vial again. The desorption solvent was collected and evaporated to dryness at 40 °C under gentle stream of nitrogen followed by reconstituting in 0.1 mL methanol for the subsequent GC-MS analysis.

Instrumental analysis

Agilent gas chromatograph 6890 plus (Agilent Technologies, Palo Alto, CA, USA) equipped with a 5973 quadruple mass spectrometer was applied for GC-MS analysis. The gas chromatograph was fitted with an HP-5ms capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The instrumental temperatures were as follows: injector temperature at 290 °C and initial oven temperature at 50 °C which was held for 1 min and then increased to 280 °C at a rate of 30 °C/min, and finally increased to 310 °C at a rate of 15 °C/min held for 4 min. The inlet was operated in

splitless mode. The temperature of the transfer line was maintained at 310 °C. As a carrier gas, helium (99.9999 %) was used at 1 mL/min (constant flow). The source and quadrupole temperatures were respectively kept at 230 and 150 °C. The electronic beam energy of the mass spectrometer was set at 70 eV. The mass selective detector was operated in electron impact (EI) mode, using selected ion monitoring (SIM). The dwell time of each ion was set at 100 ms. The GC conditions were selected to minimize the analysis time while allowing all the analytes to elute in acquisition groups containing suitable number of ions for monitoring.

Method validation

Method validation was conducted in accordance with the currently accepted U.S. Food and Drug Administration Guideline for Industry (U.S. FDA 2012). Under optimized conditions, the limit of detection (LOD) and limit of quantification (LOQ) for DEP achieved was 0.01 and 0.025 µg/L, respectively. The recover value for DEP was determined at four concentration levels (0.075, 0.25, 7.5, and 15 µg/L) as 91.5, 93.6, 92.2, and 94.3 %, respectively (Zare Jeddi et al. 2015a).

Health risk assessment

An exposure assessment and risk characterization was conducted to better understand the potential human health significance of phthalates in consumer articles. At the present time, a thorough toxicological evaluation about bottled water is not available. Therefore, in this study, daily intake of DEP achieved according to their maximum concentration in the six storage conditions. Thereafter, risk characterization calculated in six target groups including infants, toddlers, and preschool children, pregnant, lactating, and adult women.

Estimated daily exposure

Exposure assessment, as part of risk assessment, is defined as the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, or physical agents via food. Several methods can be used to estimate the intake of a food chemical, and the choice will depend on what information is available and how accurate and detailed the estimate needs to be (Kroes et al. 2002). An estimate of daily intake (EDI) to a substance was calculated by combining the arithmetic product of food-type consumption and a concentration of the substance in that food. The general procedure used to estimate intake includes the following steps: description of exposure to the various media containing the PAEs; assigning a concentration of the PAEs in each medium; and assigning an intake rate for that medium. Therefore, in this study, intake estimates of DEP were made for the daily consumption of water assuming a body weight and maximum concentration of DEP in bottled water as follows:

$$EDI = \frac{MC \times ED \times DRI}{AT \times BW}$$

Where:

EDI Estimate of daily intake in micrograms per kilogram body weight per day, MC Maximum concentration: the maximum concentration values of DEP investigated in the bottled water samples in the present study (µg/L) DRI Daily requirement intake: the recommended daily water intake basis of the body weight (L/body weight/ day).

The water consumption rates for target groups (preschooler, children, teenagers, adults, pregnant, and lactating women) were those recommended by the 2011 Exposure Factors Handbook (EFH) based on the U.S. Environmental Protection Agency (U.S. EPA) analyses of the National Health and Nutrition Examination Survey (NHANES) 2003–2006 data and Panel on Dietary Reference Intakes for Electrolytes, and Water by the Institute of Medicine (USA) (DRI 2006; U.S. EPA 2011).

EF Exposure frequency (350 days/year)

ED Exposure duration (years)

AT Average time (days: for non-carcinogenic substance, take AT=365 days/year×ED)

BW Body weight (kg)

Risk characterization

The goal of risk characterization is to compare hazard/ toxicity levels with exposure doses to determine if risk may occur under the specific scenarios. To characterize the risks for compounds in isolation, quantitative estimates of point of departures (PODs; no-observedadverse- effect level (NOAEL) or benchmark dose lower confidence limit (BMDL)) were derived from experimental studies with animals, and in a risk characterization step, these estimates were compared with exposures by calculating margin of exposure (MOE) (CHAP 2014). MOE for non-antiandrogenic phthalate methodology is used in international assessments to characterize risks to human health associated with exposure to chemicals. The MOE provides a measure of the likelihood that a particular adverse health effect will occur under the conditions of exposure.The MOE is defined as follows:

$$MOE = \frac{NOAEL}{EDI}$$

Where:

MOE, Margin of exposure in a population is a margin between the toxicity effect level and the exposure dose (unit less)

NOAEL, No-observed-adverse-effect level: The United States Environmental Protection Agency defines NOAEL as 'an exposure level at which there are no statistically or biologically significant increases in the frequency or severity of adverse effects between the exposed population and its appropriate control; NOAEL for DEP was set 1% of diet (750000 µg/kg body weight /day) (IRIS 2003). EDI, Estimated daily intake (µg/kg body weight/day). MOE needs to be compared with UFs (product of Uncertainty Factors). UF for DEP is 1000. When the MOE is equal to or greater than UF × MF (Modifying Factors=1), the need for regulatory concern is likely to be small. Additionally, there are several ways to be exposed to DEP, tolerable daily intake [TDI; µg/kg body weight/day] was calculated for all of the exposure routes. Guideline values derived using the TDI approach take into account exposures from all sources by apportioning a percentage of the TDI to drinking-water. This approach ensures that total daily intake from all sources (including drinking-water containing concentrations of the substance at or near the guideline value) does not exceed the TDI. Wherever possible, data concerning the proportion of total intake normally ingested in drinking-water (based on mean levels in food, air and drinking-water) or intakes estimated on the basis of consideration of physical and chemical properties were used in the derivation of the guideline values. Where such information was not available, an arbitrary (default) value of 10% TDI for drinking-water was used (WHO 2006). Therefore, the contribution of the daily intake of these compounds via consumption of drinking water was estimated as follows:

$$\text{Contribution via drinking water } = \left(\frac{\text{EDI}}{\text{TDI}}\right) \times 100$$

A TDI for DEP has not been set by the European Commission's Scientific Committee for Food (SCF) or the European Food Safety Authority (EFSA) but a working group of the WHO in a Concise International Chemical Assessment Document (CICAD) in 2003 proposed a TDI of 500 µg/kg bodyweight /day (WHO 2003).

Statistical Analysis

SPSS (version 20.0) was employed for statistical analysis of the results. Data are expressed as mean± SD. Before performing this statistical analysis, normality of concentrations in each group was checked using the Kolmogorov-Smirnov test. Because the distribution of data was not normal, we used nonparametric the Kruskal-Wallis H test for comparing concentrations from different storage conditions. The concentrations from the exposure periods were compared using the Friedman test. The analyses were considered statistically significant when $p < 0.05$.

Results

The findings of this study showed that conditions of storage and contact time are factors that may, to some extent, explain the release of DEP from PET-bottles into water. The results of analyses of bottled water before storage (bottled waters immediately after Production) showed that the initial levels of DEP was present in extremely low concentrations in PET-bottled water before storage but after 45 days storage at various conditions the DEP concentration was increased. The effect of various storage conditions on phthalates migration into PET-bottled water regardless of their exposure periods are summarized in Table 2.

Table 2. Result of diethyl phthalate (DEP) migration (mean±SD) in µg/L from PET into bottled waters under different storage conditions.

Storage condition	N	DEP
Control	12	0.231±0.135
Outdoor condition	24	0.352±0.195
40 °C	24	0.760±0.451
Room (25 °C)	24	0.385±0.189
Refrigerator (4-8 °C)	24	0.373±0.199
Zero (0 °C)	24	0.326±0.191
Freezing (-18 °C)	24	0.319±0.171
P-value		<0.001

DEP was detected in all PET-bottled water samples for all the storage conditions tested (Figure 1). Although Kruskal-Wallis test showed significant differences between storage conditions, but Post-Hoc results revealed only difference between 40°C with other conditions except room temperature. There is no significant difference in the concentration of the target compound between storage at outdoor conditions and low temperatures (room temperature, refrigerator and freezing). Levels of phthalates in bottled waters at the temperatures less than 25 °C relative to the initial level were not significantly different. Generally, increase in the concentration of phthalate was observed at various conditions after exposure periods (24 hr., 10, 30, 45 day) than the initial level before storage (bottled waters right after production), and toward 25°C (a common condition of keeping bottled water) (Figure 2). Therefore, this comparison demonstrated that DEP released from PET into bottle water at 40°C. Whereas, DEP release increased in freezing conditions (0°C and -18°C) a little in compare to initial level before storage. Besides, the average increment in DEP concentration at 40°C was six times higher than measured level in -18°C, and 3.7 times higher than that of refrigerator temperature.

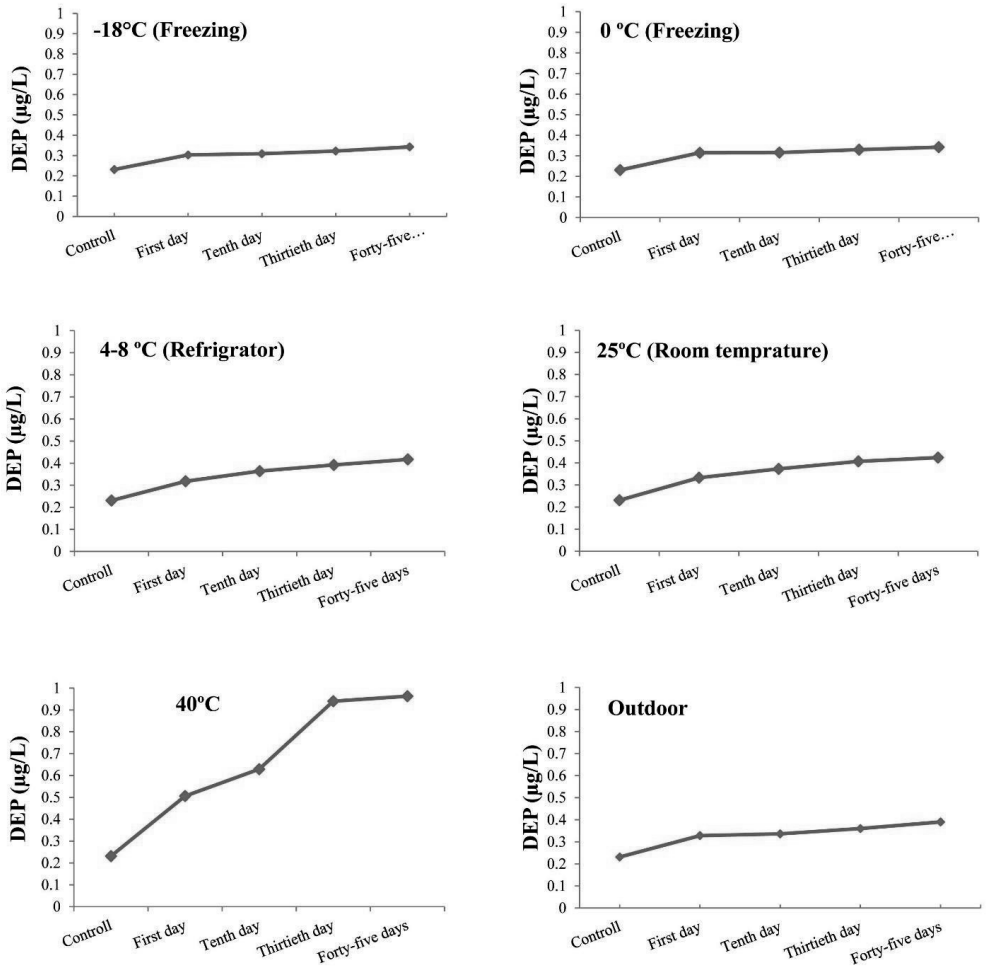

Figure1. The mean concentration of diethyl phthalate (DEP; µg/L) in bottled water at different storage conditions during the storage time.

Furthermore, the temperature, duration of time of exposure is another factor affects phthalate migration into water. Results showed that after 45 days of exposure period, a significant increase in the concentration of DEP observed. Overall, Friedman test showed significant difference between times of measurement (initial level, hour 24, days 10th, 30th and 45th). In other word, as storage time prolonged, regardless of storage conditions and brand of bottled water, concentrations of DEP augmented (Figure 1).

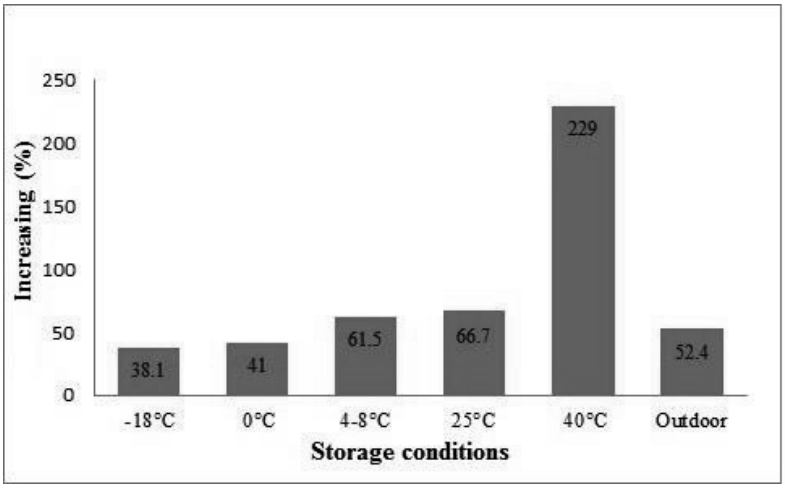

Figure 2. Increasing of diethyl phthalate concentration (%) in different storage conditions relative to the initial level before storage.

In the field of drinking water the maximum level of allowance by WHO and U.S. EPA is only determined for DEHP and not certificate for DEP; Therefore there is not a significant level for DEP in drinking water.

For an assessment of the risk associated with phthalate exposure, the phthalate exposure estimates can be compared with exposure limit values established by authorities like the European Food Safety Authority (EFSA) and the U.S. Environmental Protection Agency (U.S. EPA). This study prepared information on target substances and exposure scenario of child, teen, adults, pregnant and lactating women (due to being sensitive to the impact of the substances) and in comparison with adult women and computed MOE; these levels are shown in Table 3.

Risk assessment for DEP in PET-bottled water is based on the exposure scenario when the estimated water requirements in each age group, with the assumption that they meet their needs (DRI 2006) and the maximum concentration of DEP in different storage conditions, are available. Based on the migration data from bottled water, the total exposure (chronic daily intake) of preschooler aged 1 to 5 years, children aged 6 to 11 years, teenagers aged 12 to 18 years, adults, pregnant women, and lactating women to DEP (the most abundant phthalate after DEHP) from PET-bottled water at 40 °C (as the worst condition) was estimated to be 0.12, 0.098, 0.07, 0.06, 0.05, and 0.08 µg/kg bw/day, respectively.

After considering the daily exposure to phthalate in target women group, the MOE for DEP as non-carcinogenic toxic phthalate estimated more than 1000 in all cases (Table 3). When looking at the

contribution of water to exposure to phthalate according to daily intake, they did not exceed 0.024% of the TDI for DEP (0.004–0.024%).

Based on the guide to uncertainty analysis in environmental and health risk assessment, if screening calculations indicate that the risk is clearly below regulatory or risk levels of concern, a formal quantitative uncertainty analysis may not be necessary (Hammonds et al. 1994). Therefore, in this study, we are not to undertake a formal quantitative assessment of uncertainties.

Discussion

Migration of plasticizers from food packaging and processing products is the major contamination path of phthalates into foods. Additives such as plasticizers and antioxidants are not necessary for PET bottles. Despite this, the analysis of PET reveals some non-intentionally added substances (NIAS) produced by authorized initial reactants and additives (Bach et al. 2012). Since, phthalates are ubiquitous in the environment; therefore, DEP is the example of NIAS in PET bottle material.

In addition, the presence of NIAS has been designated as a source of this toxicological effect. Phthalates are suspected of causing health problems. The acute toxicity of phthalates is very low (LD50, 1–30 g/kg); however, the subchronic and chronic toxic effects of phthalates and their metabolites are of more importance. The government has developed regulations and guidelines for DEP. These are designed to protect the public from the possible harmful health effects of the chemical. Under laws that relate to Superfund sites, U.S. EPA has identified DEP as a hazardous substance. This decision is primarily based on the large number of Superfund sites where DEP is found. As a result, in the field of bottled water, this project has established that the amount of DEP found in bottled water depends on the period of storage (the time of contact with packaging materials) and storage temperatures. Regarding the Iranian data presented in Table 2 such as the other studies (Table 1), DEP is observed in all of the bottled water samples, ranging from 0.231 to 0.760 μg/L and after DEHP was more abundant phthalates in bottled water. But, in all conditions, DEP migration was not considerable. Nevertheless, low temperature especially freezing conditions was not a critical condition for DEP migration. So, our result confirmthe opinion of Dr. Yet Rolf Halden, Ph.D., P.E., assistant professor in the Department of Environmental Health Sciences and the Center for Water and Health at Johns Hopkinss Bloomberg School, who notes that Freezing actually works against the release of chemicals (Logomasini 2009). In light of potential health impacts associated with DEP exposure, targeted action for reduction of exposure sources may be warranted, especially for sensitive populations such as lactating women and children. Therefore, monitoring occurrence levels, identifying the sources, and determining potential risks of phthalate exposure among the sensitive populations are important. Until now, several studies have reported

the levels of phthalate exposure among children, but only limited information is available on their sources or associated risks (Kim et al. 2014; Wittassek et al. 2011). This study shows that pre-schooler (1–5 years) exposure to DEP via consumption of bottled water was higher than other target groups.

The levels of exposure appeared to be generally higher among the pre-schooler compared with the adult population. After pre-schooler and children, the exposure estimates for lactating women were higher. The level of DEP was higher in lactating women compared with pregnant and adult women because these groups have higher water requirements in relation to their body weight. However, the assessed dietary intakes to DEP from drinking bottled water in all of the target groups were far below the TDI. For example, the intake distribution for pre-schoolers in the worst-case scenario was 5000 times lower than the TDI for DEP. Therefore, with this level of DEP in bottled water, their exposure cannot exceed the TDI. In addition, contribution of the daily intake of this compound via consumption of drinking water was negligible and much lower than the default guideline value, and in the worst-case scenario only reach 0.024% of TDI (guideline derivation allocation to DEP in drinking water was <10 % of TDI).

In the field of risk characterization, the lower the MOE (margin between the toxicity effect level and the exposure dose), the more likely a chemical is to pose an unreasonable risk. The EFSA/WHO/ILSI conference concluded that the MOE approach is a useful and pragmatic option for risk assessment; this approach allows comparison between compounds to support prioritization for risk management action and, if the MOE is very large, on communication of a low level of human health concern (Barlow et al. 2006; Benford et al. 2010). In this study, the results for risk characterization for DEP showed that, the margin indicates that a particular toxicity effect level is 10–40 times higher than the expected exposure dose; therefore, there is little concern that concentrations will reach levels where toxicity is possible. However, if the toxicity level is only one time higher than the exposure dose and considering potential uncertainty in experimental measurement, there is a significant chance the exposure dose may reach the toxicity effect level. Similarly, in a study performed by Amiridou and Voutsa 2011, a health risk assessment was conducted in accordance with the guidelines indicated by U.S. EPA. It is conducted based on leaching of DEP from PET. As well as in this study, a daily consumption of 2 L of drinking water per capita by an adult of 60 kg body weight was considered. The results showed that DEP was found at a maximum concentration of 0.070 μg/L. It was found that the daily intake of DEP (0.002 μg/kg body weight/day) was far below the maximum safe dose; therefore, the factor of safety was calculated to be relatively high (Amiridou and Voutsa 2011).

Table 3. Estimation of human exposure to diethyl phthalate (DEP) via consumption of bottled water after 45 days storage under the various storage conditions.

Target Groups													
Storage conditions	**MC[1]**	**Pre-schooler (1-5 years)**		**Children (6-11 years)**		**Teenagers (12-18 years)**		**Adults**		**Pregnant women**		**Lactating women**	
		EDI[2]	TDI%[3]	EDI	TDI%	EDI	TDI%	EDI	TDI%	EDI	TDI%	EDI	TDI%
Storage at 40°C	1.80	0.121	0.024	0.098	0.020	0.07	0.014	0.064	0.013	0.053	0.011	0.080	0.02
Storage at 25°C	0.75	0.051	0.010	0.041	0.008	0.032	0.006	0.027	0.005	0.022	0.004	0.034	0.006
Storage at 4-8°C	0.70	0.047	0.009	0.038	0.007	0.027	0.005	0.025	0.005	0.021	0.004	0.031	0.006
Storage at 0°C	0.67	0.045	0.009	0.036	0.007	0.026	0.005	0.024	0.005	0.020	0.004	0.028	0.005
Storage at -18°C	0.63	0.042	0.008	0.034	0.007	0.024	0.004	0.022	0.004	0.019	0.004	0.027	0.005
Storage at Outdoor	0.73	0.049	0.0098	0.040	0.008	0.028	0.006	0.026	0.005	0.022	0.004	0.031	0.006
MOE[4*]	>1000			>>1000		>>1000		>>1000		>>1000		>1000	

1Maximum concentration [µg/L]
2 Estimated daily intake via drinking water (EDI: µg/Kg body weight/day)
3Contribution via drinking water (% TDI)
4 Margin of exposure (MOE; Unitless); Non-cancer risks are calculated by the margin of exposure approach which in the MOEs range from 7500000 to 37500000, and they were very large than an uncertainty factor (1000 for DEP). In this case, MOEs of greater than 1000 do not represent risks of concern.
*MOE was much greater than 1000 for all storage conditions.

In addition, intake of other phthalate esters (DEHP, DBP, and BBP) through the consumption of bottled water was very low and insignificant (Amiridou and Voutsa 2011; Zare Jeddi et al. 2015a, b). Consequently, based on these results, bottled water is not a critical source of exposure to phthalate esters.

The U.S. EPA has classified DEP as class D, not classifiable as to carcinogenicity (U.S. EPA 2003). Therefore, cancer slope factor was not established for this contaminant, and we cannot evaluate its excess cancer risk.

Conclusions

In the present study, six conditions of common consumption of bottled water were investigated to evaluate the release of DEP into water and potential human health risks.

Our results demonstrated that DEP migration in all of the storage condition (40 °C, room temperature, refrigerator temperature, freezing conditions (0 and −18 °C), and outdoor) was inconsequential. According to the health risk assessment, bottled water were considered safe for consumption, because the daily intake of DEP from bottled water was much lower than that reference value and also theMOE was >>1000, thus, low risk is implied. The MOE approach is both a prioritization tool and a risk assessment tool, which gives a relative indication of the level of health concern without actually quantifying the risk. In general, small MOEs indicate high concern and large MOEs show low concern. Consequently, PET-bottled water was not a major source of human exposure to DEP and from this perspective is safe for consumption. Since, as DEP is ubiquitous environmental contaminant, also other food categories and other route of exposure should be considered in order to obtain a more correct estimate of the exposure to DEP.

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CHAPTER 5

Endocrine disruptor phthalates in bottled water: daily exposure and health risk assessment in pregnant and lactating women

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Abstract

Over the last decade, the consumption of water bottled in polyethylene terephthalate (PET) has considerably increased, raising concerns over water quality and packaged materials. This study aims to investigate the levels of the anti-androgenic phthalates including bis-(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), and benzyl butyl phthalate (BBP), in bottled water and its corresponding health risks in pregnant and lactating women. The phthalate levels were measured in six different brands of bottled water exposed to temperatures ranging between −18 and 40 °C and sunlight for 45 days. The phthalate was quantified using the gas chromatography-mass spectrometry (GC-MS). In addition, the non-carcinogenic effects were assessed using hazard quotient (HQ) approach, and cumulative health risk assessment was performed on the basis of hazard index (HI) calculation. In order to assess the carcinogenic risk due to the possible carcinogen DEHP (group 2B), the excess lifetime cancer risk (ELCR) was used. DEHP and DBP contaminants were detected at different storage conditions in all of the bottled water samples during the storage time. BBP was only detected at high temperature (≥25 °C) and outdoor conditions. The maximum concentrations of all phthalates were observed when water samples were kept at 40 °C. In contrast, storage at freezing conditions had no significant effect on the concentration level of all phthalates. The estimated intake by women was between 0.0021 μg/kg body weight/day for BBP and 0.07 μg/kg body weight/day for DEHP. The highest HQ for phthalate intake via bottled water consumption was much lower than 1 (HQ < 0.004), which implies that adverse effects are very unlikely to occur. The execution of a cumulative risk assessment for combined phthalate exposure demonstrated that the HIs for anti-androgenic effect were lower than 1 in all of the conditions. Furthermore, ELCR for DEHP based on the highest detected level was found to be less than 10^{-6}, which is considered acceptable. Our results prove that the levels of phthalates in bottled water are not a health concern for pregnant and lactating women. Consequently, PET-bottled water is not a major contributor to phthalate intake for most individuals.

Introduction

There has been a constant advancement in fabricating of synthetic compounds. Although these compounds have been made to make our lives easier, their negative effects cannot be ignored. In fact, industrialization and urbanization are causing an increase in the concentrations of these chemicals in both the environment and the human body, which can seriously jeopardize human health (Diamanti-Kandarakis, Bourguignon et al. 2009, Martine, Marie-Jeanne et al. 2012, Bergman, Heindel et al. 2013). There has been a dramatic increase in the endocrine disrupting chemicals since 2000 to 2012 (Bergman, Heindel et al. 2013). Endocrine disrupting chemicals (EDCs) are a heterogeneous group of ubiquitous exogenous chemicals or a mixture of chemicals defined according to endocrine activity (De Falco, Forte et al. 2015). They are being released into our daily environment from a variety of materials found in our homes and workplaces. Phthalates are considered endocrine disrupting chemicals, with anti-androgenic properties, present in many commercial products such as personal-care products, plastic materials, food packages, detergents and paints (Sweeney, Hasan et al. 2016).

Over the last decade, the consumption of Polyethylene terephthalate (PET) bottled water has grown substantially around the globe. In addition, there is a growing tendency toward replacing tap water with PET-bottled water in developed countries (IBWA 2009, Andra, Makris et al. 2012). PET is the most widely manufactured material for food or beverage packaging. In fact, PET is being applied to produce>99% of all beverage bottles (ILSI 2000, Carneado, Hernández-Nataren et al. 2015). Allegedly, very few additives are used in manufacturing PET (ILSI 2000). However, recent studies indicate that harmful chemical compounds such as phthalate esters with carcinogenic effects including bis-(2-ethylhexyl) phthalate (DEHP) (a possible human carcinogen) and chemical compounds with anti-androgenic properties such as dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), and DEHP could migrate from plastic into packaged food and water (Amiridou and Voutsa 2011, Bach, Dauchy et al. 2012, Guart, Bono-Blay et al. 2014). Although these substances and other additives are under strict regulations (e.g., EU Regulation No. 10/2011), there are several compounds known as Non-Intentionally Added Substances (NIAS, e.g., phthalates as impurities in raw materials) which can be found in the final plastic material; this is mainly due to the complex formulations of polymers, processes and storage (EU 2011). Phthalates are not chemically bound to polymer matrix and might easily be released into the food and surrounding environment directly and/or indirectly (CDC 2009). In this regard, the safety of PET bottles has become a controversial issue (Bach, Dauchy et al. 2012), as well as a public health concern.

Pregnancy and lactation periods across the lifespan, have been recognized as potentially critical windows of vulnerability to exposure to a variety of chemicals (Bellinger 2013). Maternal

transmission of phthalates to the fetus, and neonate during the breastfeeding stage, has been reported in previous studies (Huang, Kuo et al. 2009, Wittassek, Angerer et al. 2009). In addition, the possible effects of postnatal exposure to phthalates were evaluated in babies (Calafat, Needham et al. 2004, Marsee, Woodruff et al. 2006, Swan SH, Liu F et al. 2010). While adult exposure to phthalates and other EDCs is of importance (Rupnik 2011, Bergman, Heindel et al. 2013), the exposure of fetuses and/or neonates is of primary concern since this group is extremely sensitive to the effects caused by chemicals with hormone-like properties (Schug, Janesick et al. 2011). Several animal studies reported that exposure to phthalates including DBP, BBP, DEHP, and diethyl phthalate (DEP) has been associated with reproductive developmental damage, endocrine disruption, neurodevelopmental toxicity, growth-related problems and promoted epigenetic transgenerational inheritance of adult-onset diseases (e.g., obesity, reproductive disease, and sperm epimutations) (Okubo, Suzuki et al. 2003, Borch, Ladefoged et al. 2004, Sharpe and Irvine 2004, Xu, Cook et al. 2005, Andrade, Grande et al. 2006, Heudorf, Mersch-Sundermann et al. 2007, Manikkam, Tracey et al. 2013). In addition, according to recent epidemiological studies on pregnant women, there is a correlation between the exposure to low doses of DEHP (10 µg/kg body weight/day or less when converted from urinary concentration of MEHP) and an Anogenital Distance (AGD) reduction in the male infants born to these women (McLachlan, Simpson et al. 2006, FSCJ 2013, Swan, Sathyanarayana et al. 2015). The epidemiological studies conducted in this field report measurable effects ofphthalates in humans (Sathyanarayana 2008, Wolff, Engel et al. 2008, Adibi, Whyatt et al. 2010, Jurewicz and Hanke 2011, Ferguson, McElrath et al. 2014, Neier, Marchlewicz et al. 2015). Since phthalates are ubiquitous in daily life (Miodovnik, Edwards et al. 2014), the adverse effects of ongoing exposure to phthalates on human health have raised concerns in the general population. In this regard, this issue has been studied in susceptible subjects such as pregnant women, infants, and children (Adibi, Perera et al. 2003, Bornehag, Sundell et al. 2004, Huang, Kuo et al. 2009, Zare Jeddi, Rastkari et al. 2015b).

Considering the toxicity of phthalate esters, it is essential to study the risks associated with exposure to phthalates and have a better understanding of the key sources of exposure to these compounds. Very few studies have been conducted concerning the effects of low temperature conditions (<25ºC) when compared to the effects of high temperatures and storage time on the migration of phthalates from the bottle wall into the water. Furthermore, there have been no studies regarding the associated potential risks (carcinogenic or non-carcinogenic) from bottled water consumption focusing on pregnant and lactating women, despite their high sensitivity to the adverse effects of phthalates. In order to find a suitable answer as to whether PET-bottled water jeopardizes the health of its consumers, the present study aims to: (1) evaluate levels of phthalates in bottled

water in different storage conditions (2) assess exposure in susceptible groups of the population (3) determine the contribution of bottled water to phthalates intake.

Materials and methods

Chemicals and reagents

A phthalate esters standard solution, containing three compounds dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and bis-(2-ethylhexyl) phthalate (DEHP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The experiments were conducted as follows. First, a stock solution of phthalates (100 µg/mL concentration) was prepared in methanol. Benzyl benzoate (internal standard, I.S.) was added to each sample at a final concentration of 1 µg/L. All the samples were kept at 4 °C and protected from light before being used for the experiments. All the chemicals and solvents used in this study were of analytical grade. Since phthalate esters are used in the manufacture of many laboratory products (e.g., chemicals and glassware), contamination was anticipated. In order to prevent any error of this sort, all the laboratory glassware used in this study was immersed in analytical grade acetone for at least 30 min, rinsed with ultrapure n-hexane, and then dried at 120 °C for at least 4hrs before being used.

Taking and storing the samples

In order to determine the factors influencing the migration of phthalates into bottled water, six different brands of PET-bottled water with the volume of 0.5-L frequently used in Iran were purchased from factories immediately after production. These bottles were, then, stored under the same conditions before starting the experiments. A total of twenty four samples of each brand purchased from the same production batch were prepared. In addition, one bottle of each brand was used to determine the initial levels of DEHP, DBP and BBP. The samples were then incubated under six different conditions including five sets of temperatures (-18°C (freezing), 0°C (freezing), 4 to 8°C (refrigerator), 25°C (room temperature), 40 °C and sunlight exposure.

The bottles were placed separately on the roof of the laboratory from November until February 2012 with a distance of 45 cm between them to prevent shading. It should be noted that cloudy, rainy and snowy days were not considered as part of the experiment. Also, the minimum and maximum temperatures during this experiment were 12°C and 26.5°C respectively.

The bottles of water were exposed to the aforementioned conditions for 1, 10, 30 and 45 days in order to determine the effect of storage conditions on the release of phthalates. These results were compared to the maximum residue limits established by regulatory bodies to assess the safety of bottled water.

Instrumental analysis

A method developed in a previous study was used for the quantification of phthalates (Zare Jeddi, Rastkari et al. 2015b). The instrumental analysis conducted in this study has been briefly explained here. The analysis was conducted using a gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a quadruple mass spectrometer (GC-MS). The gas chromatograph was fitted with an HP-5ms capillary column (30m, 0.25mm i.d., 0.25µm film thickness). The instrumental temperatures were as follows: injector temperature: 290°C; initial oven temperature: 50°C, which was held for 1 minute and then increased to 280°C at a rate of 30°C/min, and finally increased to 310°C at a rate of 15°C/min and held for 4 minutes. The inlet was operated in splitless mode. The temperature of the transfer line was maintained at 310°C. As carrier gas, Helium (99.9999%) was used at 1mL/min (constant flow). The source and quadruple temperatures were kept at 230°C and 150°C respectively. The electron beam energy of the mass spectrometer was set at 70eV. The mass selective detector was operated in electron impact (EI) mode, using selected ion monitoring (SIM). The dwell time of each ion was set at 100ms. The GC conditions were selected to minimize the analysis time while allowing all the analytes to elute into acquisition groups containing a suitable number of ions for monitoring.

MSPE procedure

Based on a two-step reaction method applied in a previously conducted study, the magnetic PDMS/MWCNTs-OH particles were prepared (Zare Jeddi, Ahmadkhaniha et al. 2015). The typical absorption peaks of Fe_3O_4, OH-PDMS and MWCNTs-OH in the FTIR spectra of magnetic PDMS/MWCNTs-OH particles indicate that OH-PDMS, MWCNTs-OH have both bonded successfully to the surface of Fe_3O_4 particles. In order to extract phthalate esters from water samples, first, methanol and water were successively applied to activate 10 mg magnetic PDMS/MWCNTs-OH particles; then, the activated MPs and 1g NaCl were added to 10mL of the sample. To extract the analytes, the mixture was shaken vigorously for 4.0 minutes. The magnetic adsorbent was gathered to the side of the vial (within ~90s) to discard the supernatant using an external magnet. To elute phthalate esters from the adsorbent, 2mL acetone was added to the vial and then allowed to be under vigorous vortex for 2.0min. After this step, the magnetic adsorbent was collected again using the external magnet. The desorption solvent, also, was dried out at 40°C under a gentle stream of nitrogen followed by reconstitution in 0.1mL methanol for the subsequent GC-MS analysis (Zare Jeddi, Rastkari et al. 2015b).

Quality Assurance and Control

The target analytes were determined using the classical calibration method. In the final extraction medium (0.1 mL methanol), different calibration levels of each analyte were prepared. A volume correction was performed for each sample using estimated recovery values prior for determining the original concentrations. In order to get rid of interference due to residual phthalates, preliminary studies were performed and the baseline levels of phthalates in each sample and solvent were determined and then the final concentrations were calculated after subtraction of the initial baseline values.

Method validation

The methods applied in this study were validated with respect to the currently accepted U.S. Food and Drug Administration guidelines for industry (U.S. FDA 2012).

Health Risk Assessment

In order to verify that PET-bottled water is safe for consumption from the viewpoint of carcinogenic and endocrine disrupting compounds, daily intakes of some phthalates including DBP, DEHP, and BBP were estimated based on their maximum concentrations under the various storage conditions. The risk characterization was determined based on a worst-case scenario for carcinogenic and non-carcinogenic adverse effects in 3 target groups: pregnant, lactating and adult women.

The risks posed by the studied compounds were estimated based on toxicity data collected by the Integrated Risk Information System (IRIS) of the Environmental Protection Agency (U.S. EPA) and by the World Health Organization (WHO). The health risk was calculated based on the volume of water consumed, on average, by members of the target groups. Tolerable daily intake (TDI) values estimated by the European Food Safety Authority (EFSA) and the reference doses (RfD) estimated by the U.S. EPA were used as the acceptable levels of exposure to phthalates in this study.

In order to estimate exposure to phthalates through water consumption, we followed the "forward" approach that the concentrations of phthalates in bottled waters and the rate of daily intake of water are taken into account (ILSI-Europe 2009). The estimates of daily exposure to phthalates (also referred to as intake) via water consumption can be calculated in the target groups by using the following formula (De Fátima Poças and Hogg 2007):

$$\mathrm{EDI} = \frac{\mathrm{MC} \times \mathrm{WI}}{\mathrm{BW}}$$

Where, EDI is the estimated daily intake of phthalates via drinking water (µg/kg body weight/day) and provided in units of liters per day; MC (µg/L) stands for the maximum concentration values of DBP, BBP and DEHP in the samples taken from the bottled water;
WIR, abbreviating "Water Intake Rate", is the required volume of daily drinking water for the target group members; BW is the body weight (Kg).
In this study, water consumption rates and the values for body weight (Kg) for pregnant, lactating and adult women were taken from the EPA's Exposure Factors Handbook (EFH) (2011) and the Panel on Dietary Reference Intakes for Electrolytes, & Water by Institute of Medicine (US) (DRI 2006, U.S.EPA 2011).
Non-carcinogenic health risks were assessed using the U.S. EPA Hazard Quotient (HQ) calculatedas follows (EPA 2012):

$$HQ = \frac{EDI}{RfD}$$

Where, HQ is the Hazard Quotient, EDI is the estimated daily intake via drinking water (µg/Kg body weight/day), RfD stands for the reference dose (µg/ Kg body weight /day).
It should be noted that HQ<1 indicates an absence of risk for the particular considered endpoint, whereas HQ>1 means that the exposure may be regarded as health risk.
The Hazard Index (HI) used for the estimation of the cumulative anti-androgenic risk (the phthalate syndrome) of exposure to DEHP, BBP and DBP. HI is a regulatory approach to cumulative risk assessment (CRA) based on the concept of dose-addition. It can be defined as the sum of HQs for individual chemicals with the same endpoint (Koch, Wittassek et al. 2011). For this purpose, the RfDs for anti-androgenicity (RfD-AA) have been used to evaluate the effects of combined exposures to anti-androgenic phthalates (Kortenkamp and Faust 2010).
Additionally, there are several ways in which one can be exposed to phthalates. In this regard, Tolerable Daily Intake (TDI; µg/kg body weight/day) was used and calculated to cover all of the exposure routes. The fraction of the TDI allocated to drinking water is 1% for DEHP, 10% for BBP and 10% DBP (WHO 2008).Therefore, the contribution of the daily intake of these compounds via consumption of drinking water was calculated based on the following formula:

$$\text{Contribution via drinking water} = \left(\frac{\text{EDI}}{\text{TDI}}\right) \times 100$$

Where, EDI is the estimated daily intake via drinking water (µg/Kg body weight/day), TDI is the Tolerable Daily Intake (µg/kg body weight/day), The risk of developing cancer can also be calculated by using the following formula:

Excess Cancer Risk =CSF × EDI

Where, Excess Cancer Risk is associated with the excess level of risk of developing cancer by being exposed to particular chemicals via specified routes; EDI is the estimated daily intake via drinking water (µg/kg body weight/day).

CSF stands for cancer slope factors and is used to estimate the risk of cancer associated with the oral exposure to either a carcinogenic or a potentially carcinogenic substance. The CSF for DEHP is 1.4×10^{-2} per mg/kg·day (0.014 per mg/ body weight/day)$^{-1}$ (EPA 1997).

The U.S. EPA generally considers a cancer risk value ranging between 10^{-5} and 10^{-6} to be acceptable (U.S.EPA 2012).

Statistical analysis

In the present work, R statistical software (V. 3.1) was used to analyze the regression model. Also, the data are expressed as Mean ± Standard Deviation. Multiple regressions were used for analyzing the associations between a high phthalate concentration in bottled water (high phthalate migration) and storage characteristics, the independent effect of time and the independent effect of storage temperature. The standardized B was also applied to compare the magnitude of the effects caused by temperature and storage time. The analyses were considered statistically significant when the p-value was < 0.05.

Results

Quantitative analysis

The linearity of the calibration curves was determined in the range of 0.05-20 µg/L. The Coefficient of correlation ranged from 0.992 to 0.996. The LODs were defined as three times of the standard deviation of the baseline noise (n = 6) and determined by spiking serially diluted analyte standards into a blank water sample. According to the International Conference on Harmonization of Technical Requirements for Bioanalytical Methods (ICH) guidelines for analytical method validation, for each analytes the Limit Of Quantification (LOQ) was determined as the lowest concentration on the calibration curve with a precision of 20% coefficient of variation and an accuracy of 80–120% (U.S. FDA 2012). The results showed that the LODs and LOQs for the target analytes ranged from 0.01 to 0.025 and 0.025 to 0.05 µg/L, respectively. The precision of the method was determined in terms of intermediate precision through calculating the analyte concentrations in QC samples, prepared at four levels (each six replicates) on 3 consecutive days. Interday precision values for the analytes were always <12.40%. The RSDs% of determined

responses of six replicates of QC samples were taken for the expression of the repeatability (or intraday precision). To determine the recovery, mean peak area of each analyte at each concentration level was determined for a blank water sample spiked with the analyte (n = 6). The determined value was compared with the mean value obtained from spiking the same amount of the analyte in 100 mL methanol. All these results indicate the feasibility and reliability of the developed method for determining PAEs in water samples. The selectivity of the method was confirmed by analyzing 50 different water samples from different sources. There was no interfering peak in the region of the analytes and internal standard.

Phthalates concentrations in bottled water

The effects of various storage conditions on the migration of phthalates into PET-bottled water, regardless of exposure period, are summarized in Table 1. Extremely low concentrations of DEHP and DBP were detected prior to conditioning the bottles of water (i.e., immediately after production), whereas BBP was not detectable in these samples (Table 1).

Table 1. Results of phthalates levels (mean±SD) in µg/L from PET-bottled into waters under different storage conditions.

Storage conditions	DBP	DEHP	BBP
Control*	0.135±0.078	0.217±0.092	<LOD**
Outdoor condition	0.114±0.088	0.418±0.196	0.043±0.018
40 °C	0.303±0.172	0.917±0.342	0.063±0.031
Room Temperature (25 °C)	0.116±0.095	0.411±0.161	0.020±0.004
Refrigerator (4-8 °C)	0.124±0.099	0.423±0.150	<LOD
Zero (0 °C)	0.088±0.080	0.331±0.147	<LOD
Freezing (-18 °C)	0.079±0.089	0.317±0.124	<LOD

*Before storage immediately after production.
**Limit of Detection

The effect of time duration (1 day, 10 days, 30 days and 45 days) at different storage conditions on the phthalate concentrations (µg/L) in bottled water for each phthalate is shown in Figure 1 to 3. However as shown in the Figures and Table 2, the migration of these compounds in different brands of PET-bottled water after storage under the examined conditions was inconsiderable and in the order DEHP>DBP>BBP. Therefore, DEHP migration was the highest while BBP migration was the lowest.

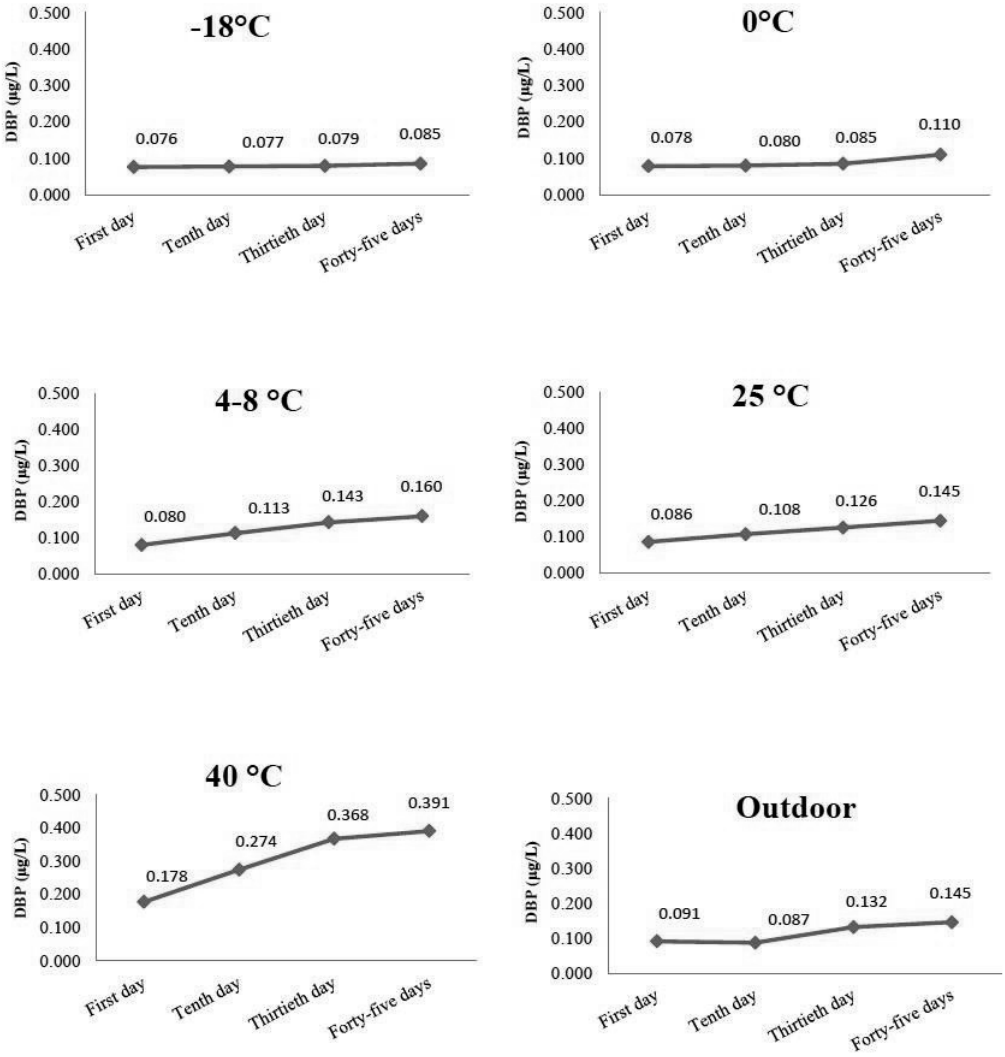

Figure 1. The effect of time duration (1, 10, 30, and 45 days) at different storage conditions on DBP concentration (µg/L) in bottled water.

According to Table 2, there is a statistically significant difference in concentrations of DBP and DEHP among the different sampled brands. In addition, the results indicate that the temperatures of storage and the duration of storage are the two main variables affecting the release of phthalates from PET-bottles. The effect of temperatures (-18 ºC to 40 ºC) and sunlight exposure on the release of the three phthalates into the water is more than the effect due to storage duration (Table 3).

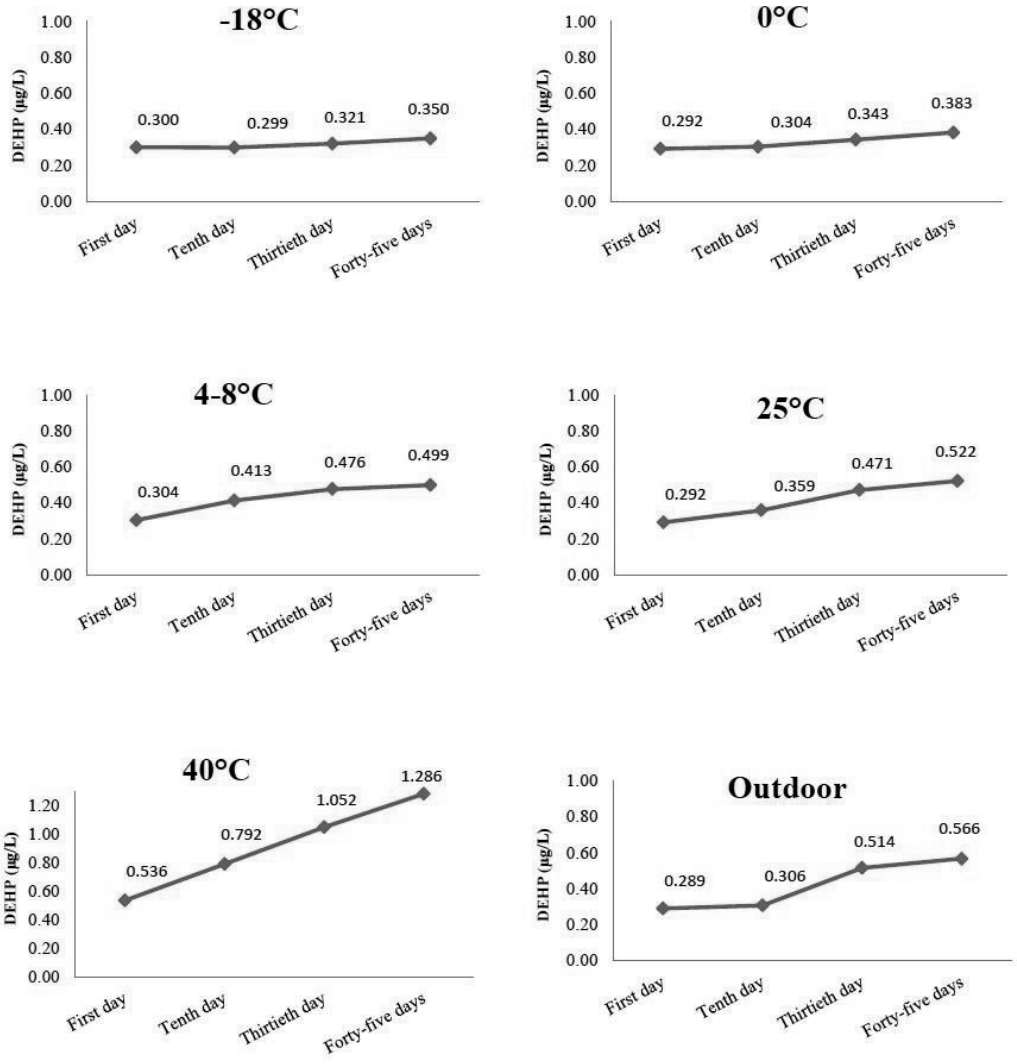

Figure 2. The effect of time duration (1, 10, 30, and 45 days) at different storage conditions on DEHP concentration (µg/L) in bottled water.

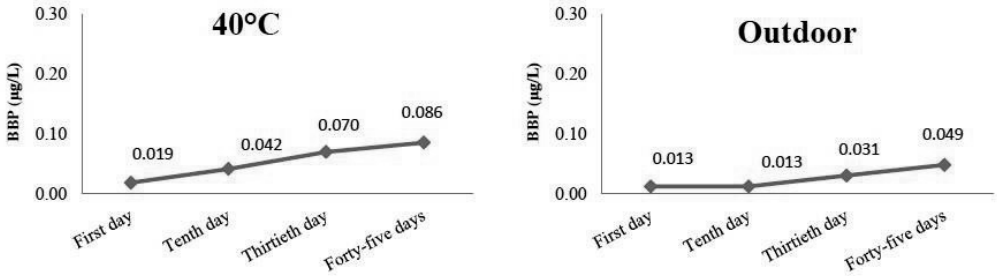

Figure 3. The effect of time duration (1, 10, 30, and 45 days) at different storage conditions on BBP concentration (µg/L) in bottled water.

On analyzing the effects of storage conditions, DEHP and DBP were detected in all samples with increased concentration during the storage time, whereas BBP was not detectable at low temperatures (i.e., under 25 °C) even in end of the storage time. In other words, its concentration was below the limits of detection (less than LOD). However, temperature had the largest effect on the migration of BBP. In fact, a unit increase in the temperature caused the release of 6.2×10^{-4} units of BBP, as noted in Table 3.

	Bottled waters						P-value
	A	B	C	D	E	F	
DBP	0.331±0.134	0.162±0.132	0.110±0.098	0.053±0.021	0.035±0.019	0.130±0.046	<0.001
DEHP	0.612±0.292	0.414±0.310	0.701±0.264	0.316±0.199	0.400±0.232	0.370±0.225	<0.001
BBP	0.024±0.024	0.027±0.031	0.017±0.012	0.020±0.018	0.017±0.013	0.024±0.024	0.768

Table 2. Phthalates concentrations (mean±SD) in six brands of PET-bottled water at various conditions after 45 days storage.

Among the three forms of phthalate esters, DEHP was the most abundant of the phthalates in all of the samples and in all storage conditions. Nevertheless, DEHP concentrations were significantly below the maximum contaminant level (MCL) set by the U.S. EPA standard or the WHO guidelines (6/8 µg/L), which is also shown in Figure 4. The concentration of DEHP in all tested samples in this study are closely in accord with those reported by previous studies in this field (Schmid, Kohler et al. 2008, Amiridou and Voutsa 2011, Greifenstein, White et al. 2013, Keresztes Szilvia , Enikő et al. 2013). It is noteworthy that the maximum contaminant level goals (MCLG) set by the U.S. EPA for DEHP in water is zero (U.S. EPA 2009).

Table 3. The independent effects of temperature and day of storage on phthalate migration.

	B	standardized B	*p* value
DEHP			
(Intercept)	0.0808009		0.0478
Day	0.0059512	0.355415	<0.001
Temperature	0.0088837	0.5702099	<0.001
DBP			
(Intercept)	0.0056088		0.804
Day	0.0016417	0.2068683	0.006
Temperature	0.0032838	0.4447298	<0.001
BBP			
(Intercept)	-4.97E-03		0.133
Day	4.06E-04	0.3191632	<0.001
Temperature	6.20E-04	0.5232442	<0.001

The concentrations of the phthalates measured in bottles of water stored at temperatures less than 25°C were not significantly different from each other or their initial level. However, by conducting experiments over several exposure periods (24hrs, 10days, 30days, and 45days) and raising the temperature to 40°C during these tests, we observed that the concentrations of DBP, DEHP and BBP increased substantially by 935.8%, 349.1% and 333% respectively when compared to the initial concentrations of these chemicals. Also, the corresponding amounts at 25°C (a common condition at which bottled water is kept), were 207.5%, 92.4% and 49.5% of DBP, DEHP and BBP respectively. It should be noted that the average concentration of DEHP at 40°C was higher than its measured concentration at -18°C by 200%, and at refrigerator temperature by 119%. In addition, under the same conditions applied on conducting the previously mentioned experiment, the concentrations of DBP were higher than their corresponding amounts at -18 ºC by 956% and at refrigerator temperature by 300%. BBP concentration at 40°C increased from its level at room temperature (25°C) by 275% and under outdoor conditions by 40%.

Besides the temperature, storage time is another factor studied in the present work. A significant increase in the concentration of phthalates was observed after 45 days. 0.006 units per day of DEHP were released during the storage period; the related result is given in Table 3. Therefore, it can be inferred that, second to the temperature, duration of time is a factor of high importance.

Estimated daily intake and risk

In this study, we estimated the daily intake of phthalates from the maximum amount of phthalates measured in bottled waters. These levels are presented in Table 4. Based on the results regarding the concentrations of the three types of phthalates found in bottled water, we estimate that the chronic

daily intake of DEHP (most abounded phthalate) from PET-bottled water stored at 40 ºC (the worst scenario) is 0.056µg/kg body weight/day for adults, 0.047µg/kg body weight/day for pregnant women and 0.07 µg/kg bw/day for lactating women. The HQ calculated did not exceed 0.004 (Table 4). In other words, the calculated HQs in all cases were much less than 1; in addition this value was lower than 0.2 for single route of exposure to these chemicals. Therefore, it can be inferred that the exposure is not expected to cause adverse effects. Because a HQ value of less than 0.2 for any given pathway is often considered acceptable (Health Canada 2010). The Hazard Index (HI) for lactating women came to a value of 0.003, below 1, in the worst condition (40°C), indicating that anti-androgenic effects are unlikely for combined exposures to the three types of phthalates at maximum exposure level (Table 4).

When considering the contribution of water to the daily intake of phthalates, the results showed that daily intake of phthalates from bottled water did not exceed 0.14% of the TDI for DEHP in lactating women. The nearest concentration of an individual phthalate to the TDI was observed for DBP (0.3% of the TDI).

Furthermore, the excess lifetime cancer risk, especially in lactating women, caused by exposure to DEHP via drinking water was most strongly associated with storing bottled water at 40ºC, compared with the other storage conditions. However, in comparison to the established criterion (less than 10^{-6}), the calculated excess lifetime cancer risk in lactating women is negligible.

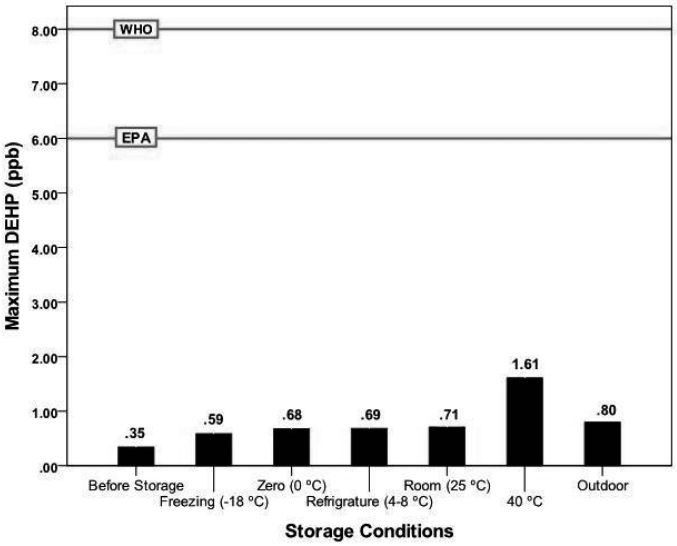

Figure 4. The maximum concentration of DEHP (µg/L) in bottled water in different storage conditions.

Table 4. Estimation of exposure to endocrine disruptor phthalates via consumption of bottled water after 45 days storage under the different storage conditions in adult, pregnant and lactating women.

	Storage at 40°C			Storage at 25°C			Storage at 4-8°C			Storage at 0°C			Storage at -18°C			Storage at Outdoor		
	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP	DEHP	DBP	BBP
Detected maximum concentration [µg/L]	1.61	0.702	0.130	0.711	0.328	0.250	0.687	0.375	-	0.678	0.248	-	0.589	0.274	-	0.802	0.302	0.071
EDI[1]																		
Adult	0.07	0.025	0.004	0.025	0.012	0.009	0.024	0.013	-	0.024	0.009	-	0.021	0.01	-	0.03	0.011	0.002
Pregnant	0.05	0.021	0.004	0.03	0.01	0.007	0.021	0.011	-	0.02	0.008	-	0.017	0.008	-	0.024	0.01	0.002
Lactating	0.08	0.03	0.006	0.04	0.02	0.011	0.03	0.02	-	0.03	0.011	-	0.025	0.012	-	0.034	0.013	0.003
MCL[2]	8/6	-	-	8/6	-	-	8/6	-	-	8/6	-	-	8/6	-	-	8/6	-	-
Calculated safety factor (WHO /EPA)	5/3.7	-	-	11.3/8.4	-	-	11.6/8.7	-	-	11.8/8.8	-	-	13.6/10.2	-	-	9.9/7.5	-	-
TDI[3]	50	10	500	50	10	500	50	10	500	50	10	500	50	10	500	50	10	500
Contribution via drinking water (%)																		
Adult	0.11	0.25	0.0008	0.05	0.1	0.002	0.05	0.1	-	0.05	0.08	-	0.04	0.09	-	0.06	0.1	0.0004
Pregnant	0.09	0.2	0.0008	0.04	0.1	0.002	0.04	0.1	-	0.04	0.08	-	0.034	0.09	-	0.05	0.1	0.0004
Lactating	0.14	0.3	0.001	0.06	0.2	0.002	0.06	0.2	-	0.06	0.11	-	0.05	0.1		0.07	0.13	0.0006
RfD[4]	20	100	200	20	100	200	20	100	200	20	100	200	20	100	200	20	100	200
RfD-AA[5]	30	150	300	30	150	330	30	150	330	30	150	330	30	150	330	30	150	330
HQ[6]																		
Adults	0.003	0.0002	0.00002	0.001	0.0001	0.00004	0.001	0.0001	-	0.001	0.00008	-	0.001	0.00009	-	0.002	0.0001	0.00001
Pregnant	0.003	0.0002	0.00002	0.001	0.0001	0.00004	0.001	0.0001	-	0.001	0.00008	-	0.001	0.00009	-	0.002	0.0001	0.00001
Lactating	0.004	0.0003	0.00003	0.002	0.0002	0.00005	0.002	0.0002	-	0.002	0.0001	-	0.002	0.0001	-	0.005	0.0002	0.00002
HI[7]																		
Adult		0.002			0.0008			0.0007			0.0007			0.00073			0.0011	
Pregnant		0.002			0.0011			0.0007			0.0007			0.00073			0.0011	
Lactating		0.003			0.002			0.0011			0.0011			0.0011			0.0015	
SF[8]	1.4×10^{-2}	-	-	1.4×10^{-2}	-	-	1.4×10^{-2}	-	-	1.4×10^{-2}	-	-	1.4×10^{-2}	-	-	1.4×10^{-2}	-	-
ECR[9]																		
Adult	7×10^{-7}	-	-	3×10^{-7}	-	-	3×10^{-7}	-	-	3×10^{-7}	-	-	32×10^{-7}	-	-	4×10^{-7}	-	-
Pregnant	7×10^{-7}	-	-	4×10^{-7}	-	-	3×10^{-7}	-	-	3×10^{-7}	-	-	3×10^{-7}	-	-	4×10^{-7}	-	-
Lactating	1×10^{-6}	-	-	6×10^{-7}	-	-	4×10^{-7}	-	-	4×10^{-7}	-	-	4×10^{-7}	-	-	6×10^{-7}	-	-

[1]Daily intake via drinking water (EDI: µg/kg body weight/ day)

[2]Maximum concentration limit (MCL) basis of WHO drinking water Guidelines value (µg/L)/ U.S. EPA standard (µg/L)

[3]Tolerable daily intake (µg/kg bw/day)

[4]IRIS RfD chronic non-carcinogenic effect risk (RfD, µg/kg bw/day)

[5] Reference Dose for Anti-Androgenicity (RfD-AA, µg/kg bw/day)

[6] Hazard Quotients (HQ) (unitless)

[7] Hazard Index (HI) (unitless)

[8] U.S.EPA Oral Slope Factor= 1.4×10^{-2}/mg/kg/day

[9]Excess Cancer Risks (ECR) (unitless)

Discussion

Phthalates are considered to be estrogenic and anti-androgenic endocrine disruptors, which can migrate from plastic packaging into food and water. In other words, these compounds can leak into the contact material, and water bottled in PET containers. Moreover, humans can be easily exposed to phthalates through water (Kim, Yang et al. 2011). So the aim of this study was to clarify existing concerns about the effect of temperature and storage of bottled water on the migration of phthalates, and secondly to assess the daily intake of phthalates and potential carcinogenic and non-carcinogenic risk to vulnerable groups (pregnant and lactating women).

The results of the present study regarding the role of the two abovementioned factors (storage temperature and storage time) in releasing phthalates into bottled water are consonant with the results of the previously conducted studies(Schmid, Kohler et al. 2008, Amiridou and Voutsa 2011, Greifenstein, White et al. 2013, Keresztes Szilvia , Enikő et al. 2013). Keresztes et al. reports that no clear trend could be established for phthalate leaching when water samples were kept at higher temperatures (maximum 60°C) (Keresztes Szilvia , Enikő et al. 2013), whereas the results reported in most of the previous studies showed that the dissolution of phthalates increases at temperatures greater than 40°C. In addition, based on the results of our study, increased duration of storage plays a minor role in the release of phthalate from bottles stored at low temperature.

In a study conducted by Al-Saleh et al. (2011), the phthalate concentrations were evaluated in mineral waters bottled with PET containers and stored in three different conditions. The levels of DMP, DEP, BBP and DEHP in stored bottled waters at 4°C for 1 month were reported to be significantly higher than the levels related to the other two storage modalities (i.e., room temperature for 2 months and outdoors (more than 45 C) for three months). However, the opposite trend was observed for DBP, especially when water was stored at room temperature (Al-Saleh, Shinwari et al. 2011). These results are in contrast to our findings. It may be due to the absence of a standard analysis method for detection of phthalates in bottled water in their analysis. Phthalates are ubiquitous contaminants and the major challenge in phthalate studies is to control the contaminations. Chemicals do not diffuse as readily in very low temperatures limiting chemical release (Zare Jeddi, Rastkari et al. 2015c).

Although the detected concentrations were far below toxic levels, considering the extensive use of bottled waters in both developed and developing countries, it is wise to pay adequate attention to the intake of phthalates via drinking water in pregnant and lactating women. The daily intakes of phthalates in pregnant women were more that lactating women and non-pregnant adult women. This could be attributed to increased water demand during lactation. In all cases, the exposure was clearly below the values set by the EFSA's TDI and the U.S. Environmental Protection Agency

(U.S. EPA) RfD (EFSA 2005a, EFSA 2005b, EFSA 2005c, TEACH 2007). Overall, the exposure of lactating women to DEHP is approximately 714 to 2900 times lower than the TDI value (50 µg/kg body weight/day) (EFSA 2005c).

To the best of our knowledge, this is the first risk assessment study for phthalates in pregnant or lactating women in Iran. Several studies on phthalate exposure through other routes have been conducted. In this regard Wormuth et al. (2006) studied the total intake of phthalates in Europeans using a multi-pathway approach. In this study, phthalates and other chemicals were detected in 99–100% of pregnant women (Wormuth, Scheringer et al. 2006).

In biomonitoring studies, it has been suggested that pregnant women are exposed to multiple chemicals simultaneously (Huang, Kuo et al. 2007). Therefore, the early parts of an infant's life are regarded as the most vulnerable periods of exposure to phthalates (NAS 2008). Considering the fact that human can be exposed to phthalates through different sources, the aim of the present study was to identify if bottled water is the main source of phthalates intake. Our findings show that exposure to phthalates through PET-bottled water in three target groups is very low and can be considered safe, even in the worst storage conditions.

As given in Table 4, the calculated HQ for DEHP, DBP and BBP in water at 40ºC compared to that under other storage conditions was higher; but even in these conditions, the health-related risk to the vulnerable groups was very low and negligible. The intakes of individual phthalates via drinking water were also found to be much less than currently published RfD benchmarks (Table 4). For example, the currently published RfD for DEHP on U.S. EPA's IRIS database is 20µg/kg/day (U.S.EPA 2012), whereas our estimation regarding the intake of DEHP in lactating women from drinking water stored in a refrigerator (4ºC - 8ºC) was 0.03µg/kg body weight/day (0.15% RfD). In a study conducted by Zare Jeddi et al. (2015) on the intake of DEHP in children (considered the most vulnerable group) via drinking water kept in a refrigerator (4ºC -8 ºC), the result was only 0.1µg/kg body weight/day (0.5% RfD) which is slightly higher than our finding (Zare Jeddi, Rastkari et al. 2015b). Very low-level exposure to phthalates has been well documented in adults (Montuori, Jover et al. 2008, Schmid, Kohler et al. 2008, Amiridou and Voutsa 2011). In addition, Clark (2003), in a study aimed to assess the exposure pathways, reported that drinking water, in general, represents less than 0.2% of exposure to phthalates (i.e. DEP, DBP, and DEHP) (Clark 2003).

The execution of a cumulative risk assessment based on hazard indices showed no cause of concern for pregnant, lactating and adult women. However, a future cumulative risk assessment should consider the simultaneous exposure to all chemicals that have anti-androgenicity effects. If this is

not done, it is likely that we have substantially underestimated cumulative risks from anti-androgens.

Furthermore, there are discrepancies in the classification of DEHP as to its carcinogenicity. According to EU Risk Assessment on DEHP *(JRC European Commission Report 2008)*, it is not genotoxic in *in vitro* and *in vivo* studies and is not carcinogenic (tumors observed are likely caused by peroxisome proliferation in rodents). In addition, according to International Agency for Research on Cancer (IARC), there is inadequate evidence in humans for the carcinogenicity of DEHP, hence it is classified as "possibly carcinogenic to humans". The U.S. EPA's Integrated Risk Information System (IRIS), however, states that information on the carcinogenic effects of DEHP in humans is not available and classifies it as Group B2 (U.S.EPA 2012, IARC 2013). Additionally, a literature review of DEHP genotoxicity and potential carcinogenic mechanisms reported that this chemical can produce the mentioned effects at concentrations below those inducing apoptosis or necrosis. These effects include damage to DNA and chromosome, increased transformation, reversal of apoptosis in tumor cell lines and nuclear receptors, increased cancer progression and gene expression changes observed at low concentrations (Caldwell 2012).

The carcinogenic risk posed by the concentration of DEHP was found to be far below the accepted risk level (10^{-6}) for cancer risk. For reference, the concentration of DEHP in drinking water corresponding to an excess estimated lifetime cancer risk of 1 in 1,000,000 is 0.028 µg/L. In fact, the carcinogenic risk posed by the highest concentration of DEHP in bottled water is negligible.

This research did have some limitations. We could not analyze all brands of bottled water which are being sold in Iran, although popular brands were used to conduct this study. Since the sample monitoring period was 45 days, the findings were limited to this time span. In addition, potential seasonal variations in phthalate levels were not examined and were not reported within the context of this study. Furthermore, other forms of EDCs were not analyzed in this study. While the present study focused only on exposure via water, the use of phthalates in many consumer products has been recognized within the scientific and regulatory community as an important issue for human exposure.

Conclusion

The present study on release of phthalates from PET-bottles into the water, confirms that all examined storage conditions especially freezing ones, are safe and do not result in the release of dangerous levels of hazardous contaminants (DBP, DEHP and BBP) into the bottled water. In addition, the observed levels of phthalates in bottled water were not significant in terms of health-related issues and should not be considered as a matter of concern, because daily exposure to phthalates through the consumption of bottled water has been fairly fewer than the toxic range. Consequently, PET-bottled water is not a major contributor to phthalates intake among most of the individuals. Moreover, we suggest keeping the bottled water at a temperature less than 25°C and far away from direct sunlight to minimize the level of exposure.

Human beings can be exposed to several phthalates, simultaneously. Nevertheless, the risk caused by cumulative effects of exposure to several phthalates requires to be considered. Therefore, understanding exposures to mixtures across the life span (cumulative risk assessment) is critical for improving risk assessment and chemical safety.

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CHAPTER 6

The role of phthalate esters in autism development: A systematic review

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Abstract

Background: Available evidence implicates environmental factors in the pathogenesis of autism spectrum disorders (ASD). However, the role of specific environmental chemicals such as phthalate esters that influence ASD risk remains elusive. This paper systematically reviews published evidences on association between prenatal and/or childhood exposure to phthalate and ASD.

Methods: Studies pertaining to systematic literature search from Scopus, PubMed, PsycInfo and Web of Science prior to December 2015 were identified. The authors included studies which assessed the effect of exposure to phthalates on occurrence of ASD. This comprehensive bibliographic search identified five independent studies. Each eligible paper was summarized with respect to its methods and results with particular attention to study design and exposure assessment. Because of the heterogeneity in the type of included studies, different methods of assessing exposure to phthalates and the use of different statistics for summarizing the results, meta-analysis could not be used to combine the results of included studies.

Results: The results of this systematic review have revealed the limited number of studies conducted and assessed phthalate exposure. Seven studies were regarded as relevant to the objectives of this review. Two of them did not measure phthalate exposure directly and did not result in quantitative results. Out of the five studies in which phthalate exposure was mainly measured by the examining biomarkers in biological samples, two were cohort studies (one with positive results and another one with not clear association). Among the three case control studies, two of them showed a significant relation between exposure to phthalate and ASD and the last case control study had negative results. Indeed, this case control studies showed a compromised phthalate metabolite glucuronidation pathway, as a probable explanation of mechanism of the relation between phthalate exposure and ASD.

Conclusions: This review reveals evidence showing a connection between exposure to phthalates and ASD. Nevertheless, further research is needed with appropriate attention to exposure assessment and relevant pre and post-natal cofounders.

Introduction

Since the industrial revolution, synthetic chemicals have been increasingly manufactured in order to be used in almost every product with which we are in contact. From a scientific perspective, recent data have shown that nearly all the people regardless of age and sex are being exposed to hundreds of these man-made chemicals worldwide (Meeker, 2012). It has been proved that nearly two hundreds of these chemicals are neurotoxic in humans; and even worse, based on laboratory analysis, more than 1000 of such compounds can potentially be neurotoxic (Schwartzer et al., 2013). However, less than 20% of high-volume chemicals have been screened for potential neurodevelopmental toxicity during early development (Landrigan, 2010). It should be noted that human brain, at its early developing stage, is highly vulnerable and sensitive to the damages caused by environmental neurotoxicants. In fact, exposure of the brain to neurotoxicants at this stage could damage this vital organ in a way which is far worse than what it does to an adult brain (Grandjean and Landrigan, 2006; Weiss, 2000). This susceptibility roots from the fact that during the 9 months of prenatal life, the human brain develops from a strip of cells along the dorsal fetal ectoderm into a complex organ consisting billions of precisely located, highly interconnected and specialized cells. In fact, exposure to environmental chemicals, especially endocrine disruptor chemicals (EDCs), during the brain growth spurt (BGS) in prenatal period, has been suggested to be a possible causal factor for neurodevelopmental disorders (Colborn, 2004; Kim et al., 2010). In this regard, autism spectrum disorders (ASD) and attention-deficit/ hyperactivity disorder (ADHD) could be the outcomes of exposure to these chemicals (Miodovnik, 2011; Tanida et al., 2009). The BGS period usually begins during the third trimester of pregnancy and continues throughout the first two years of life (Kim et al., 2010). Although the involvement of genetic abnormalities in developing ASD is well-accepted, it is widely believed that a single genetic risk factor cannot cause ASD. In other words, the most likely cause of ASD might be genetic susceptibility besides the exposure to environmental neurotoxic compounds (Hertz-Picciotto et al., 2006). In fact, this hypothesis provides a plausible explanation for the rapid increase in the incidence of ASD over the past few decades (Hertz-Picciotto and Delwiche, 2009).

ASDs are comprised of a broad spectrum of heterogeneous, neurodevelopmental disorders (Ashwood et al., 2006). Previously, disorders which were considered as part of the autism spectrum were divided into the following discrete categories: Autistic Disorder, Asperger's Disorder, and Pervasive Developmental Disorder, Not Otherwise Specified (PDD-NOS), as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). The DSM-V, published in May 2013, combined the previous categorical disorders into a single category of

"Autism Spectrum Disorder," with varying degrees of severity depending on the amount of support required by an individual. Because no medical or biological marker exists for ASD, the diagnosis is mostly based on behaviours (APA, 2013). Thus ASD, similar to the one first described in 1943 by Kanner, is a complex developmental disability with social, cognitive, and communicative deficits (Kanner, 1943). The symptoms of autism usually appear before a child reaches the age of three and last throughout the life (CDC, 2012). From a social point of view, some children with ASD have difficulty in understanding the fact that others think differently from the way they do, and in coordinating attention with a social partner. Regarding the cognitive deficit, some children have weak central coherence and executive dysfunction and these continue into adulthood in some individuals (Mendes, 2013).

In a scientific statement published by the Endocrine Society in 2009, it was argued that endocrine disruptors indeed pose a "significant concern for public health" (Diamanti-Kandarakis et al., 2009). Recently, due to proven adverse effects on human health, concerns over a class of chemicals namely, phthalates has also emerged (Myers, 2012). Phthalates with a di-ester structure are additive polymers applied as plasticizers to produce high volumes of synthetic chemicals (Miodovnik et al., 2014a). In fact, these chemicals are being used to provide flexibility, durability, and solubility and can be found in a wide range of products used in daily life (Lyche et al., 2009); many of these products do not require labeling of phthalates as an ingredient (Dodson et al., 2012). Currently, over a dozen forms of phthalates are in commerce among which di(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DiNP), butylbenzyl phthalate (BBP or BBzP), diisoheptyl phthalate (DiHP), di-n-butyl phthalate (DBP or DnBP) and diethyl phthalate (DEP) are the most commonly produced forms (Miodovnik et al., 2014a). Concerns over human exposure to phthalates root from the fact that these compounds do not form a covalent bound with the polymer matrix. In other words, phthalates may leach or outgas into their surroundings. Humans are exposed to phthalates via ingestion, inhalation and dermal exposure during their whole lifetime including intrauterine development. (Heudorf et al., 2007; Zare Jeddi et al., 2015). In fact, it is not surprising that metabolites of some phthalates can be detected in saliva, urine, amniotic fluid and breast milk (Fromme et al., 2007; Koch et al., 2006; Koch et al., 2011; Völkel et al., 2014). Since, the potential consequences of human exposure to phthalates have raised concerns among the general population, these compounds have been studied in terms of their effects on susceptible subjects, including pregnant women, infants and children (Jurewicz and Hanke, 2011).

Ubiquitous environmental contaminants (phthalates in particular) can be potential risk factors for the pathogenesis of ASDs while interfering with neurological development (Schug et al., 2015;

Ventrice et al., 2013). Carbone et al. (2013) have conducted a study that shows DEHP has anti-androgenic effects, and this can be related to anxiogenic-like effects in rats. This finding shows that the endocrine effects of phthalates can lead to other neurological/psychiatric diseases (Carbone et al., 2013). Furthermore, EDCs can interfere with the thyroid's hormonal functions and in turn result in neurodevelopmental outcomes (De Cock et al., 2012). Regarding this, it can also be implied that exposure to exogenous agents, particularly during critical prenatal or early post-natal windows of development, might interfere with the expression of genetic susceptibility (Hertz-Picciotto and Delwiche, 2009; McDonald and Paul, 2010).

So far, among the environmental neurotoxicant factors, a number of epidemiological studies have been conducted in order to evaluate the possible association between exposure to phthalate and the risk of developing autism in human subjects. However, to the best of our knowledge, there is no thorough study in which the issue at hand is fully discussed and studied. There are Review articles such as those conducted by Kalkbrenner et al., (2014), Rossignol et al., (2014) and Marijke de Cock et al., (2012) and other related reviews investigating the relationship between environmental contaminants, including phthalates and other endocrine disruptors and autism (De Cock et al., 2012; Kalkbrenner et al., 2014; Matelski and Van de Water, 2016; Rossignol et al., 2014; Sealey et al., 2016). However, they did not fully discuss the existing data on phthalates and risks of ASD. Moreover, in these reviews, the limitations and strengths of related studies were not taken into account. Thus, the present study aimed to systematically review the previous studies on exposure of humans to phthalates which had resulted in autism in order to help producing more reliable evidence for future studies.

Material and Method

Search strategy and selection criteria

The present systematic review was conducted by means of following the preferred reporting items for systematic reviews and meta-analyses guidelines. To identify pertinent articles which have been published up to December, 2015, specific terms relating to exposure in combination with outcome-related keywords were used to search the literatures in multiple international databases, including Pub Med (www.ncbi.nlm.nih.gov), ISI Web of Science (www.isiknowledge.com), Scopus (www.scopus.com), PsycInfo (www.ebscohost.com/academic/psycinfo) databases, and Google Scholar. The authors formulated the search strategy by employing a combination of the following concepts: "Autism spectrum disorder", "phthalate", and all of their possible variations and synonyms and the use of Boolean operators, such as "OR" to explode and "AND" to combine. The final terms used in the search strategy were:

((("Autism spectrum disorder*") OR (autism) OR ("autistic disorder*") OR ("Pervasive Development* Disorder*") OR (PDD) OR (ASD)) AND ((*phthalate*) OR ("*phthalic acid*") OR (plasticizer*) OR ("endocrine disrupter*") OR ("*phthal* ester*) OR (plastic*) OR ("polyvinyl chloride*)" OR ("polyethylene terephthalate*") OR (PET) OR (PVC))).

Moreover, to ensure that relevant papers were not missed, the reference lists of retrieved articles were screened for additional relevant studies. The researchers have searched and located the papers in which those most relevant studies have been cited (forward citation). Also, grey literature was searched on the World Health Organization (http://www.who.int/en), U.S. FDA (http://www.fda.gov/) and Health Canada (http://www.hc-sc.gc.ca/ahc-asc/pubs/index-eng.php) websites in order to identify relevant missed articles. The authors did not impose any restrictions on the time of publication or language, study design and publication status.

Study selection and eligibility criteria

Having removed duplicates, two authors independently screened titles and abstracts to ensure that articles met the inclusion criteria and irrelevant papers were excluded. Where uncertainty arose regarding the eligibility of an article from its abstract, the authors retrieved the full-text version of the article and evaluated it against the inclusion criteria. Also, discrepancies were resolved through consultation and consensus building. Finally, the full text of identified papers was deeply explored in order to be sure that only relevant papers were selected to be included in the review for quality assessment and data extraction.

Inclusion criteria

In our review, we included studies which met the following criteria: (a) original articles (b) all observational (i.e., cohort, case-control and cross-sectional) studies; (c) studies with assessment of pre- or post-natal exposure to phthalate esters (PEs) through a biomarker of exposure; (d) publications were only included if the outcome measured and reported in those studies was related to autism and not autistic-like disorders or other health outcomes; (e) Studies conducted on human subjects.

Exclusion criteria

Review articles, hypothesis papers, conference papers and letters to the editor which did not present unique or new data were excluded from this study. Publications of animal models were also excluded. The researchers also excluded articles that their outcomes were related to autistic-like but not ASD or other behavioral disorders.

Figure 1 shows the process of selecting relevant papers for our systematic review based on the PRISMA flow diagram (Moher et al., 2009).

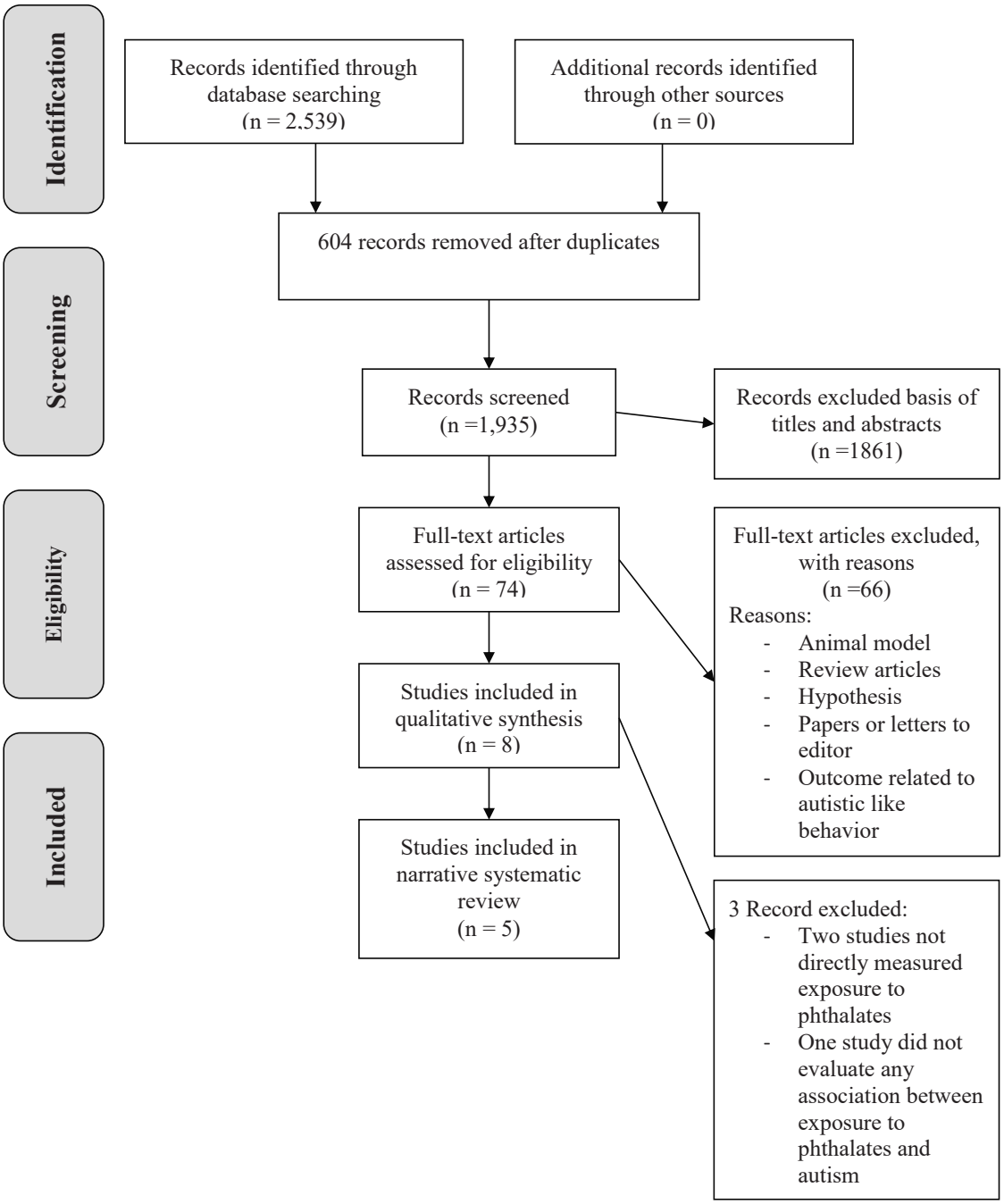

Figure 1. PRISMA flow chart of publications examining estimated phthalate esters relation to autism spectrum disorder (ASD)

Quality assessment

The methodology of each eligible paper was assessed using a checklist based on Strengthening and Reporting of Observational Studies in Epidemiology (STROBE) statement to assess the methodological quality of the observational studies (Daneshparvar et al., 2016; Olmos et al., 2008; Ricci-Cabello et al., 2010; Scales et al., 2008). (Von Elm et al., 2008). This tool was initially developed to assess clarity in reporting research results of the observational studies. The STROBE tool uses a systematic approach to appraise three broad areas namely, study validity, an evaluation of methodological quality and presentation of results, and assessment of external validity.

Of 22 items listed in the checklist, 9 items that were related to the methods section were selected; in other words, these selected items can be used to assess the different aspects of methodology in an observational study (Appendix A). It should be mentioned that the authors equipped each question in the modified checklist with "Yes" or "No" answers and scored them with 1 and 0, respectively; therefore, the final score was in the range of 0 to 9. After performing the assessment, the methodological quality was classified according to the following procedure: articles which had their final scores in the range of 0–3, 4-6, and 7-9 were respectively considered with low, medium and high methodological qualities.

Data extraction and abstraction

In addition, a pre-designed standard data collection form was used to systematically extract the data from each selected study. The required data to be extracted from each article was the general characteristics of the study (i. e. first author's name, year of publication, study location, study design, type of study and study period), as well as the characteristics of the study population (i. e. age and sex of studied participants, the sample size, type of exposure, exposure measurements, outcome scales, and effects studied). Two reviewers (MZJ) and (LJ) extracted data independently whilst another (MY) checked the extracted data from all eligible papers.

Statistical analysis

Study outcomes were summarized using narrative and quantitative methods. Because of the heterogeneity in type of the studies which were included, different methods of assessing exposure to phthalates, different scales for detecting autism and also using different statistics for summarizing the results, meta-analysis could not be used to combine the results of included studies.

Result

Bibliographic Search

A total of 2,539 records have resulted from the combined database searches. After duplicates were removed, 1,861 of them were excluded in the initial screening of manuscript titles and abstracts. Then, by screening the full texts of the remaining 74 articles according to the inclusion/exclusion criteria, seven studies were regarded as relevant to the objectives of this review. Five studies (three case-controls and two cohorts) (Braun et al., 2014; Kardas et al., 2015; Miodovnik et al., 2011; Stein et al., 2013; Testa et al., 2012) which were related to analyzing biochemical markers in association with the autism and phthalates exposure, were selected (Figure 1). However, of those seven, two studies (Larsson et al., 2009a; Philippat et al., 2015) were excluded as they did not use bio-monitoring approach (direct measurement). One of them was a Swedish cohort study on association of ASD and type of flooring material as polyvinyl chloride (PVC) (Larsson et al., 2009b). Larsson et al. (2009) did not directly implicate the evaluation of possible link between phthalates exposure and autism. Thus, phthalate metabolites were not measured in biological samples of the patients. Indeed, assessment of the exposure to phthalate was based indirectly on the questionnaire data and eventually, they reported that ASD was significantly associated with PVC as flooring material (in the parent's bedroom). In the second investigation, Philippat et al., (2015) studied phthalate concentrations in the house dust in association with the risk of developing ASD or developmental delay (DD). Participants were a subset of children from the case–control study of CHARGE (Childhood Autism Risks from Genetics and the Environment). Similar to the pervious article, in this study phthalate metabolites were not measured in biological samples of children with ASD. Instead, Philippat et al., measured the concentration of five phthalate esters in the dust collected from the child's home using a high volume small surface sampler. This study reported that detection frequency of phthalates in the home dust was 63 % for DMP, 92 % for DEP and 99 % for DEHP, DBP and BBzP. However, none of the dust phthalate concentrations was associated with the risk of ASD. In addition, they found no association of vinyl flooring with the diagnosis of ASD (Philippat et al., 2015). One of the possible explanations is that, vinyl flooring was found in 37 % of residences in CHARGE study as compared to the Sweden cohort in which vinyl flooring has been reported in 52 % of the children's bedrooms and 45 % of the parents' bedrooms. However, due to inconsistency in method of autism and phthalate exposure measurement we had to eliminate this study.

It is to be note that, there was one article in the United States and Mexico involving 71 deciduous teeth, primarily molars and canines (without cavities or fillings), from children with ASD who were chosen from IRB-approved pilot studies on autism through the University of Texas Health Science Center (UTHSCSA) (Palmer et al., 2015). The mentioned article has not assessed any association between exposure to phthalate and ASD. Instead, this study objective was to evaluate the use of deciduous teeth as a new biological sample for measuring early life exposure to semi-volatile organic chemical metabolites such as monoethyl phthalate (MEP), mono-n-butyl phthalate (MnBP), monoisobutyl phthalate (MiBP), monobenzyl phthalate (MBzP), and mono-2-ethylhexyl phthalate (MEHP) in autistic children. In this report, detection rates of all phthalate monoester metabolites except MBzP (0-6%) were between 36-100% (Palmer et al., 2015). This report provided evidence that deciduous teeth can be used as a useful medium for measurement of early life exposure to organic contaminants biomarkers in epidemiological case-control studies. However, they did not provide any information regarding the relationship between early life exposure to these chemicals and ASD.

Narrative Analysis

We reviewed various aspects of the included studies and presented the results narratively. As mentioned earlier, due to the large variation in the type of studies and methods used to assess exposure and outcome, we could not combine the results using Meta-analysis.

Overview of the type of study

Tables 1 to 5 show the characteristics of studies included in this review. Across the relevant studies, three were case-control studies (Kardas et al., 2015; Stein et al., 2013; Testa et al., 2012) and the other two were prospective cohort studies (Braun et al., 2014; Miodovnik et al., 2011).

In all of the included studies, phthalate exposure was mainly measured by the examining biomarkers in biological samples; for instance, HPLC electrospray ionization MS (HPLC-ESI-MS), and isotope dilution-liquid chromatography mass spectrometry–mass spectrometry (ID-LC-MSMS) were applied in order to measure the concentration of phthalate in urine and serum samples (Table 1 and 2). In addition, autism characteristics in participants were assessed using different methods. As shown in Table 1, selected cohort studies (Braun et al., 2014; Miodovnik et al., 2011) used the Social Responsiveness Scale (SRS). The SRS is a well-validated tool for quantitative autism spectrum assessment particularly for social impairment identification while it is highly correlated with gold standard diagnostic instruments such as the autism diagnostic observation schedule

(ADOS) and Autism Diagnostic Interview-Revised (ADI-R) (Daniels et al., 2012). It should be noted that in these cohort studies, the mean change in SRS score was considered as an indicator of the severity of autistic behavior. However, in case-control studies (Kardas et al., 2015; Stein et al., 2013; Testa et al., 2012), ASD were assessed by gold standard tools including ADOS or ADI-R and Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV TR) criteria (Table 2).

Quality assessment

Based on the quality assessment procedure of the five selected studies, using the mentioned criteria, the researchers found that only one study (Braun et al., 2014) illustrated high quality. The other four studies had medium quality in terms of predefined quality assessment criteria (Table 3).

Effects of Quantified exposure to phthalate on autism

Miodovnik et al. (2011) conducted a multiethnic cohort study on 404 primiparous women who delivered at Mount Sinai Hospital between May 1998 and July 2002, with follow-ups to 2009.

Table 1. Summary of cohort studies' characteristics and exposure- outcome assessment methodology

Study	location	Autism data source	Birth years	Sample size	Sex / age	Exposure measurement	Exposure timing	Outcome Assessment
Miodovnik et al., (2011)	New York City, New York, USA	The Mount Sinai Children's Environmental Health Study	1998-2002	137	Gender not specified / 4 to 9 years of age	Laboratory analyzed for 10 individual high and low molecular phthalate metabolites* in Maternal spot urine samples were collected during pregnancy	Mid-late pregnancy between 25 and 40 weeks (mean of 31.2 weeks)	Social Responsiveness Scale (SRS) when child was 7-9 years
Braun et al. (2014)	Cincinnati, Ohio metropolitan area, USA	The Health Outcomes and Measures of the Environment (HOME) Study	2003-2006	222	Gender not specified / 4 and 5 years of age	8 Phthalate metabolites** measured in twice maternal urine samples with sensitive and specific gas chromatography mass spectrometry and ceratinine standardized	Pregnancy around 16 and 26 weeks of gestation	Social Responsiveness Scale (SRS) at ages 4 and 5 years

- HMWphthalates =DEHP metabolites [MECPP, mono (2-ethyl-5-carboxypentyl) phthalate; MEHHP, mono (2-ethyl-5-hydroxylhexyl) phthalate; MEOHP, mono (2-ethyl-5-oxohexyl) phthalate; and MEHP, mono (2-ethylhexyl) phthalate]; MBzP, monobenzyl phthalate; and MCPP, mono (3-carboxypropyl) phthalate.
  LMW phthalates =MMP, monomethyl phthalate; MEP, monoethyl phthalate; MBP, monobutyl phthalate; and MiBP, mono-isobutyl phthalate.

** MBP, mono-n-butyl-phthalate; MBzP, monobenzyl phthalate; MCPP, mono(3-carboxypropyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEP, monoethyl phthalate; MiBP, monoisobutyl phthalate.

Table 2. Summary of Case-Control studies' characteristics and exposure-outcome assessment methodology of studies included in the review

Study	Location	Autism data source	Population	Sex (%) / age	Exposure measurement	Outcome Assessment
Testa et al (2012)	Siena, Italy	Staff of Children Neuropsychiatric Department	48 with autism 45 healthy controls	36boys, 12girls/ age at examination: 11±5 years 25 boys, 20 girls / age at examination: 12±5 year	Urinary concentrations of the primary and secondary metabolites of DEHP [di-(2-ethylhexyl) phthalate] by HPLC-ESI-MS (HPLC electrospray ionization MS), was applied to urine spot sample.	All the patients with ASD, diagnosed by Diagnostic and Statistical Manual of mental disorders (DSM IV) and evaluated using ADOS (autism diagnostic observation schedule), ABC (autism behavior checklist) and CARS (childhood autism rating scale) scores entered the study.
Stein et al (2013)	USA	Pediatric Neurology and Pediatrics clinical practices at University of Medicine and Dentistry of New Jersey (UMDNJ), New Jersey Medical School	50 children with autism 53 healthy controls	76% boys / age at examination: 10.26 ± 3.83 55% girls / 10.74 ± 4.03	The concentration of free phthalates and total phthalates in the collected spot urine samples between 10:00 a.m. and 4:00 p.m.was measured by isotope dilution-liquid chromatography mass spectrometry–mass spectrometry (ID-LC-MSMS) using minor modifications.	All autistic subjects were under the care of the pediatric neurologist and the diagnoses were made by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV TR); 52 % of the subjects were further confirmed by Autism Diagnostic Interview-Revised, and/or Autism Diagnostic Observation Scale-Generic criteria.
Kardas et al (2015)	Kayseri, Turkey	Children's Hospital of Erciyes University Medical School (Kayseri, Turkey) between May, 2012 and May, 2013.	48 children with autism 41 healthy subjects	27 boys, 21 girls/ age at examination: 7.54 + 2.92 24 boys, 17 girls/ age at examination: 7.47 + 2.79	MEHP and DEHP concentrations were determined by using high performance liquid chromatography in serum sample.	The diagnosis of autism was made according to the criteria of the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM IV) and the Autism Behavior Checklist.

In this study, they focused on social behavior of the school-aged children with respect to the prenatal exposure to phthalates. Through linkage between maternal exposure to phthalates and children who were diagnosed with ASD in the New York City, at the last childhood evaluation, when the child was between the ages of 7 and 9 (n= 137), a survival analysis of the time to diagnosis in children was used to estimate the incidence of ASD according to the SRS completed by mothers. In this article, the maternal spot urine samples were analyzed by 10 phthalate's metabolites and expressed as sum of the high-molecular-weight (>250 Da), namely, monoester metabolites (∑HMW) and low molecular-weight (<250 Da) known as monoester metabolites (∑LMW). The reason for this type of classification is that the phthalate metabolites within each grouping demonstrate similar molecular structure, biological activity and sources of exposure as the parent diester. Metabolites and methods of detection which have been used in the studies are also shown in Tables 1 and 3. Compared with the original birth cohort (n= 404), the median urinary concentrations of the low and high molecular phthalates metabolites were similar in the women who returned for follow-ups (n = 137). Accordingly, there were no significant differences with respect to median urine concentrations of phthalate metabolites between the original birth cohort and those at 7–9 year follow-ups. In addition, among the 137 children in follow-up stage of the cohort study, based on the SRS score, 106 children were in the normal range of social impairment, 25 children had mild social impairment and the other six children had scores higher than 75 which were strongly associated with a clinical diagnosis of ASD. Regarding the prenatal LMW phthalate urine biomarkers in relation to SRS T-scores at age 7-9 years, in thirty-one (22.6%) children who met the threshold level of "Mild to Moderate" (SRS T-score of 60–74) and "Severe" (SRS T-score≥ 75) social impairment in the studied population, the LMW phthalate metabolite concentrations were 460 µg/L (n= 25) and 1260 µg/L (n= 6), respectively. It implied that relative to the healthy children with normal SRS T-score, children with mild and severe social impairment had highest concentrations of phthalates. In general, the scores of few children exceeded the cut-off value which identifies children with clinically significant social impairments. On one hand, any increase in the log-unit of LMW phthalate metabolite concentration was associated with higher SRS scores; and among the investigated LMW phthalate metabolites, only MEP was found to be statistically significant (Table 4). On the other hand, no consistent association between SRS scores and HMW phthalate metabolites was found. Furthermore, although a positive association between ASD and the LMW phthalate level was observed, statistical significant difference was not found (LMW phthalates, p= 0.09; HMW phthalates, p= 0.54).

The second study in which the phthalate exposure and its adverse birth outcomes were measured was conducted from March 2003 to January 2006 (Braun et al., 2014). In the prospective birth

cohort study on mothers and their children participating in the Health Outcomes and Measures of the Environment (HOME), a study from Cincinnati Ohaio was aimed at assessing the association between low-level environmental chemical exposure and children's growth and development. Two spot urine samples were taken from each pregnant woman (n= 389) who were between 16 to 26 weeks of their pregnancy (Table 1). The following 222 mother–child pairs (57%) completed the SRS when their children were 4 (n= 184), 5 (n= 205) years of age and at both 4 and 5 years of age (n= 135). Of the entire 135 participants, the scores of only 22 children were higher than 60 (SRS score≥ 60) and the rest were in the normal range. They also measured the concentrations of eight urinary phthalate metabolites expressed in μg/g creatinine which were similar among women with and without follow-ups when their children were 4 or 5 years old (Table 4). It should be mentioned that all the phthalate metabolites were associated with the autistic symptom scores.

In three case-control studies performed by Testa et al., (2012), Stein et al., (2013), and Kardas et al., (2015) the association of phthalates exposure to ASD were also investigated. In the study conducted by Testa et al., (2012) on an Italian sample, it was tried to evaluate the levels of the primary and secondary metabolites of DEHP in children with ASD by using innovative chemically reversed approach (Testa et al., 2012). This small nested case control study was conducted on 48 children with ASD and 45 children without ASD as the control group. As shown in Table 2, all the scores regarding 48 patients with ASD who were diagnosed by DSM-IV and evaluated using ADOS and CARS (Childhood Autism Rating Scale) were captured in the study. Healthy controls (HCs) were randomly chosen from outpatients who had no pathological symptoms. Determination method of urinary concentrations of DEHP metabolites (MEHP and 6-OH-MEHP [mono-(2-ethyl-6-hydroxyhexyl) 1, 2-benzenedicarboxylate], 5-oxo-MEHP [mono-(2-ethyl-5-oxohexyl) 1, 2-benzenedicarboxylate] and 5-OH-MEHP [mono-(2-ethyl-5-hydroxyhexyl) 1,2-benzenedicarboxylate]) is shown in Table 2. Table 5 shows results of the mentioned study. As shown in this table, urinary excretion of 5-oxo-MEHP (p= 0.005), 5-OH-MEHP (p= 0.0224) and MEHP (p= 0.0312) was significantly higher in autistic patients, compared with the gender- and age-comparable HCs. This study demonstrated a strong association between phthalates exposure and risk of ASD.

Table 3. Outcome and quality assessment of studies, limitations and benefits of articles.

References	Results	Strengths of Study	Limitations of Study	Quality
Testa et al. (2012)	Indicate that specific DEHP metabolites are statistically significantly increased in autistic children.	- Patients with Rett syndrome, X- fragile syndrome, inborn errors of metabolism, 21 trisomy, tuberous sclerosis and gene microdeletions were excluded from the present study. - Urinary creatinine was measured in the children mg/kg per 24 h) and data unadjusted for creatinine.	- Single spot urine specimens were collected in the morning. - Medical history of patient did not screen. - Important perinatal and neonatal potential confounders being appropriately accounted for, limiting potential bias with respect to the prognostic factor of interest has not been investigated. - Genetic data on subjects or their parents has not been collected.	Medium
Stein et al. (2013)	There are an association between phthalate metabolism and autism. So that, the degree of glucuronidation was lower with the autistic group. Although this study shows a compromised phthalate metabolite glucuronidation pathway, this does not necessarily mean that phthalates are directly linked to ASD.	- Medical history and comorbidity data were collected in the autistic subjects. - All subjects were carefully screened for signs of infection or inter-current illness on the day of specimen acquisition, and subjects with acute illness were excluded. - The dietary intake history within 24 h of sampling was recorded, including that of medication and vitamin intake. - Urinary concentration data are frequently normalized to, or controlled for creatinine to control for differences in urine dilution. - Multiple regression analyses were conducted in which, sex, age, BMI, and creatinine were entered simultaneously as covariates with the metabolite indices	- This study included ASD children with or without comorbidity. - Single spot urine specimens were collected - Time of urine sample collection was wide (between 10:00 a.m. and 4:00 p.m). - Genetic data on subjects or their parents has not been collected. - Important perinatal and neonatal potential confounders being appropriately accounted for, limiting potential bias with respect to the prognostic factor of interest has not been investigated.	Medium

Table 3. Continue.

References	Results	Strengths of Study	Limitations of Study	Quality
Kardas et al (2015)	In this study, serum MEHP and DEHP levels were found to be significantly higher in the autism spectrum disorder group when compared to healthy controls (P = .000).	- Patients with pervasive developmental disorder-not otherwise specified, Asperger's syndrome, Rett syndrome, or Childhood disintegrative disorders were excluded. - Patients were excluded if they had genetic disorders, including chromosomal abnormalities, fragile X syndrome, tuberous sclerosis, and neurofibromatosis type 1. - Subjects with acute infection were excluded. - Plastic products have not been used during blood sampling. - Relationship between variables include age, gender, residence and duration of breast feeding with phthalates have been evaluated in both groups.	- Single spot serum specimens were collected, while the time and condition of sampling was not mentioned. - Secondary metabolites did not measured in other biological sample. - Genetic data on subjects or their parents has not been collected. - Medical history of patient did not screen.	Medium
Miodovnik et al. (2011)	maternal urinary MEP concentrations during pregnancy were associated with higher SRS scores in 7- to 9-year-old children	- Urinary concentrations of the biomarkers were examined both as micrograms per liter and corrected for urine dilution as micrograms per gram creatinine (mg/gC). - The following were considered as potential confounders or covariates*.	- Outcome assessment did not rely on the clinical diagnosis of autism but only on symptoms common to the disorder. - Single spot urine specimens were collected. - Mode of measurement of urinary concentration of phthalates has not been mentioned.	Medium

*maternal age (continuous variable), maternal IQ, marital status at the time of follow-up (single caretaker versus living with both parents), maternal education (less than high school versus more than a high school), child race (non-Hispanic white, non-Hispanic black, or Hispanic), sex, child IQ, exact age at examination, and urinary creatinine.

** Maternal demographic and prenatal factors, including maternal age at delivery, race, marital status, education, parity, insurance status, employment, household income, and prenatal vitamin use were obtained using structured interviews and chart reviews conducted by trained research staff.

The other case-control study was aimed at comparing the efficiency of conjugation reactions of DEHP metabolites as a detoxification mechanism in a group of children with documented ASD against healthy children as a control group (Stein et al., 2013). In this study, random spot urine specimens were collected from 50 children with ASD and 53 age-matched HCs between 10:00 a.m. and 4:00 p.m. As shown in Table 2, all ASD subjects were under the care of the pediatric neurologist and the diagnosis was made by the DSM-IV TR. In this regard, 52 % of the subjects were further confirmed by ADI-R, and/or ADOS. Control children were screened for medical and developmental disorders during their well-child visits in addition to chart review, and only those which were free of any chronic or recurrent medical disorders were considered healthy and included in the study. The metabolites measured were mono-2- ethylhexyl phthalate (MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-OH MEHP) and mono-(2-ethyl-5-carboxypentyl phthalate (5-CX MEPP). Based on the results of this study, total MEHP, total 5-OH MEHP and total 5-oxo MEHP in ASD group were lower than those of the control group (Table 5); and, total 5-cx MEPP in ASD children were higher than those in the control group. However, there was no significant difference in urinary concentration of 5-cx MEPP between autistic group and control group. As 5-cx MEPP accounted for more than 90% of the total metabolites of DEHP, therefore, sum of the total amount of DEHP metabolites (free plus conjugated) was similar between case and control groups. In other words, although this study shows a compromised phthalate metabolite glucuronidation pathway, this does not necessarily mean that phthalates are directly linked to ASD. Perhaps, a decreased capacity for glucuronidation in ASD children caused a little higher amount of total free phthalates metabolites in their urine than healthy children (Alabdali et al., 2014; Stein et al., 2013).

The Third case control study was conducted by Kardas et al. (2015) on 48 children with ASD without comorbidity and 41 healthy subjects as controls in Turkey between May, 2012 and May, 2013 (Table 2). In this study, serum MEHP and DEHP levels were found to be significantly higher in the ASD group when compared to healthy controls (P = .000). No significant relationship was detected between gender, residence and duration of breast feeding and MEHP and DEHP within each group (Kardas et al., 2015).

All of these research studies have a number of methodological strengths and limitations regarding outcome-exposure assessment and confounding factors analysis which are summarized in Table 3.

Table 4. Median maternal urinary concentration of phthalate metabolites in cohort studies and unadjusted and covariate adjusted associations (β [95% CI]) between SRS Total score in children and maternal gestational urinary phthalate concentrations.

Study		Median (Interquartile Range)[1]	Unadjusted β [95% CI]	Adjusted model β [95% CI][2]
Miodovnik et al. (2011)	**MEP**	380 (137-1010)	-	1.38 (0.23, 2.53)
	MBP	36 (16-75)	-	1.37 (-0.43, 3.17)
		Median (95% median CI)[3]	**Unadjusted β [95% CI]**	**Adjusted model β [95% CI][4]**
Braun et al. (2014)	**MEP**	133 (25-1010)	1.3 (-1.1, 3.6)	-0.9 (-2.7, 1.0)
	MBP	26 (9.5-75)	0.8 (-1.7, 3.3)	-0.4 (-2.3, 1.5)

[1] µg/ml
[2] Adjusted for child race, sex, caretaker marital status and urinary creatinine.
[3] µg/g creatinine
[4] Adjusted for maternal age (continuous, years), parity (0, 1-2, and 3+), prenatal vitamin use (daily, 1-6 times/week, and never or rarely), maternal IQ (continuous), depressive symptoms during pregnancy (continuous), HOME score (continuous), and gestational serum cotinine concentration (continuous log10 transformed).
MBP-Mono-n-butyl phthalate, MEP-Monoethyl phthalate, µg/g creatinine

Discussion

Over the last three decades, global concern about the public health risk factors attributed to the environmental pollution has been increasing. It can be said that like an iceberg which has only one-tenth of its volume visible, of the thousands of chemicals currently being used, only a small fraction has been identified as neurotoxicants for the human developmental processes. Moreover, many of these chemicals are likely to have stronger effects on fetuses and children, compared with adults. In fact, this visible part, which is the result of previously conducted studies in this field, may only be a small part of a bigger potential problem (Grandjean and Landrigan, 2006).

Although, several researches have been conducted to assess the effects of genetic, environmental and immunological factors on the development of ASD, there is still much to be done regarding the understanding of the ASD etiology (i. e. a phenomenon termed "etiological heterogeneity") (Kalkbrenner et al., 2014). Extensive research in rodent models has shown that phthalates primarily act as anti-androgens which impair testosterone production in Leydig cells (De_falco et al., 2015; Foster, 2005). Furthermore, it is proposed that phthalates may disrupt the endocrine system by interfering with thyroid homeostasis through various mechanisms, including alteration of transcriptional activity of the sodium/iodine symporter, inhibition of the binding of triiodothyronine (T3) to purified thyroid receptors, and inhibition of T3-induced cell proliferation (Boas et al., 2012; Ghisari and Bonefeld-Jorgensen, 2009). Likewise, involvement of phthalates in neurodevelopmental disorders is supported by animal studies that indicate the role of phthalate

induced hypothyroidism in decreased intellectual capacity and development of ASD (Miodovnik et al., 2014b).

Table 5. Comparisons of urinary excretion of secondary metabolites for DEHP in autistic and control groups in case-control studies.

Study	Size		Scale	Effect Median (95% median CI) in µg/ml*		P-value
	With autism	Control		With autism	Control	
Testa, et.al (2012)	48	45	5-OH-MEHP	0.18 (0.037-0.399)	0.04 (0–0.124)	0.0224
			5-Oxo-MEHP	0.096 (0.04–0.17)	0.04 (0.015–0.079)	0.005
			MEHP	0.055 (0–0.11)	0.028 (0–0.059)	0.0312
Stein, et.al (2013)	**With autism**	**Control**	**Scale**	**Effect** Median (25th , 75th) in µg/ml*		**P-value**
	50	53	5-OH-MEHP	0.005 (0.002-0.014)	0.008 (0.004-0.012)	0.12
			5-Oxo-MEHP	0.003(0.001-0.006)	0.004 (0.002-0.006)	0.21
			MEHP	0.011 (0.009-0.014)	0.013 (0.009-0.018)	0.06
Kardas et al (2015)	**With autism**	**Control**	**Scale**	**Effect** Mean + SD in µg/ml		**P-value**
	48	41	MEHP	0.47 + 0.14	0.29 + 0.05	.000
			DEHP	2.70 + 0.90	1.62 + 0.56	.000

MEHP monoethylhexylphthalate, 5-OH-MEHP 5-hydroxy-methylethylhexyl phthalate, 5-oxo MEHP 5-oxo-methylethylhexyl phthalate
*Mann–Whitney U Tests

In this review, the researchers have brought together the existing body of evidence regarding the effects of phthalate exposures on ASD. Findings from this study may highlight the fact that so far, a limited number of studies attempted to assess the phthalate exposure during pregnancy and childhood as an ASD risk factor. Among the five retrieved studies on human subjects from three different countries, three were case-control, while the other two studies were cohort.

Cohort studies are very important as they show the full impact of gestational exposure to phthalates by measuring the maternal urinary concentrations of phthalate metabolites. During this critical period, even low doses of EDCs which may have little effect on adults can have devastating effects on the unborn, neonate and the child. Many substances easily penetrate the placenta during prenatal development and because the fetal blood-brain barrier is not fully formed, toxicants can enter this vital organ and interfere with its development. This can occur through direct toxicity or interference with a variety of cell-signaling mechanisms, including the endocrine system (Colborn, 2004).

In both cohort studies, among the phthalate metabolites, high concentrations of MEP were found in all patients. However, Braun et al., (2014) reported that gestational MEP concentrations in either

adjusted or unadjusted models for co-pollutant confounding had an inverse association with SRS scores (Table 3). The study showed that the beta coefficients for prenatal urine MEP, in relation to total SRS scores was -0.5 (95% confidence intervals -2.2, 1.3) in fully adjusted model as compared with the displayed betas which changed in SRS scores among children born to women with detectable vs. non-detectable levels of these chemicals [β = -0.2 (95% confidence intervals -1.9, 1.5)]. In contrast, as shown in Table 3, in the first prospective birth cohort of 137 mothers and their children, Miodovnik et al., (2011) reported that maternal urinary MEP concentrations during pregnancy were positively associated ($p< 0.05$) with severe social impairments (SRS $\geq$ 75) in 7 to 9 year-old children in adjusted model for race, sex, caretaker material status and urinary creatinine of the child [β=1.38 (0.23-2.53)]. Since in the investigated studies different target groups and confounding variables were presented to the model and different indices were reported as the central tendency and dispersion of results (i.e. median with quartiles and mean with confidence intervals), there was a high level of heterogeneity in the findings of the included studies and that prevented us from combining the results of these studies.

In addition to the hereditary factors, one of the most important points that should be taken into consideration is to avoid under or over estimation of the association between the exposure and neurodevelopment endpoints (Polańska et al., 2013). In the field of ASD particularly, the important risk factors reported by previously conducted studies were pregnancy and delivery complications, low birth weight, too small for gestational age, duration of maternal bleeding during pregnancy, maternal depression, umbilical-cord complications, excess gestational weight gain, maternal prenatal medication (psychiatric medication), being first born versus third or later (birth order), low 5-minute Apgar score, feeding difficulties, meconium aspiration, neonatal anemia, ABO or Rh incompatibility, hyper bilirubinemia and maternal diabetes before and during pregnancy (Gardener et al., 2009; Gardener et al., 2011; Guinchat et al., 2012; Lyall et al., 2014; Xu et al., 2014).

Moreover, it is well known that the prenatal period risk factors, including maternal and paternal ages, demographic variables, geographic coordinates, the socio-economic status, physical activity, smoking status (Smoking during pregnancy and second-hand exposure to smoke) and nutrient deficiencies, have also been reported to be potential risk factors for psychopathology and behavioral problems of the offspring (Dietert et al., 2011; Kolevzon et al., 2007; Sandin et al., 2012). It should also be taken into consideration that mothers' inadequate knowledge about environmental/lifestyle contaminants and greater exposure to environmental contaminants may be associated with ASD (Kim et al., 2010). However, in retrieved studies, only the prenatal risk factors were mentioned among the pre-, peri- and neonatal risk factors. In other words, the perinatal and neonatal risk factors have been ignored.

Since the critical period for the development of a human brain is believed to be between fourth week of pregnancy and the time of delivery, and considering that the sensitivity to damage extends up to early childhood (Schug et al., 2015), exposure to chemicals during critical windows of development can lead to neurodevelopmental diseases. However, all the windows are not equal. Regarding the two cohort studies, Braun et al., (2014) measured phthalates exposure two times at the second trimester, (Weeks 16 and 26), but Miodovnik et al., (2011) evaluated exposure to phthalates during the second and third trimesters of pregnancy (mean of 31.2 Weeks). Although, available evidence from neuroanatomical, animal and epidemiological studies show the prenatal and early postnatal origins of ASD, but the accurate critical windows of susceptibility to neurotoxicant chemicals for ASD have not been fully elucidated. Therefore, in order to provide a better insight for the specific effects of maternal phthalates exposure and to identify critical periods of exposure, it is of interest to assess the exposure to phthalates at several time points during and after pregnancy than only one time point exposure measurement.

In the cohort studies, the way of assessing the exposure to phthalates was based on the measurement of maternal urinary biomarkers. However, in the case control studies, the exposure measurement was based on comparison of the phthalate metabolite concentrations in the urine of ASD children with the control group. Regarding the evidence provided and methods of measuring the phthalates exposure, it can be argued that the case-control studies failed to obtain exact data on maternal exposures in pregnancy period.

Among the three case-control studies, Testa et al. (2011) reported that urinary concentrations of two DEHP metabolites (5-OHMEHP and 5-oxo-MEHP) in the ASD group were significantly higher than control group. Consistent with this study, Kardas et al. (2015) reported that serum MEHP and DEHP levels were significantly higher in the ASD group as compared to controls (Kardas et al., 2015). In contrast to previous case control studies, Stein et al. (2013) did not show any association between phthalates and ASD. However, they reported that despite similar phthalate exposure levels, ASD children had decreased glucuronidation of DEHP (i. e. measured by urinary metabolites) in comparison to the control group, Glucuronidation is notably a significant pathway involved in the metabolism of xenobiotics and lower glucuronidation might lead to a decreased detoxification capacity for phthalates (Stein et al., 2013). Generally, case-control and cross-sectional studies cannot determine the causal relationship regarding the issue at hand, because the temporal relationship between disease occurrence and exposure cannot be established (Song and Chung, 2010). In addition, a single case-control study in which a single biomarker is measured is not appropriate. In other words, biomarkers have usually short half-life and there is a long period between exposure to phthalates and its outcomes.

Since phthalates, as non-persistent compounds, have short biological half-lives, phthalate metabolites only represent exposure for a few days instead of the entire relevant period of development (Kalkbrenner et al., 2014). Therefore, a single urinary measure of biomarkers for phthalates does not suffice, and, it may not adequately reflect long-term exposure level to these chemicals. It is believed that in the case of phthalates, since ongoing exposure to these chemicals is expected, urinary phthalate metabolites appear to be stable over a period of days to months (Miodovnik et al., 2014b). However, a single measurement during pregnancy, delivery or childhood period may not be sufficient in determining the window at which the exposure occurred. In the evaluated studies, two-time urine sample measurements from pregnant women were taken only in one perspective cohort study (Braun et al., 2014).

Several concerns and limitations have emerged from this topic. First, the issue is related to the small number of studies with similar health endpoints. In addition to the paucity of evidence, the findings were inconsistent which this inconsistency could be explained by differences in the collection time of urine samples, instruments of measurement and health endpoints across the studies. The potential for misclassification of the exposure due to the short biological half-life of phthalates in humans is the next point. It is believed that single urinary concentrations can only reflect the recent exposures over the past 6 to 12 hours. Therefore, a single spot urine sample cannot accurately classify long-term exposure (over weeks, months or years), since data shows that exposures are often episodic and vary over time (Braun et al., 2010).

In addition, many studies only assessed phthalate exposures during specific periods of time (e. g. during gestation (in cohort studies) or early childhood (in case-control studies)). Since there are several critical time periods during development, the precise timing at which the exposure to a toxicant is most disruptive is unclear. It is also of concern that the timing of spot urine collection was different in these studies. Therefore, this difference may account for the inconsistent results.

Finally, information from extensive body of literature on the association between phthalate exposure in prenatal and postnatal period and ASD is limited. Thus, the researchers could not apply meta-analysis as well as graphical or statistical methods to assess any publication- related biases. Therefore, there is need to conduct large, well-designed prospective cohort studies and in doing so, the relevant pre-, peri- and neonatal confounders and characterization of the exposure should be taken into consideration.

Conclusions

ASD is a serious neuro-developmental disorder with heterogeneity in the behavioral symptoms and it affects the functions and structures of the brain (Ratajczak, 2011). It is clear that there is no single or universal cause of ASD; rather, many environmental and genetic factors are likely to be involved. Although, over the last decade, potential contributions of environmental chemicals and conditions to the etiology of ASD have been the subject of current researches and speculations, the field is still at its early stages. Till now, a few studies support a potential role for phthalate exposures in relation to ASD. Considering the insufficiency of the number of identified studies and the heterogeneous methods used in these studies, it is difficult to provide a definite conclusion. In this review, the authors have provided a useful summary of existing research findings. This review reveals evidence showing a connection between exposure to phthalates and ASD. Nevertheless, results of the retrieved studies confirm the shortage of knowledge in this important area and confirm that the major limitations of the existing studies are related to both the exposure and outcome assessments. Therefore, the association between exposure to phthalates and ASD requires further well-designed pregnancy cohort studies in order to aid understanding and validation of the findings from population-based studies.

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CHAPTER 7

Biomonitoring and subsequent risk assessment of combined exposure to phthalates in Iranian children and adolescents

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Abstract

The aims of this study were to estimate the exposure pattern and daily intake of five different phthalates, dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), diethylhexyl phthalate (DEHP), dimethyl phthalate (DMP), and diethyl benzyl phthalate (DEP), and to assess the health risks from combined exposure to the anti-androgenic phthalates including DEHP, DBP and BBP in Iranian children. To this end we determined the internal exposure of 56 Iranian children and adolescents aged 6 to 18 years by analyzing seven urinary metabolites of five phthalates. Using urinary excretion fractions (F_{UE} values), urinary concentrations of the various metabolites were converted to estimated daily intakes (EDIs) for the respective phthalates. The hazard quotient (HQ) approach was used to evaluate the potential risk posed by exposure to the single phthalates. Assuming additive effects, the hazard index (HI) was utilised for assessing the combined risk of anti-androgenic phthalates. Furthermore, the maximum cumulative ratio (MCR) was applied to quantify the degree to which a single phthalate drives the risk from combined exposure. The phthalate metabolites detected indicated exposure to BBP, DBP and DEHP previously identified as priority chemicals. The EDI values derived from the biomonitoring data ranged from 0.01 µg/kg body weight (bw)/day for BBP, to17.85 µg/kg bw/day for DEHP. The risk assessment revealed that not only the exposure to the individual phthalates, but also the combined exposure did not raise a safety concern (HI values in the surveyed participants averaged 0.2). The range of MCR values in the participants varied from around 1 for most individuals to around 2 in some individuals, indicating that the combined exposures were dominated by one and in some cases by two of the three anti-androgenic phthalates, especially DBP and/or DEHP. It is concluded that biomonitoring data indicate that the overall combined exposure of Iranian children and adolescents to phthalates does not raise a concern, while reduction of exposure is best focused on DEHP and DBP that showed the highest HQ.

Introduction

Phthalates are diesters of phthalic acid with a wide variety of industrial applications. There is an interest in the safety evaluation of phthalate exposure because these compounds are ubiquitous environmental contaminants, resulting in widespread human exposure, with varied toxicological endpoints. Several phthalates have been cataloged as ubiquitous environmental endocrine-disrupting chemicals (EDCs) and that is why there is a strong demand for their reliable determination. Phthalates with endocrine-disrupting properties, such as di (2-ethylhexyl phthalate (DEHP), dibutyl phthalate (DBP) and butyl benzyl phthalate (BBP), are suspected to interfere with developmental androgen action, possibly leading to adverse effects on reproductive function (Jacobs et al., 2017; K Khetan, 2014). Phthalates have been classified as "chemicals of concern" by the U.S. EPA (EPA, 2012). In addition, the European Chemicals Agency's Committee for Risk Assessment (RAC) recommends that the trends in exposure to phthalates (in consumer products, body burden based on biomonitoring, content in and migration from articles, etc.) should be monitored (HBM4EU, 2018; ECHA, 2012). Certain phthalates, like dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), diethyl phthalate (DEP), dimethyl phthalate (DMP), and diethylhexyl phthalate (DEHP) are omnipresent in the environment. Phthalates have numerous industrial applications and uses including food packaging, personal-care products, pharmaceuticals, medical devices, building materials, nutritional supplements, cleaning materials, solvents, adhesives, paints, lacquers, insecticides, children's toys, and children's school supplies (ECB, 2008; ECHA, 2013; NRC, 2008). In these products phthalates are not chemically bound to the polymer matrices and therefore might easily migrate to the food and surrounding environment which leads to exposure of humans by multiple routes including ingestion, inhalation and dermal uptake throughout their lifetime beginning in fetal stages (Bruinen de Bruin et al., 2007; CDC, 2009; Navarro et al., 2010).

Several animal studies reported that exposure to DBP, BBP, DEP, and DEHP has been associated with reproductive developmental damage, endocrine disruption and neurodevelopmental toxicity, and the European Food Safety Authority (EFSA) determined the critical effects of DEHP, BBP and DBP to relate to reproduction as derived from data from reproduction/developmental toxicity studies (Andrade et al., 2006; Borch et al., 2004; Fisher, 2004; Heudorf et al., 2007; Okubo et al., 2003; Sharpe and Irvine, 2004; Xu et al., 2005). In addition, epidemiological studies, in spite of their limitations, suggest that there are strong and rather consistent indications that phthalates may affect reproductive outcome and children's health (Jurewicz and Hanke, 2011). Although phthalates have short biological half-lives, and are quickly excreted from the body as their respective metabolites (e.g. monoesters), particular consideration has been given to phthalates for years, especially due to their ubiquitous existence in the environment, the size of the population exposed

and their endocrine-disrupting properties (EC, 2011; Jurewicz and Hanke, 2011). Upon exposure, phthalates can undergo metabolism in two stages in the human body namely phase I (hydrolysis, oxidation) catalyzed by esterases and lipases and phase ∏ (conjugation) catalyzed by uridine 5′-diphosphoglucuronyl transferase enzyme. Lower molecular weight phthalates such as DEP, DMP and DBP will undergo phase I biotransformation to become hydrolytic monoester metabolites while DEHP with more carbon atomes in alkyl chain first will hydrolyze into a monoester and that can further be oxidized to secondary oxidized metabolites. Hydrolytic monoesters and secondary oxidized metabolites are further metabolized through phase ∏ biotransformation to produce glucuronide conjugates.

Young children, as a result of their proportionally higher rates of breathing, eating and drinking, are likely to be more exposed per unit of body weight than adults (Gaspar et al., 2014). A Canadian study reported children to display significantly higher urinary concentrations of metabolites of DEHP, DBP and BBP than adolescents and adults (Saravanabhavan et al., 2013). Similar results were also observed in German school children and the US National Health and Nutrition Examination Survey (NHANES) (CDC, 2012; Koch et al., 2011).

Human bio-monitoring (HBM) is an important tool in the investigation of phthalate exposure and risk assessment since it reflects the phthalate body burden by measuring specific metabolites especially in the urine (Calafat and McKee, 2006; Colacino et al., 2010). Although phthalate metabolites can be detected in several body fluids such as amniotic fluid, breast milk, saliva and seminal plasma (Braun et al., 2013; Johns et al., 2015), the presence of enzymes such as esterases in these matrices can cleave phthalates converting the phthalates samples from external sources into their monoesters. In general, in epidemiological studies urine has been considered the matrix of choice for non-persistent chemicals, such as phthalates because urinary concentrations of especially their metabolites are usually considerably adequate biomarkers of exposure.Therefore, the measurement of phthalate metabolites in urine as a valuable approach in environmental epidemiology studies, represents an integrated measure of exposure to phthalates from all possible known and unknown sources and routes, and incorporates individual variability in exposure profiles (Christensen et al., 2014; Johns et al., 2015). Forward and backward methods can be used for exposure assessment with the latter one being based on interpretation of HBM data (Gurusankar et al., 2017). Forward anaylsis uses measured intake doses to predict body burden while backward (reverse) analysis uses urinary HBM data to reconstruct past exposure by calculating estimated daily intake (EDI) (McLanahan et al., 2013). One of the simplest methods to convert urinary HBM concentrations into exposure doses (e.g., EDIs) is based on the fractional urinary excretion (F_{UE}), defining the fraction of the dose that ends up as a defined biomarker in a relevant matrix. Thus, the

F_{UE} can be used for reverse dosimetry and convert the urinary level of a biomarker into an oral dose level. In this approach a correction for urinary dilution can be made applying the urine volume-adjustment method or the creatinine adjustment approach (Gurusankar et al., 2017).

When considering the use of urinary biomarkers for estimation of phthalate exposure it is also of importance to consider combined exposure, since disregard of combination effects may lead to underestimation of risks (Kortenkamp, 2014). For instance with a mixture of two phthalates, DBP and DEHP, which act through a common mode of action by suppressing testosterone synthesis, the combined effects were shown to be additive (Howdeshell et al., 2007). This illustrates the importance of cumulative risk assessment (CRA), considering the effects of combined exposure. In the case of phthalates, chronic co-exposure may constitute a risk of anti-androgenic effects during the stages of puberty due to hormonal changes and development of reproductive organs (Hartmann et al., 2015). Therefore, the U.S. National Research Council in recent recommendations has reported that phthalates meet the conditions necessary to warrant a mixture risk approach (NRC, 2008).

The aim of the present study was to determine the extent of exposure to the phthalates BBP, DBP, DEHP, DEP and DMP, for the first time, among children in Iran, and to estimate for this population the risk of exposure to the individual phthalates as well as to the combined exposure to the anti-androgenic phthalates BBP, DBP and DEHP.

Material and method

Study population and sample collection

This study is a cross-sectional study conducted among children and adolescent, ranging from 6 to 18 years in Tehran, Iran between September and November 2015. Fifty-six healthy children were included in the study by random selection. Random house addresses for recruiting children and adolescents and collecting urinary samples were selected by a weighted approach based on the Tehran population density using Arc GIS software (Figure 1). From this initial list, house addresses for actual sampling were individually approached. For collection of the required spot urine samples, one resident per household was randomly chosen among the children to participate in this study. If there was no child at the mentioned age range in the randomly selected house, the next selected house was interviewed to find an eligible case. Urine samples were collected in a sterile polypropylene cup. Samples were shipped on dry ice to the laboratory for the quantification of concentrations of phthalate metabolites. The subsets of the sample population were stratified by age (pre-reproductive: 6–11; reproductive: 12–18), and gender (girl and boy), as these factors have been indicated to have potential influence on phthalate exposure levels (Qian et al., 2015). Demographic

and anthropometric measures were assessed by trained health worker interviewers. Verbal and written assent was obtained from children and their legal guardians. Further, this study was approved by the Research Ethics Committee of Tehran University of Medical Sciences.

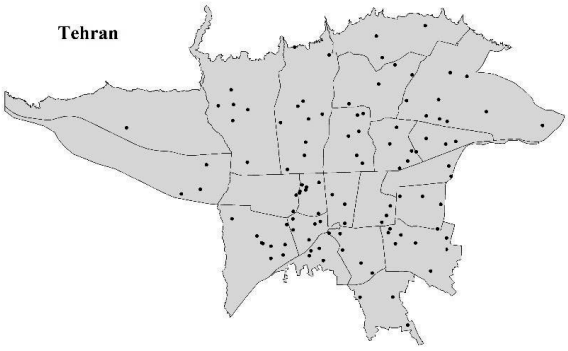

Figure 1. Location map of the study area showing the sampling sites.

Target compound and analysis

Collected spot urine samples were analyzed for seven phthalate metabolites of five phthalates, namely, monobutyl phthalate (MBP) for DBP, monobenzyl phthalate (MBzP) for BBP, monoethyl phthalate (MEP) for DEP, monomethyl phthalate (MMP) for DMP, mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono(2-ethyl-5-oxohexyl) phthalate (MOEHP) for DEHP. All the investigated phthalate metabolites (99.9% purity) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Ammonium acetate (98%), 3-bromobenzoic acid (3- BrBA) (internal standard, I.S.) (99.9% purity), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), β-glucuronidase from Helix pomatia type H-2 and derivatization reagent TMCS (chlorotrimethyl-silane) and BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) in pyridine (1:10:10) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Multiwalled carbon nanotubes (MWCNTs, length 5.0–30 μm, diameter 30–60 nm), were obtained from Nanoshel (Panchkula, India) (Rastkari and Ahmadkhaniha, 2013).

Phthalate metabolites analysis

The chemicals, reagents, applied methods for sample treatment and instrument analysis were reported in our previous study (Rastkari and Ahmadkhaniha, 2013). Briefly, two milliliters of human urine sample spiked with internal standard (10 μg/L) was buffered with 1.0 M ammonium

acetate solution (100 µL, pH 6.8). To ensure complete deconjugation of phthalate metabolites, 20µL β-glucuronidase from Helix pomatia type H-2 were added to the urine (Dirtu et al., 2013). The sample was sealed in a glass tube at 37 °C and gently mixed for 90 min. The mixture was then acidified with phosphate buffer (1 M, pH 2, 1 mL). Then multiwalled carbon nanotube-magnetic nano particle (MWCNT–MNP) composite (100 µL of suspension 10 mg mL^{-1}) (Rastkari and Ahmadkhaniha, 2013) and 100 mg NaCl were added and the mixture was vigorously vortexed for 3.0 min to extract the analytes. Then, an external magnet was applied to gather the magnetic adsorbent. After precipitation of the magnetic sorbent the supernatant was discarded followed by the addition of 5.0 mL isopropanol to elute phthalate metabolites from the adsorbent with vigorous vortexing for 2.0 min. The magnetic adsorbent was gathered using an external magnet afterward. The desorption solvent was collected and evaporated to dryness at 50 ºC under a gentle stream of nitrogen. Then, 50 µL of derivatization reagent TMCS (chlorotrimethyl-silane) and BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) in pyridine (1:10:10) was added to the residue. The sample was mixed and kept at 65 ºC for 30 min. Finally, 1 µL of the resulting solution was injected into the GC–MS that was an Agilent gas chromatograph 6890 plus (Agilent Technologies, Palo Alto, CA, USA) equipped with a 5973 quadrupole mass spectrometer. One blank, one repeat and one quality control (QC) recovery sample of high and low concentration were included in each analytical run to monitor for accuracy and precision. Blank samples, contained 3-bromobenzoic acid (3-BrBA) as internal standard (I.S.) and the concentration of blank samples was required to be less than twice the method detection limit (Rastkari and Ahmadkhaniha, 2013). Blank samples and QC samples were analyzed at the beginning, middle and at the end of the sample queue (EQUAS, 2012).

Some studies indicate that β-glucuronidases from Helix Pomatia may contain environmental levels of phthalates that may lead to phthalate metabolites measurement errors in urine (Albro et al., 1982; Tranfo et al., 2013). However, the results of the blank samples of the present study and from our earlier study (Dirtu et al., 2013) revealed that using the above mentioned enzyme did not influence our QA/QC results. The limit of detections (LODs) varied for each phthalate metabolite and ranged from 0.025 to 0.050 µg/L (Table 1).

Table 1. Molecular weights and urinary excretion fractions for phthalate metabolites.

Parent phthalate Compound	Phthalate monoester metabolite	Limit of detection (LOD) (µg/ L)	Molecular weight of diester parent compound, g/mole (MW_{parent})	Molecular weight of metabolite, g/mole ($MW_{metabolite}$)	Urinary excretion fraction (FUE, 24-h), expressed as percent (%)
Di (2-ethylhexyl phthalate) (DEHP)	Mono-(2-ethylhexyl) phthalate (MEHP)	0.050	390.56	278.34	6.2
	Mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)	0.050	390.56	294.35	14.9
	Mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP)	0.050	390.56	292.33	10.9
Dibutyl phthalate (DBP)	Monobutyl phthalate (MBP)	0.040	278.34	222.24	84
Butylbenzyl phthalate (BBP)	Mono-benzyl phthalate (MBzP)	0.050	312.36	256.25	73
Diethyl phthalate (DEP)	Monoethyl phthalate (MEP)	0.025	222	194	69
Dimethyl phthalate (DMP)	monomethyl phthalate (MMP)	0.030	194	180	69

*The FUEs are taken from the following studies: DEHP (Anderson et al., 2011); DBP (Koch et al., 2012); BBP (Anderson et al., 2001); DiNP (Anderson et al., 2011); DiBP (Koch et al., 2012).

Daily Intake Estimation

Based on the spot urine phthalate metabolite concentrations (expressed in µg/L urine), the estimated daily intake (EDI) in µg/kg bw-day of the main phthalates was determined. The EDIs of the target phthalates (DEP, DEHP, DBP, DMP, and BBP) were calculated for each child normalized based on urine creatinine extraction. The EDI was estimated using the following equation (1) (Christensen et al., 2014; Qian et al., 2015):

$$\mathrm{EDI} = \frac{\mathrm{UC_{Cr\text{-}adj}} \times \mathrm{CE}}{\mathrm{F_{UE}} \times 1000_{\mathrm{mg/g}}} \times \frac{\mathrm{MW_{Parent}}}{\mathrm{MW_{Metabolite}}} \quad (1)$$

Where EDI is the estimated daily intake of phthalate (µg/kg bw-day), $UC_{Cr\text{-}adj}$ is the phthalate metabolite concentration adjusted for creatinine ([µg/ml urine]/[g creatinine/ml urine]), CE is the daily (24-h) creatinine excretion normalized to body weight (mg/kg bw-day); and F_{UE} is the fractional urinary excretion of the metabolite determined over the full-time course of urinary excretion (24 hour excretion) in relation to ingested parent phthalate over 24 h after exposure ('unitless'); 1000 is the unit conversion factor creatinine (mg/g); MW_{parent} and $MW_{metabolite}$ are the molecular weights of parent phthalate and the metabolite (mg/mole) of each phthalate, respectively. Values related to F_{UE}, MW_{parent} and $MW_{metabolite}$ are displayed in the Table 1. The CE was calculated

based on the equations from (Mage et al., 2008) for children and adolescents (aged 6–18 years) by using demographic information (age, gender, height, and weight) (Mage et al., 2008).
We calculated DEHP exposures based on each individual metabolite and in addition based on the sum of the three measured urinary DEHP metabolites, MEHP, MEHHP and MEOHP in µg/g creatinine. Since the calculation of the EDI for DEHP is based on multiple metabolites, the following equation was used (2) (Qian et al., 2015):

$$\sum_{i=1}^{j} EDI = EDI_i \times \frac{\mathrm{F_{UEi}}}{\sum_{i=1}^{j} F_{UEi}} \quad (2)$$

Where i is the i^{th} metabolite of a phthalate, j is the number of metabolites for a phthalate, and F_{UEi} is the fractional urinary excretion of the i^{th} measured DEHP metabolite and the amount of DEHP taken up ('unitless').

In addition, the relative metabolic rate (RMR) of DEHP metabolism was calculated to investigate the possible differentiations in DEHP metabolism among population groups. The formation of the three DEHP metabolites analyzed in this study occurs in a stepwise metabolic pathway (Koch et al., 2005) including DEHP conversion to MEHP (1st step), MEHP hydroxylation to MEHHP (2nd step) followed by MEHHP oxidation to MEOHP (3rd step). Based on quantification of the 3 DHEP metabolites two relative metabolic rate (RMR) values can be determined. The first relative metabolic rate (RMR1) is representative for the rate of MEHP hydroxylation to MEHHP and calculated by dividing a molar concentration (µmol/µL) of MEHHP as a product over that of MEHP as a precursor. Similarly, the second relative metabolic rate (RMR2) is representative for the rate of MEHHP oxidation to MEOHP and calculated by dividing a molar concentration (µmol/µL) of MEOHP over that of MEHHP (Song et al., 2013).

Mixture Risk Assessment

Hazard quotients (HQs) were calculated for quantifying potential risks for children from exposure to single phthalates, which is defined as the ratio between the EDI and their health-based guidance values and/or acceptable level of exposure listed in Table 2 and comprising the Tolerable Daily Intake (TDI), the Reference Dose (RfD)and the Reference Dose for Anti-Androgenicity (RfD-AA) established by EFSA, U.S. EPA and Kortenkamp and Faust (2010). The value of HQ is described in equation 3 for participant i and phthalate j:

$$HQ_{ij} = \frac{EDI_{ij}}{\mathrm{AL}_j} \quad (3)$$

The health-based guidance values and/or acceptable level of exposure is denoted AL in equation 3 and 4.

To assess combined exposures to multiple phthalates, the Hazard Index (HI) was used, which is based on the dose addition concept (Hertzberg et al., 2018; Fox et al., 2017; NRC 2008). The following equation was applied to determine the values HI for participant i and phthalate j for N phthalates (4):

$$HI = \sum_{i=1}^{n} \frac{EDI}{AL} \quad (4)$$

Where EDI is the estimated daily intake of phthalate (µg/kg bw-day), and n is the number of substances in the mixture. The selected RfVs used to construct the HQ and HI on the basis of the same toxicological endpoint (anti-androgenic effects) are two different sets which include: 1) The tolerable daily intake (TDI) values as defined by the European Food Safety Authority (EFSA) for DBP, BBP, and DEHP based on testicular/germ cell toxicity (EFSA, 2005a,b,c) and, 2) The reference doses for anti-androgenicity (RfD-AA) established by Kortenkamp and Faust for DBP, BBP, and DEHP (Kortenkamp and Faust, 2010).

The United States Environmental Protection Agency Reference Doses (U.S. EPA RfDs) were not used, because the RfDs for DBP, DEHP and BBP have not been defined based on anti-androgenic effects as the most sensitive endpoint (IRIS EPA 1987a,b, 1988). The EFSA TDIs, the RfD-AA values and the U.S. EPA RfD values for DBP, DEHP and BBP are presented in Table 2. Thus, two different HIs were estimated based on two types of reference values. DEP and DMP were not included in the combined risk assessment because these two phthalates do not exhibit endocrine disrupting effects similar to those observed for DBP, BBP and DEHP. Indeed, predominantly negative results on the oestrogenic or antiandrogenic effects of DEP and DMP have been reported and no endocrine-related adverse effects of DEP and DMP on the male reproductive system have been observed and these phthalates are regarded safe compared to other phthalates (ECHA 2015; (EC, 2007; NRC, 2008a). Nevertheless, they may contribute significantly to other adverse effects for example neurodevelopmental disorders (Jeddi et al., 2016).

Table 2. Related health-based guidance values and toxicological target for dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and di (2-ethylhexyl phthalate) (DEHP).

Phthalate	Toxicological Target			Toxicity health-based guidance values		
	EFSA TDI	U.S. EPA RfD	RfD-AA	EFSA TDI (µg/kg bw/day)[1]	U.S. EPA RfD (µg/kg bw/day)[2]	RfD-AA (µg/kg bw/day)[3]
DBP	Germ cell development and mammary gland changes	Increased mortality	Suppression of testicular testosterone production	10	100	100
BBP	Anogenital distance change	Increased liver-to-body weight and liver-to-brain weight ratios	Suppression of testicular testosterone production	500	200	330
DEHP	Testicular toxicity and developmental toxicity	Increased relative liver weight	Nipple retention	50	20	30

[1] EFSA, 2005a,b,c
[2] Kortenkamp and Faust, 2010
[3]IRIS EPA 1987a,b, 1988

The present study also applied the Maximum Cumulative Ratio (MCR) approach, which is the ratio of the combined risk measure HI divided by the HQ of the chemical with the highest risk contribution (HQ_{max}) (Price and Han, 2011). Indeed, this tool can be used to determine which chemicals are the drivers of the combined risk (Han and Price, 2013). This approach helps to prioritize chemicals for mitigation strategies as well.

The maximum HQ among the investigated phthalates in the current study was determined taking into account three phthalates (j=3), and the maximum HQ (HQ_M) was determined as indicated in equation 5:

$$HQ_{M,i} = Max\ HQ_{ij} \quad (5)$$

The value of MCR for a participant i in an exposed population is defined as (Reyes and Price, 2018):

$$MCR_i = \frac{HI}{HQ_{M,i}} \quad (6)$$

The values of MCR will vary across participants in an exposed population ranging from 1 to N (i.e. MCRi ∈[1,N]), where N is the number of chemicals considered in the assessment. When ratio values for a mixture are close to 1, one chemical is responsible for nearly all of the individual's combined risk. A value of N indicates that, the individual receives an equitoxic dose from all chemicals (Reyes and Price, 2018).

Statistical Analysis

Appropriate descriptive statistics were used for description of all metabolite concentrations, demographic variables, RMR, HQs, HIs and MCRs. Nonparametric Mann-Whitney U test was implemented to compare median of target variables across sex and age groups. Principal component analysis (PCA) was applied to investigate the potential exposure sources and Spearman's rank correlation coefficient were used for investigation correlations between metabolites. Data were analyzed using STATA Version 12.0. Statistical significance was set at $p < 0.05$. Concentrations of phthalate metabolites below LOQ were set equal to a value of ½ LOQ, and concentrations <LOD were set to 0.

Results

Urinary phthalate metabolite concentrations

This study is based on data from 56 participants (24 males and 32 females) aged 6 to 18 years. All phthalate metabolites (except for MBzP, the metabolite of BBP, in four out of the total 56 spot urine samples) were detected in concentrations above the respective LODs, which indicated the ubiquitous exposure of Iranian children to phthalates. Descriptive statistics and statistical comparisons across gender and age groups of seven phthalate metabolites are presented in Table 3. The median unadjusted and creatinine-adjusted levels of MBP, MEHP, MEHHP, MEHOP, MMP in girls (n = 32) were slightly lower than those in boys (n = 24). For MEP and MBzP, the highest concentrations were measured in girls with statistically significant differences compared to values for boys.

Table 3. Urinary concentrations of phthalate metabolites (in μg/L (μg/g creatinine)) in Iranian children and adolescents population (n=56).

Phthalate metabolites	Group	*n*	GM	5th	50th	95th	Max	*P*-value [a]
MEHP	**All**	56	2.81 (2.82)	0.48 (0.53)	3.3 (3.3)	9.1 (11.5)	11.6 (13.2)	0.406 (0.579)
	≥6~<12	22	2.9 (3.02)	0.54 (0.55)	4.0 (3.6)	7.83 (8.72)	9.1 (11.5)	
	≥12~≤18	34	2.8 (2.7)	0.485 (0.53)	3.2 (3.3)	9.3 (12.1)	11.6 (13.2)	
	Boys	24	3.70 (3.61)	1.0 (0.65)	4.9 (4.1)	9.1 (12.1)	11.6 (13.2)	0.213 (0.127)
	Girls	32	2.35 (2.33)	0.32 (0.32)	2.8 (2.6)	7.83 (7.2)	9.3 (9.5)	
MEOHP	**All**	56	18.14 (18.12)	3.62 (3.6)	17.5 (19.3)	65.1 (73.8)	79.43 (99.4)	0.470 (0.233)
	≥6~<12	22	19.5 (20.3)	4.0 (3.7)	21.43 (24.2)	58.3 (46.2)	63.7 (121.1)	
	≥12~≤18	34	17.3 (16.9)	3.6 (2.2)	16.62 (18.0)	69.33 (82.7)	79.43 (99.4)	
	Boys	24	19.32 (19.1)	4.0 (3.7)	19.9 (20.4)	60.64 (82.74)	79.43 (99.4)	0.823 (0.881)
	Girls	32	17.3 (17.43)	3.62 (2.2)	16.74 (18.9)	65.1 (53.3)	69.33 (64.4)	
MEHHP	**All**	56	26.73 (26.7)	6.4 (3.91)	24.1 (28.6)	80.5 (100.24)	129.85 (135.3)	0.387 (0.227)
	≥6~<12	22	28.9 (30.02)	14.31 (13.64)	27.03 (35.54)	74.8 (60.5)	95.7 (68.6)	
	≥12~≤18	34	25.4 (24.8)	6.4 (3.9)	21.92 (25.3)	80.5 (100.25)	129.85 (33.8)	
	Boys	24	29.03 (28.7)	10.61 (8.9)	30.9 (31.05)	95.7 (121.13)	129.85 (135.3)	0.602 (0.740)
	Girls	32	25.12 (25.3)	6.40 (3.91)	22.24 (26.8)	78.82 (61.9)	80.3 (78.03)	
MEP	**All**	56	27.5 (27.4)	13.1 (9.6)	28.2 (30.4)	53.5 (62.4)	62.1 (68.6)	0.491 (0.579)
	≥6~<12	22	30.2 (31.4)	18.7 (17.7)	28.7 (29.9)	53.5 (66.1)	62.1 (68.6)	
	≥12~≤18	34	25.8 (25.1)	6.3 (3.4)	26.9 (31.6)	51.2 (59.2)	54.9 (62.4)	
	Boys	24	23.5 (23.3)	13.1 (9.7)	25.5 (26.5)	53.5 (47.0)	54.9 (62.4)	0.012 (0.025)
	Girls	32	30.8 (31.1)	13.6 (9.6)	35.4 (34.2)	51.2 (66.1)	62.1 (68.6)	
MBP	**All**	56	36.4 (36.4)	12.8 (9.7)	42.9 (38.7)	70.3 (84.2)	72.3 (105.9)	0.502 (0.880)
	≥6~<12	22	34.8 (36.2)	14.4 (13.1)	42.9 (41.6)	61.2 (71.3)	64.1 (72.0)	
	≥12~≤18	34	37.5 (36.5)	12.8 (9.8)	45.0 (36.9)	71.9 (98.1)	72.3 (105.9)	
	Boys	24	37.2 (36.8)	14.1 (9.8)	47.3 (39.2)	71.9 (98.1)	72.3 (105.9)	0.6429 (0.868)
	Girls	32	35.9 (36.2)	12.8 (13.5)	38.5 (38.7)	69.7 (78.2)	70.3 (84.0)	
MMP	**All**	56	15.9 (15.8)	4.3 (4.3)	17.4 (15.8)	42.8 (48.2)	43.4 (53.8)	0.356 (0.737)
	≥6~<12	22	14.5 (15.1)	5.2 (4.5)	15.2 (15.7)	34.2 (40.3)	40.0 (41.6)	
	≥12~≤18	34	16.8 (16.4)	4.3 (4.3)	19.3 (16.0)	43.3 (51.5)	43.4 (53.8)	
	Boys	24	15.8 (15.6)	4.3 (3.8)	19.1 (16.7)	40.0 (42.6)	42.7 (53.8)	0.8946 (0.973)
	Girls	32	15.9 (16.1)	4.9 (4.5)	15.1 (15.2)	43.3 (48.2)	43.4 (51.5)	
MBzP	**All**	56	2.0 (2.0)	<LOQ	2.2 (2.3)	5.0 (5.4)	5.1 (6.2)	0.063 (0.043)
	≥6~<12	22	2.4 (2.5)	0.7 (0.6)	3.4 (3.1)	5.0 (6.0)	5.1 (6.2)	
	≥12~≤18	34	1.8 (1.7)	<LOQ	2.1 (2.0)	4.4 (5.2)	5.1 (5.4)	
	Boys	24	1.8 (1.7)	0.7 (0.6)	2.0 (2.0)	4.5 (4.1)	5.1 (4.6)	0.0354 (0.043)
	Girls	32	2.2 (2.2)	<LOQ	3.3 (2.8)	5.0 (6.0)	5.1 (6.2)	

Creatinine adjusted level of each phthalate metabolite was shown in the parentheses (μg/g); GM: geometric mean; LOQ: limit of quantification. LOQ for MzBP is 0.25 μg/L.

a Mann-Whitney U test ; p-value for adjusted level of each phthalate metabolite was shown in the parentheses.

Median levels of phthalate metabolites appeared not considerably different between the age groups and differences were indeed shown to be not statistically significant. This trend was consistent also for the creatinine-adjusted values (Table 3). In this study, the geometric mean (GM) concentration of the sum of DEHP metabolites (Σ3DEHP metabolites) was 47.7 µg/L with a decreasing order in the level of the three DEHP metabolites that varied as follows: MEHHP > MEOHP > MEHP. In addition, MBP and MEP were the most abundant metabolites, with GM values of 36.4 and 27.5 µg/L respectively. MBzP showed the lowest level in both unadjusted and creatinine adjusted models with a GM value equal to 2.0 µg/L. Table 4 depicts correlation coefficients among several selected urinary phthalate metabolites. As shown, statistically significant Spearman correlations ($p < 0.05$) were identified between some of the metabolites. Our study displayed a strong correlation between the primary metabolite of DEHP, MEHP, and the two oxidative metabolites of DEHP including MEHOP and MEHHP as well as between these two secondary metabolites of DEHP. A moderate correlation was indicated between MBP and MEP or MMP, and between MBzP and MEP or MBP. The observed correlations were correlated positively (p-value<0.05; Table 4), an observation that may indicate combined exposure.

Table 4. Correlations between urinary phthalate metabolite concentrations in (µg/g creatinine) among the Iranian children and adolescant.

	MBP	MEP	MMP	MEHP	MEOHP	MEHHP
MBzP	0.263*	0.540*	0.025	-0.003	0.127	0.021
MBP		0.277*	0.625*	-0 .063	-0.000	0.043
MEP			0.006	0.187	0.163	0.119
MMP				0.026	-0.024	0.007
MEHP					0.731*	0.798*
MEOHP						0.896*

*Correlation is significant at 0.05 levels (p<0.05)

In addition, PCA was performed for the seven phthalate metabolites (MMP, MEP, MBP, MBzP and the three DEHP metabolites). The results of PCA are summarized in Figure 2. Three significant (Eigen values >1.000) factors were retained and they can explain about 83% of the data variability. As shown in Figure 2, seven investigated phthalates are in three clouds. The first cloud is represented by MBzP and MEP, the second by the three DEHP metabolites (MEHHP, MEOHP and MEHP) and the third one by MMP and MBP. This clustering in the PCA analysis might point at a similar molecular origin, combined exposure, and or/ similar ADME (absorption, distribution, metabolism and excretion) characteristics.

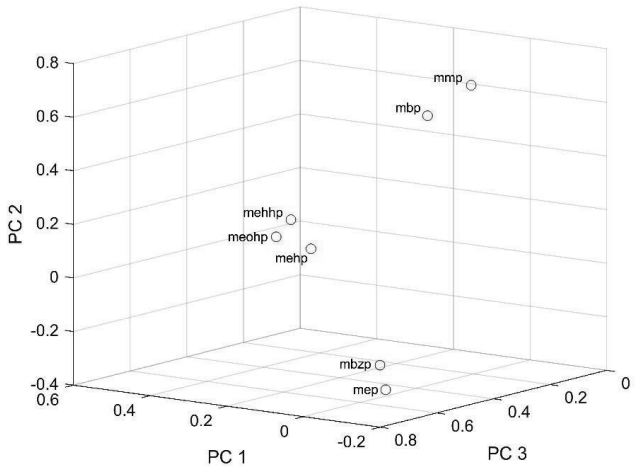

Figure 2. Principal component analysis (PCA) of the levels of the seven phthalate metabolites detected in urine samples from Iranian children and adolescent

In this study, we included all samples in the relative metabolic rate (RMR1 and RMR2) calculation because all three metabolites of DEHP were detected in 100% of the samples. The RMR1 and RMR2 arithmetic mean for children were 13.4 and 0.72, respectively. Mean of RMR1 in girls (13.6) was slightly (but not statistically significant) higher than that in boys (13.4) and also no gender- or age-based differences were observed for either RMR1 or for RMR2.

Daily intake estimations and cumulative risk assessment

The estimation of individual daily phthalate intakes among the study population was performed according to the creatinine adjusted model. The median EDIs of BBP, DMP, DEP, DBP, and DEHP for all investigated children and adolescents were 0.06, 0.8, 1.0, 1.1, and 3.4 µg/kg body weight/day, respectively. Comparing boys and girls in the whole age range, the EDIs of DBP, DEP and BBP for girls were slightly higher than those for boys with statically significant differences for DEP. Concerning age, no statically significant differences were found among daily intake values for all investigated phthalates. On the basis of the EDI values for each participant, the risks (HQs as well as HI) associated with phthalate exposure based on TDI values and RfD-AA values were characterized. Results obtained are summarized in Table 5. In this study, HQ reflects the risk value for a single phthalate, while HI shows the risk value obtained for cumulative exposure of the anti-androgenic phthalates BBP, DBP and DEHP. As shown in Table 5, median HIs for cumulative

exposure based on both health-based guidance values (TDI and RfD-AA) are below 1. HIs ranged from 0.03 to 0.70 and from 0.02 to 0.62 based on EFSA TDI and RfD-AA approaches, respectively, and the HIs did not exceed 1 for any of the surveyed participants.

The MCR calculated among the 56 participants ranged from 1.09 to 2.32. Because in the present study, three phthalates were considered, MCR values can theoretically range between 1 to 3. An MCR value close to 1 indicates that one chemical had a dominant influence on the participant's value of HI. The fact that all MCR values are between 1.1 and 2.32 indicates that for none of the exposed participants the three phthalates had the same influence on the participant's value of HI, since this would have resulted in an MCR value of 3. That is, for each subject, a subgroup of phthalates had a dominant influence on the participant's value of HI. The collective internal doses of all participants were driven by either DBP or DEHP. Among investigated phthalates, BBP did not produce HQ_M for any participants. Approximately, 73% of combined HI-TDI could be attributed to DBP's metabolite MBP while based on the HI-RfD-AA the sum of DEHP metabolites makes up the whole RfD-AA.

Table 5. Hazard quotients (HQ) and Hazard Index (HI) based on TDI (EFSA) and RfD-AA for children and adolescents.

		HQ TDI					**HQ RfD-AA**				
		Min	Median	95_p	max	N>1	Min	Median	95_p	max	N>1
DBP	**All**	0.02	0.11	0.25	0.31	0	0.001	0.01	0.02	0.03	0
	≥6~<12	0.02	0.11	0.21	0.22	0	0.002	0.01	0.02	0.02	0
	≥12~≤18	0.02	0.10	0.27	0.31	0	0.002	0.01	0.02	0.02	0
	Boys	0.02	0.10	0.30	0.31	0	0.002	0.01	0.03	0.03	0
	Girls	0.03	0.11	0.22	0.11	0	0.003	0.01	0.022	0.01	
BBP	**All**	0.00002	0.0001	0.0003	0.0005	0	0.00002	0.0002	0.0004	0.0007	0
	≥6~<12	0.0002	0.0001	0.0004	0.0005	0	0.00002	0.0002	0.0006	0.0007	0
	≥12~≤18	0.00001	0.0001	0.0003	0.0003	0	0.00002	0.0002	0.0005	0.0005	0
	Boys	0.00002	0.0001	0.0002	0.0002	0	0.00004	0.0002	0.0003	0.0003	0
	Girls	0.00001	0.0002	0.0004	0.0005	0	0.00002	0.0002	0.0006	0.0007	0
DEHP	**All**	0.01	0.10	0.31	0.40	0	0.02	0.11	0.50	0.60	0
	≥6~<12	0.01	0.10	0.33	0.36	0	0.02	0.14	0.55	0.60	0
	≥12~≤18	0.01	0.11	0.30	0.32	0	0.016	0.11	0.51	0.54	0
	Boys	0.01	0.10	0.35	0.40	0	0.02	0.13	0.58	0.60	0
	Girls	0.01	0.11	0.22	0.23	0	0.015	0.11	0.37	0.40	0
HI	**All**	0.03	0.20	0.56	0.70	0	0.02	0.13	0.52	0.62	0
	≥6~<12	0.03	0.20	0.54	0.60	0	0.03	0.14	0.57	0.62	0
	≥12~≤18	0.03	0.20	0.57	0.63	0	0.20	0.12	0.50	0.55	0
	Boys	0.03	0.20	0.65	0.70	0	0.03	0.13	0.60	0.62	0
	Girls	0.04	0.20	0.44	0.35	0	0.02	0.12	0.40	0.40	0

N: number of participants

Discussion

In this study, we used a human biomonitoring approach to determine EDI values for five different phthalates in Iranian children and adolescents, based on phthalate metabolite levels in urine spot samples, and performed an associated risk assessment. For this risk assessment the EDI values obtained were compared to two sets of health-based guidance values as acceptable levels of exposure, derived based on anti-androgenic effects as the critical endpoint, including TDI values established by EFSA (EFSA 2005a,b,c) and RfD-AA values determined by Kortenkamp and Faust (Kortenkamp and Faust, 2010) using the HQ approach. The evaluation also included a combined risk assessment for exposure to phthalate mixtures using the HI approach.

The results of this study show that Iranian children are ubiquitously exposed to certain phthalates included in the study, namely DEHP, DBP, DEP and DMP. Our findings showed that the levels of the urinary biomarkers for phthalate exposure varied in the order MBP> MEP > DEHP metabolites>MMP>MBzP. For comparison the results from other studies on exposure of children to phthalates as reported in the literature on a worldwide scale are presented in Figure 4. The urinary metabolite patterns obtained in our study are especially similar to the urinary phthalate metabolite patterns reported for children from other countries such as Taiwan, China, Brazil and Greece Huang et al., 2015; Myridakis et al., 2015; Rocha et al., 2017; Wang et al., 2014). All the reviewed studies were population-based cross-sectional studies conducted among minors (< 18 years old) after 2010. Consistent with our results, among all studies the lowest urinary concentrations were observed for MBzP. This reveals that children have relatively lower exposure to BBP compared to other phthalates, and that this is the case in several countries over the world, probably reflecting that the application and sources of exposure to BBP are comparable (ECHA 2010). The urinary metabolite patterns in our study revealed also differences to urinary metabolite patterns reported in several of the other countries for which data were available (Figure 4). Such differences may reflect differences in patterns of exposure in different countries due to country specific use patterns for phthalates in relevant products, differences in food consumption habits, and/or differences in socio-economic strata (EFSA 2011). These differences may also reflect changes in the phthalate content of specific products over time, and further may be the result of public pressure and political regulations (Johns et al., 2015). For example in 2004, the European Union (EU) has banned the use of certain phthalates including DEHP, DBP and BBP from cosmetics and food packaging (Directive 2004/93/ EC) and in 2005 from all toys and childcare products (Directive 2005/84/EC) (Davies, 2015). Furthermore, comparison of data from two studies in Denmark with sampling in the period 2006 to 2008 or more recent in 2011 revealed a decreasing trend in phthalate exposure (Mieritz et al.2013; Frederiksen et al. 2012, 2013). Reported urinary concentrations for BBP, DBP and DEHP

in the studies conducted by Frederiksen et al. and Mieritz et al. (sampling time between 2006-2008) are 1.5, 6.8 and 2 times higher than the results of the study in 2011. This decreasing trend in phthalate exposure also becomes apparent when comparing two studies in the USA one with 2005-2006 and one with 2009-2010 as sampling periods (CDC 2018). Recently, Koch et al., investigated the time trend of phthalates exposure using urinary samples from the German Environment Specimen Bans (ESB) regularly taken in the time frame of 1988 until 2015. They showed that the exposure to certain phthalates (DEHP, DnBP, BBP) has decreased (Koch, 2016).

This reduction in phthalate exposure is likely attributable to prohibition of usage of specific phthalates. In addition, dietary habits and life styles play an important role in exposure to phthalates (Wang et al., 2014). One remarkable difference in the urinary phthalate metabolite pattern between the Iranian children and data from several other countries was the fact that the Iranian samples revealed the presence of MMP, the metabolite of DMP. This metabolite was also observed to a significant extent in 6 to 18 year old children in China and Taiwan (Huang et al., 2015; Shen et al., 2015). The possible sources of this DMP exposure are not clearly known. A study conducted in China suggested that the concentrations of DMP in milk products, instant noodle, cakes, cookies and salt eggs were higher than those in other foods (Guo et al., 2012). DMP was detected in some food samples such as yogurt, fish, and spice from Europe and North America as well (Wormuth et al., 2006).

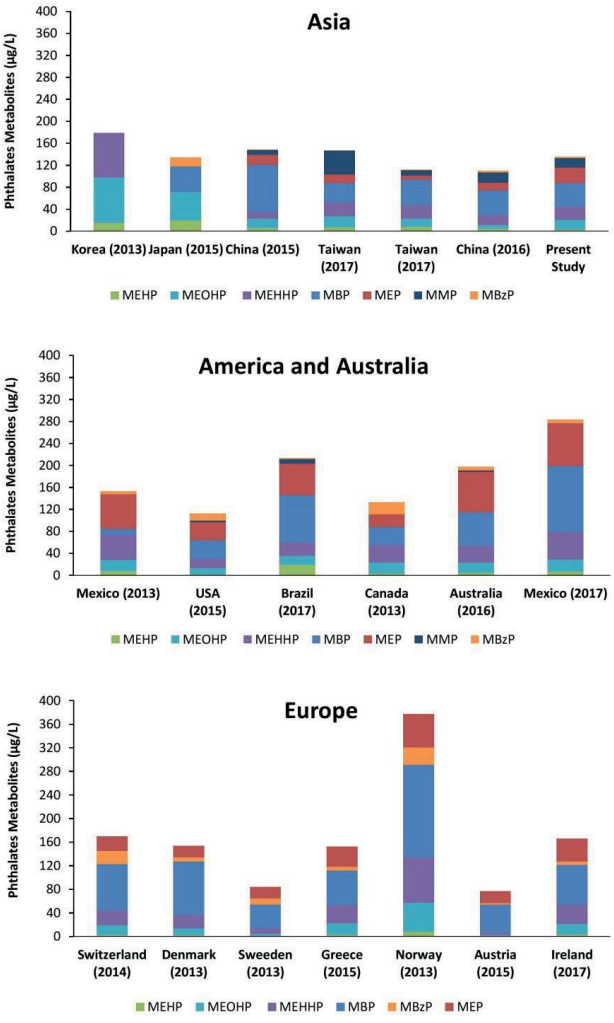

Figure 4. Median urinary concentrations (μg/L) of phthalate metabolites in children and adolescents throughout the world. All the reviewed studies were conducted among children and adolescents (< 18 years old) with sampling time after 2010.

Related references based on regions are as follows: Asia: (Bamai et al., 2015; Huang et al., 2017; Lewis et al., 2013; Song et al., 2013; Wang et al., 2015; Wu et al., 2017) Chen et al. (2017), America: (Lewis et al., 2013; Ramos et al., 2016; Rocha et al., 2017; Saravanabhavan et al., 2013; Yang et al., 2017; CDC 2015) Europe: (Bertelsen et al., 2013; Carlstedt et al., 2013; Frederiksen et al., 2013; Hartmann et al., 2015; Larsson et al., 2014; Myridakis et al., 2015).

According to the results of the current study and in line with results from related studies, the concentrations of the oxidative metabolites of DEHP (MEHHP and MEOHP) appeared to be excreted in several-fold higher concentrations than MEHP the metabolite resulting from hydroxylation of DEHP (Figure 4). Although metabolic abilities may differentiate between age groups, previous studies already showed that these oxidative metabolites could be more sensitive biomarkers for monitoring exposure to DEHP than MEHP (Barr et al., 2003). In addition, previous studies reported that children had a particularly faster relative metabolic rate (RMR) than adults, specifically for the first step of DEHP metabolism (RMR1: ratio MEHP/MEHHP) (Song et al., 2013). The results from the present study also corroborate that RMR1 is higher than RMR2, because the transformation of MEHP to MEHHP (as expressed by RMR1) appears to be positively related with age implying a reduced ability of DEHP metabolism at lower ages (Barr et al., 2003; Myridakis et al., 2015; Kasper-Sonnenberg et al., 2012).

Furthermore, the urinary biomarker patterns confirmed the observation that Iranian children, like children in other countries, are simultaneously exposed to mixtures of phthalates. The PCA analysis indicated grouping of some phthalates (DEHP, DBP, DMP) and thus pointed at combined exposure from similar sources, like food packaging material and several consumer products, leading to combined exposure to DBP, DMP, and DEHP, and or/ similar ADME (Wormuth et al., 2006). In contrast, MEP and MBzP were positively correlated with PC3, which could be an indication of the same origin of exposure via for example personal care-hygienic products and cosmetics (Bao et al., 2015) which may explain why the concentrations of these two phthalates were higher in girls with statistically significant differences compared to values for boys. Generally, multiple phthalates correlate with one another if they are used in the same applications and thus share similar sources of exposure, and again there may be some other existing unknown sources of exposure (Johns et al., 2015).

In a review conducted by Smith et al., aiming to prioritize hazardous chemicals in children's products based on the U.S. Children's Safe Product Act (CSPA) database, the relationships between phthalate exposure and adverse health effects was investigated (Smith et al., 2016). Four endpoints including endocrine disruption, reproductive and developmental toxicity, carcinogenicity and neurotoxicity were selected as relevant health endpoints in their framework. The analysis confirmed

that toxicity drives a substantial part of the differences in health effects caused by the chemicals. Phthalates, including DEHP, BBP, and DBP were found to raise a concern because of reproductive and developmental toxicity, which would be in line with their activity as anti-androgens (Smith et al., 2016).

Across the whole study population of the present study, the highest median phthalates intakes were for DEHP within the range of 0.58 to 17.85 µg/kg body weight/day and DBP within the range of 0.2 to 3.1 µg/kg body weight/day. Median BBP daily intakes were lowest, ranging from 0.01 to 0.23 µg/kg body weight/day.

The phthalate exposure profile was consistent with previous studies. These studies also revealed that levels of most urinary phthalate metabolites detected for children were found to decrease with increasing age (Becker et al., 2009; CDC, 2013; Koch et al., 2007). However, no significant differences were found between the two age groups in our study.

According to the results of our study, DEHP is the compound with the highest median HQ of 0.14 when based on the RfD-AA for anti-androgenic effects, whereas based on the TDI approach DBP with a median of 0.11 is the compound with the highest HQ value. However, since all HQ values, and also the HI values for combined exposure were markedly below 1.0 it can be concluded that for none of the surveyed participants the HQ and HI values raised a concern. It is also of interest to note that for the risk assessment performed, HQs and HIs were not calculated based on U.S. EPA RfDs, since these health based-guidance values are not based on endpoints that share an underlying mode of action. However, even when using U.S. EPA RfD values, EDI values for investigated phthalates in the present study would remain far below these health based guidance values and thus corroborate that the exposure does not raise a concern. This result is in contrast to those from a study conducted among German children (n = 239, 2–14 years old), in which some individuals showed DEHP exposure estimates based on HBM data that exceeded the U.S. EPA RfD for DEHP (20 µg/kg body weight/day) (Wittassek et al., 2007). More so, in Seoul, using the U.S. EPA RfD value, approximately 3–8% of elementary school children (n = 39, aged 9–12 years) showed a HQ greater than 1.0 for DEHP exposure, using HBM data for exposure estimation (Kim et al., 2014). The HI approach used in the present study for combined risk assessment has been previously used for combined risk assessments on phthalates in the literature. In a study conducted by (Søeborg et al., 2012) of the 129 Danish children and adolescents, 19 children exceeded the HI value of 1.0 determined based on EFSA TDI values for the anti-androgenic phthalates (DBP, BBP and DEHP), while one child exceeded the HI value of 1.0 based on the RfD-AA values (Søeborg et al., 2012). Dewalque et al. (2014) also reported HI values based on TDIs exceeding the value of 1.0 in 25% of the children in a study on phthalate exposure of 52 male and female children (1–12 years) in

Belgium (Dewalque et al., 2014). A study conducted among Austrian children aged 7-15 years on cumulative risk assessment for combined phthalate exposure demonstrated that in 4.2% of children the HI values calculated based on TDIs were more than 1.0 (Hartmann et al., 2015). In the present study, the HQ values for DBP based on EFSA-TDI values were higher compared to the estimated HQs based on RfD-AA values. This discrepancy is due to the fact that the underlying RfD-AA value used for DBP by (Kortenkamp and Faust, 2010) is 10 times higher than the relevant EFSA-TDI value (Table 2). However, DEHP was associated with the highest HQ value (~ 0.6) for Iranian children in both RfD-AA and EFSA-TDI approaches. Thus, obviously, the HQ and HI values and resulting conclusions may to some extend depend on health-based guidance values used to calculate these values.

Regarding the MCR approach, the cumulative exposures of concern mainly originated from one of the three anti-androgenic phthalates including DBP and DEHP. The MCR approach has been applied to biomonitoring data on mixtures of dioxin-like chemicals (Han and Price, 2013), exposures to mixtures of chemicals in water (Han and Price, 2011; Price and Han, 2011; Silva and Cerejeira, 2015; Vallotton and Price, 2016), and mixtures in residential indoor air (De Brouwere et al., 2014). A recent study conducted by (Reyes and Price, 2018) was the first publication that used the MCR approach in a biomonitoring study on phthalates collecting data on six phthalates. The results of that study showed that HI values in the surveyed participants averaged 0.15 (HI<1.0). Only 21 (0.8%) of the participants had HI values >1.0 (Reyes and Price, 2018). Reyes and Price, reported that for about 43% of these participants with HI > 1.0, a potential risk would have been overlooked if only single chemical based risk assessment (HQ) rather than a combined exposure approach (HI) was performed. In addition, the MCR calculated among the participants ranged from 1.1 to 3.6, which indicated that a single or a subgroup of phthalates like DEHP and DBP had a dominant influence on the participant's value of HI (Reyes and Price, 2018). Also in the present study the HI values were dominated by specific phthalates, being DBP when determining HI values based on TDI values, and DEHP when determining HI values based on RfD-AA values.

Altogether, it can be concluded that, in line with other studies, our subjects were not exposed to single phthalates, but rather to a mixture of phthalates. In a previous study prioritizing chemicals and products, DBP, BBP and DEHP were identified as the highest priority chemicals based on both exposure and toxicity scores (Smith et al., 2016). Metabolites of these priority phthalates were also detected in the urinary samples of the present study and were shown to contribute to the combined HI values. This corroborates that biomonitoring data indicate that the overall combined exposure to phthalates of Iranian children and adolescents does not raise a concern, while reduction of exposure is best focused on DEHP and DBP that showed the highest HQ.

Conclusions

This work investigated human biomonitoring derived phthalate exposure data for a population of Iranian children and adolescents ranging from 6 to 18 years of age analyzing urinary biomarkers for five phthalates. The data indicated that the Iranian children and adolescents were exposed to a mixture of phthalates and a subsequent risk assessment revealed that none of the surveyed participants had HQ and HI values that raised a concern. The phthalates exposure pattern for the study population of Iranian children appeared comparable to the results reported for children from other parts of the world with the greatest similarity being found for children from China, Taiwan, Brazil and Greece. Although combined exposure to anti-androgenic phthalates did not exceed the acceptable level of exposure, aside the investigations of phthalates in this study, people typically come into contact with several chemicals with anti-androgenic properties, which may also contribute to combined anti-androgenic effects. This indicates that a risk assessment of combined exposure including other anti-androgenic chemicals would be required to determine whether combined exposure to anti-androgenic chemicals is below acceptable levels.

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CHAPTER 8

Summary, General Discussion and Future Perspective

In the context of the proposal of the Iranian Ministry of Health and Medical Education in response to a public concern about exposure to phthalates via consumption of bottled water, phthalate concentrations in Iranian bottled water stored under various conditions were analysed. In this study, bottled water was considered because it attracted remarkable attention as a main source of drinking water in part owing to the water scarcity in Iran and the perception of higher quality, purity and safety of bottled waters compared to tap water. Among the possible contaminants in bottled waters, including formaldehyde, acetaldehyde, antimony, ultraviolet (UV) stabilizers and phthalates (Harunarashid et al., 2017), phthalates were chosen as the chemicals of potential concern in this study, since this group of substances is classified as endocrine disruptor chemicals with reproductive and developmental toxicity. The high and regular consumption of bottled water, the presence of phthalates in PET bottles as impurities originating from their manufacturing, the uncertainty about the impact of storage conditions of PET bottled water on migration of phthalates into the water, and the potential health effects of phthalates together raised a public concern.

Based on the measured levels, investigations were performed in the present research, in order to assess the exposure and related risk posed by drinking bottled water for the Iranian population (adults, pregnant women, and children). In addition, a risk assessment for combined exposure was performed based on urinary levels of phthalates measured in children to assess the risks resulting from the total exposure to phthalates from all routes and sources.

In the following sections, first the results obtained in this research will be discussed, after which future perspectives will be presented.

Chapter 1 of the thesis presents an introduction to the topic and the outline of the thesis. Chapter 2 of the thesis describes the development of a method to extract phthalates from bottled water by applying surface-functionalized magnetic particles (MPs) as the adsorbent of Magnetic Solid-Phase Extraction (MSPE). In this study, MPs along with polydimethylsiloxane and Multi-Walled carbon nano-tubes were used, mainly because of their excellent adsorption capability. MPs are able to completely disperse in sample solutions and to adsorb the analytes, which facilitates high extraction efficiency. In the method applied, the MPs were collected and separated from the liquid phase by applying an external magnet, which greatly simplifies the SPE procedure (Xu et al., 2012). By combining the MSPE with Gas Chromatography-Mass Spectrometry (GC-MS), a reliable, sensitive, and cost-effective method for the simultaneous determination of the main phthalates (DEHP, DBP, DEP, DMP, BBP) was developed. The factors which could influence the extraction were investigated in depth. Phthalate quantification with good precision and reliability is a real challenge (Net et al., 2015). Phthalates are ubiquitous in the laboratory and likely present in water, organic solvents, ambient air, glassware and plastic materials used for the analysis (Russo et al., 2015).

Therefore, the primary issue for phthalate quantification is not the trace analysis itself but the risk of secondary contamination during the analytical procedure, which can often lead to false positives or overestimated results (Marega et al., 2013).

In this study, a fume hood with a purified air filter dedicated to phthalate analysis was applied. All the materials handled during sampling and sample treatment were made of glass, aluminium or stainless steel. Prewashing of the laboratory material and equipment was done with acetone followed by drying at 120 °C for at least 4 h. In addition, high quality (i.e. HPLC grade) solvents including n-hexane, dichloromethane and methanol were used. All these preparations were essential to avoid secondary contamination that may occur during sampling, sample preparation, extraction and/or instrumental analysis thereby preventing overestimation of phthalate levels due to sample contamination (Reid et al., 2007).

The results indicated that under optimized conditions, the limits of detection (LODs) and limits of quantification (LOQs) were in the range of 0.01-0.025 and 0.025-0.05 µg/L, respectively. Also, the calibration curves were linear ($r^2 \geq 0.992$) over the concentration ranges from 0.05 to 20 µg/ L. In addition, a satisfying reproducibility was achieved when evaluating the intra- and inter-day precisions with relative standard deviations (RSDs) amounting to less than 11.71% and 12.40%, respectively. The recoveries of the five phthalates ranged from 91.5 % to 97.8 % with the RSDs being less than 10.64 %. DMP, DEP, DBP and DEHP were detected in most of the water samples. Based on the results obtained, it was concluded that the MSPE-GC-MS method developed in the current study provides a new option for the determination of phthalates in water samples. Compared with the conventional SPE procedure, MSPE is a simple method, with low consumption of organic solvent at the same time being time and labour effective, because it does not require any prior sample preparation thereby reducing the risk of secondary contamination (Xu et al., 2012; Harunarashid et al., 2017).

In general, MSPE is classified as a green analytical chemistry (GAC) technique for sample preparation with very good sensitivity (Net et al., 2015). MSPE coupled with GC/MS allows low LOQs in the range of 3.1–37 ng/L for 16 phthalates (Luo et al., 2012).

Based on the method developed in chapter 2, the occurrence and concentrations of common phthalates (DBP, BBP, DEP, and DEHP) were investigated in PET bottled water locally produced in the Iranian market and stored under various common storage conditions. Results of these studies are reported in Chapters 3, 4 and 5. In Chapter 3, three phthalates including DBP, BBP and DEHP were measured in bottled water samples, immediately after purchasing, and after being stored at room temperature (25±5 °C), in a refrigerator (4±1 °C), in a freezer (-18°C and 0°C), at 40°C and outdoor under sun exposure to investigate the factors that could potentially affect the phthalates

leaching from the PET plastic bottles into the water. Samples were stored up to 45 days and the release of DBP, DEHP and BBP was measured in the first 24 hours and on day 10, day 30, and day 45 in each storage conditions. It was shown that an increase in temperature and/or in the duration of storage affects phthalate migration. The highest concentrations of all phthalates were observed when bottled water samples were kept at 40 °C for 45 days. DEHP in bottled water was the most abundant phthalate under all storage conditions, although the observed level of DEHP in the worst case scenario (40 °C for 45 days) was still much lower than the DEHP maximum concentration limit (MCL) in bottled water (MCL= 6 µg/L) set by the U.S. Food and Drug Administration (U.S. FDA). In the present study, DEHP concentrations in bottled water after 45 days of storage were 26.83% of the FDA DEHP MCL upon storage at 40°C and 9.8% of the U.S. FDA DEHP MCL upon 45 days storage at -18°C (Figure 1). The MCL is the maximum permissible concentration of a contaminant in water. Since DEHP is a possible human carcinogen (group 2B), U.S. EPA established the non-enforceable maximum contaminant level goal (MCLG) at zero (U.S. EPA 1995). However, based on analytical feasibility, EPA also suggested an MCL of 6 µg/L to be applied for DEHP in bottled and drinking water. This level is an enforceable standard for a contaminant based on analytical methods/treatment (U.S. EPA 2018). According to the U.S. EPA, long-term chronic exposure to DEHP above the MCL of 6 µg/L may have the potential to cause damage to the liver and testes, adverse reproductive effects, and cancer in humans (U.S. FDA 2011).

WHO set a guideline value of 8 µg/L for DEHP allocating 1% of the tolerable daily intake (TDI) to drinking water (WHO 2011). The TDI of 25 µg/kg body weight/day for DEHP was derived using the NOAEL of 2.5 mg/kg body weight/day based on peroxisome proliferation in the liver in rats (Morton 1979; WHO 2003). It is of interest to note that at present this endpoint may no longer be considered relevant for human risk assessment (IARC 2011). Figure 1 presents an overview of how the actual levels of DEHP detected in the study described in Chapter 3 of the thesis in bottled water stored under various conditions, compare to these limits available for DEHP in drinking water. This clearly illustrates that under all conditions DEHP values remained below these MCLs.

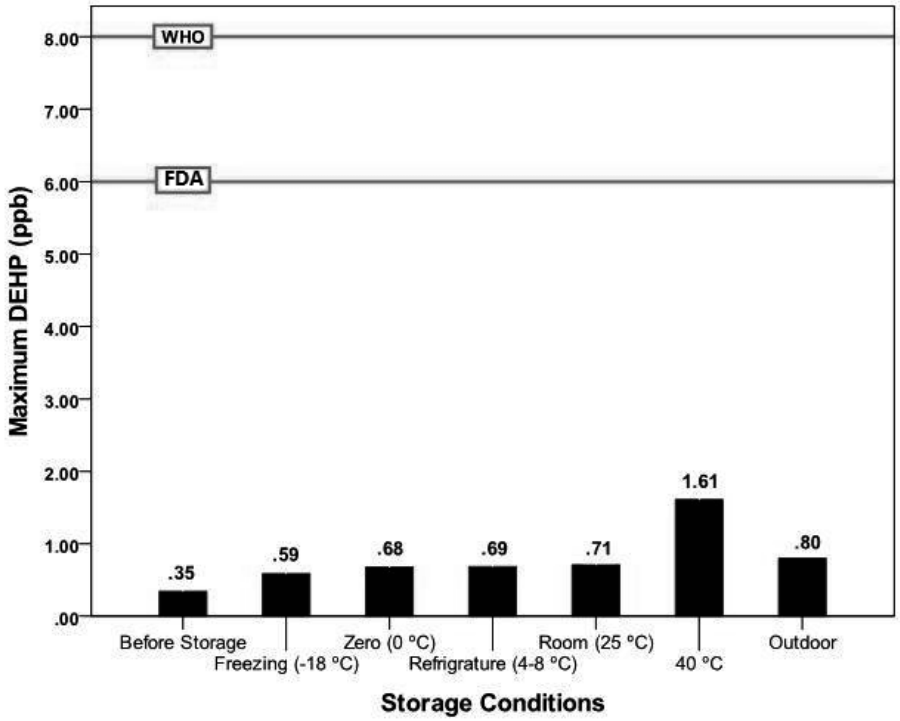

Figure 1. Overview of the level of DEHP (µg/L) in bottled water stored for 45 days under various conditions in comparison with maximum concentration limits derived by U.S. FDA, (equal to the limit suggested by U.S. EPA for tap water) and WHO (For further details see chapter 3).

None of the agencies (U.S. EPA, U.S. FDA, WHO) have established MCLs for DBP, BBP, DEP or other phthalates in drinking and/or bottled water.

As it is common for vendors and distributors in Iran to transport and store bottled waters outdoors under direct sun exposure for extended periods, or for families to freeze bottled water for children's backpack in the warm seasons, these conditions were also included in the studies (Figure 1). The levels of phthalate compounds measured showed that the migration of phthalate compounds during storage under these conditions is limited. Nevertheless, the results indicate that contact time with packaging material (PET) under all temperatures causes phthalates to be released into water from PET bottles (Chapter 3 and 4).

It is worth mentioning that among common phthalates, BBP was only detected in the samples stored at high temperature (≥25 °C) and outdoor under direct sun exposure.

When comparing the concentrations of DBP, BBP and DEHP with initial levels in the bottled water, the results demonstrate that the release of phthalates was not substantial under all storage conditions especially at low temperatures (<25 °C) and under freezing conditions (Figure 2).

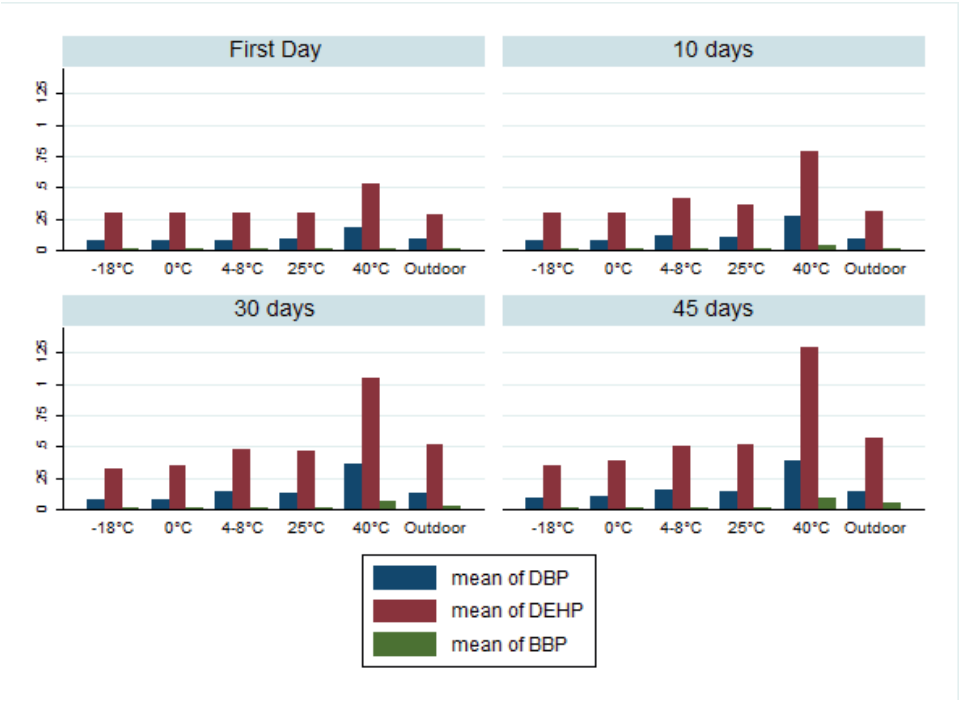

Figure 2. The effect of PET-bottled water storage conditions on phthalate migration (µg/L) with increasing storage time.

These observations are consistent with previous reports showing a slightly increasing trend in the concentrations of phthalates as a function of storage time and temperature and that phthalate concentrations in bottled water are attributable to migration of phthalates from the respective plastic bottles (Zaki et al., 2018; Cincotta et al., 2018; Selvaraj et al., 2016; Dumitrascu 2012). Phthalates are not typically used in PET manufacturing and their presence therefore, is non-intentional, likely resulting from the wide use of phthalates in various industries and their ubiquitous presences as environmental contaminants (Cincotta et al., 2018).

In addition in Chapter 3, based on the measured concentrations of phthalates, a phthalate exposure assessment through PET bottled water consumption was performed for children in Iran. To this end, the maximum concentrations of DBP, BBP and DEHP obtained throughout the study were used to calculate the estimated daily intake (EDI) representing the worst-case exposure scenario. The level of concern for non-carcinogenic effects of individual phthalates is determined by calculating a hazard quotient (HQ) as the ratio between the level of exposure (EDI) and an acceptable level of exposure (reference dose, RfD or TDI) (EPA 2000). An HQ < 1.0 raises no concern whereas an HQ>1.0 indicates a concern because the EDI exceeds the TDI or RfD.

The EDI of phthalates for children from the Iranian PET bottled water ranged from 0.01 µg/kg body weight/day for BBP to 0.24 µg/kg body weight/day for DEHP which is far below their respective

RfD values resulting in a maximum HQ of 0.012 for the worst condition (40°C after 45 days). Furthermore, in order to assess the carcinogenic risk due to exposure to the possibly carcinogenic DEHP (group 2B), the excess lifetime cancer risk was calculated by multiplying the cancer slope factor for the substance by an estimated daily intake (EDI). The slope factor is defined as the upper-bound estimate of the probability that an individual will develop cancer if exposed to a chemical for a lifetime of 70 years and is expressed in $(mg/kg\ body\ weight/day)^{-1}$ (IRIS EPA 1987). The European Commission's Scientific Committee on Occupational Exposure Limits (SCOEL) assigns non-genotoxic carcinogens (such as tumour promoters) and non-DNA reactive genotoxic carcinogens into a group of carcinogens with a threshold mode of action (EC/ECHA 2017). DEHP is not genotoxic and considered a carcinogen that acts by a threshold mode of action. Its carcinogenic risk can be evaluated by linear extrapolation form the tumor data (EC/ECHA 2017).

The EDI value resulting from consumption of the DEHP containing bottled water by children, was calculated to result in a negligible cancer risk of 6.5×10^{-7} upon life time exposure. This is lower than the target reference risk defined by U.S. EPA, WHO or national regulatory bodies, who typically consider a risk ranging from 10^{-6} to 10^{-4} acceptable for carcinogens in drinking water (Cotruvo 1988; WHO, 2001).

A 10^{-6} increased cancer risk represents an increased lifetime risk of 1 in 1,000,000 for developing cancer due to lifetime exposure to a substance (Kelly et al., 1991). The concept of 10^{-6} was originally defined by the U.S. FDA (1977) as a screening level of "essentially zero" or de minimis risk (Kelly et al., 1991).

In chapter 4, concentrations of DEP were measured in bottled water kept under various storage conditions, similar as those used in chapter 3 for DEHP, DBP and BBP, and the resulting risks of consumption of this water for children but also for other age groups were evaluated. The results indicate that storage duration and storage temperature also slightly influences the release of DEP from PET bottles into water. In comparison to the initial level of DEP in bottled water samples, the migration of DEP appeared negligible under most storage conditions, especially at low temperatures (<25 °C) and freezing conditions, being in line with what has been reported in literature. In this chapter the margin of exposure (MOE) approach as a method of risk characterization was applied to assess the potential risk of exposure to DEP via consumption of bottled water. The MOE is defined as the ratio between the NOAEL determined from an experimental animal study and the estimated level of human exposure (Williams et al., 2000). The MOE quantifies the distance between the exposure level and the NOAEL (EPA 2000). Determination of an acceptable MOE relies on the judgment of the regulatory authority and varies with factors such as nature/severity of the toxicological endpoint observed, completeness of the database, and size of the exposed population

(Williams et al., 2000). The value of the MOE that is associated with a concern for toxic effects is generally expressed as the product of the applicable uncertainty and modifying factors that the Agency considers for non-cancer effects. For compounds which have a substantial toxicological database, MOE values of 100 (uncertainty factor of 100) or more are generally considered to indicate that the potential for causing adverse health effects is negligible (Williams et al., 2000).

In the present study, MOEs were calculated using the NOAEL of 750 mg/kg body weight/day from a sub-chronic rat study (IRIS 1987c). The level of exposure to DEP via consumption of bottled water in different age groups based on the worst-case scenario exposure assessment was as follows: pre-school children> children> lactating women > teenagers> adults> pregnant women. The EDIs resulting from intake of DEP via consumption of bottled water resulted in MOEs that were generally much higher than 1000 (uncertainty factor for DEP) (IRIS EPA 1988), indicating a low concern. It can be noted that using the MOE of 1000 is essentially comparable to deriving a TDI form the NOAEL using an uncertainty factor of 1000 and then comparing the EDI to the TDI. MOEs much higher than 1000 indicate that all EDIs would be below the TDI thus derived.

Among investigated phthalates in the present thesis, DBP, BBP and DEHP have all been shown to be anti-androgenic, based on the following observations: decreased fetal testosterone production; reduced male anogenital distance; and increased nipple retention in male offspring, which is an early marker of anti-androgenic effects, seen consistently in connection with the other effects (ECHA 2017). In addition to the anti-androgenic effects, DBP, DIBP and DEHP induce changes in germ cell differentiation (multinucleated germ cells), which are considered to occur independent of fetal testosterone reduction (Borch et al. 2006, Lambrot et al. 2009).

Also of interest is that all phthalates may have this mode of action pointing at the need for a combined exposure and risk assessment. This was investigated in chapter 5. To this end dose additivity is the method of choice, given that combined toxicity of multiple chemicals act through dose addition when the chemicals have a similar mode of action or through response addition when chemicals have a dissimilar/independent mode of action (EFSA 2013).

For combined risk assessment of the anti-androgenic activity of the phthalates in chapter 5 the hazard index (HI) approach was used to evaluate this combined effect (ECHA 2017). Based on dose additivity, the HI is presented as the sum of individual HQs with the same adverse effect and mode of action.

In Chapter 5 the cumulative health risks in pregnant and lactating women posed by combined exposure to anti-androgenic phthalates (BBP, DBP, and DEHP) via consumption of bottled water were estimated based on the measured concentrations of mentioned phthalates in bottled water stored under various conditions. To this aim, the cumulative health risk assessment was performed

on the basis of an HI calculation for anti-androgenicity (AA) (using the RfD-AA). The estimated intake by pregnant and lactating women was between 0.0021 µg/kg body weight/day for BBP and 0.07 µg/kg body weight/day for DEHP. Using these values, the highest HQ obtained for individual phthalate intake via bottled water consumption in pregnant and lactating women based on the U.S. EPA RfD values was much lower than 1 ($HQ \leq 0.004$), and cumulative risk assessment for combined phthalate exposure demonstrated that the HIs for anti-androgenic effects were also lower than 1 ($HI \leq 0.003$) which implies that adverse effects are unlikely to occur.

The excess lifetime cancer risk for DEHP calculated for pregnant and lactating women based on the highest detected level and resulting EDI amounted to a carcinogenic risk of 6.5×10^{-7} above background levels, which is less than 10^{-6}, raising no concern.

The exposure to individual phthalates via bottled water consumption as percentage of their respective TDI values was determined to assess whether consumption of the bottled water on itself is expected to be safe. The exposure to phthalates via bottled water consumption was at maximum 0.5, 0.024, 1.1, and 0.004% of the TDI values for DEHP, DEP, DBP and BBP, respectively, indicating that consumption of this water is safe. A study in 2017 on Egyptian bottled water reported that the contribution of bottled water consumption to phthalate daily intake amounted to around 0.16 and 0.72% of the TDI values of DBP and DEHP, respectively, indicating a similar exposure via bottled water to DEHP, but a higher exposure to DBP, compared to our study. Also the authors of that study reported that there should be no adverse health effects through consumption of bottled water even at the maximum concentrations detected for these chemicals (Zaki et al., 2018).

In Chapter 6 of the thesis it was additionally investigated whether the phthalate exposure would be a factor contributing to the development of autism spectrum disorders (ASD) by means of a systematic review. Several studies have investigated the association between exposure to phthalates and autism but the use of different study designs and other parameters often presents a challenge to conclude on the possible role of phthalates in ASD development. Autism is a life-long neurodevelopmental condition characterized by persistent difficulties in social communication and interaction with various genetic and environmental risk factors (APA 2013). Autism is also associated with an increased risk for other neurodevelopmental and psychiatric conditions (Pan 2014; Simonoff et al. 2008). Available evidence implicates environmental factors with especially exposure to endocrine disruptor chemicals in the pathogenesis of autism (Moosa et al., 2017; Posar A et al., 2017). A median global prevalence of 62 cases per 10,000 individuals is reported based on epidemiologic studies of ASD (Elsabbagh et al., 2012). The prevalence of 6.26 per 10,000 is determined for autistic disorder in Iran (Samadi et al., 2012). Thus, the rates obtained for Iran are

much lower than those reported for the USA (Bertrand et al., 2001) and England (Baird et al., 2006), with rates of up to 40 per 10,000 for children with autistic disorder. Several factors might explain the lower prevalence of autism in Iran including:

1- A diagnosis of disability is likely to be seen as stigmatizing in Iranian culture (Samadi, 2008).
2- Parents may under-report the child's difficulties to assessors even though they are aware of them to avoid being referred to a special school which is not readily available.
3- Tools that are used in Iran mainly rely on parental reports, with limited time and opportunity for assessors to observe and interact with the child and for them to make consensus decisions (Baird et al., 2006).

Owing to importance and increasing prevalence of ASD, finding environmental causes of this disorder is of interest (Picciotto et al., 2018). To shed more light on this issue, Chapter 6 of the thesis aimed to systematically review published evidence on the possible association between prenatal and/or childhood exposure to phthalates and ASD.

This review was conducted on published peer-reviewed journal articles listed in Scopus, PubMed, PsycInfo and Web of Science prior to December 2015. Studies that were included were those that assessed the effect of pre- or post-natal exposure to phthalates on occurrence of autism and non autistic-like disorders. Each eligible paper was summarized with respect to its methods and results with particular attention to study design and exposure assessment.

The results of this systematic review revealed that only a limited number of studies has addressed phthalates in relation to autism. Seven studies were regarded as relevant for the review. A total of five studies met the inclusion criteria and were included in the review. Of the 5 studies, two studies were cohort studies both in the U.S.A. and three were case-control studies conducted in the U.S.A., Italy and Turkey. Study populations ranged from children aged 4 years to 12 years. Among these cohort and case-control studies, different screening administration methods were used.

The two cohort studies measured phthalate metabolites in maternal urine samples. One of the cohort studies reported positive results regarding maternal urinary MEP, a metabolite of DEP, concentrations during pregnancy associated with ASD diagnosis in 7- to 9-year-old children (Miodovnik et al., 2011) whereas the other study showed only poor association for MEP with ASD (Braun et al., 2014). Among the three case-control studies, two showed a significant relation between exposure to DEHP metabolites and ASD and the last case-control study showed a compromised phthalate metabolite glucuronidation pathway, as a probable explanation or mechanism underlying the relation between phthalate exposure and autism although this does not necessarily mean that phthalates are directly linked to ASD.

Because of the heterogeneity in the type of included studies, different methods of assessing exposure to phthalates and the use of different statistics for summarizing the results, a meta-analysis could not be used to combine the results of included studies.

The review revealed some equivocal evidence suggesting a possible connection between exposure to phthalates and ASD. Nevertheless, further comprehensive research is needed with appropriate attention to exposure assessment and relevant pre and post-natal confounders. In general, finding ASD etiology is complex and currently, environmental health investigators are innovating 'exposomic' approaches (Picciotto et al., 2018). However, for ASD research, the principal obstacle to applying environment-wide analyses (statistical methodologies) is the lack of large amounts of data collected during relevant time windows in robust samples sizes (Picciotto et al., 2018).

To the best of our knowledge, exposure to phthalates is poorly studied and understood in healthy Iranian children. Therefore, in chapter 7, an observational study was designed to estimate the exposure pattern and total daily intake of five common phthalates, and to assess the health risks of combined exposure to anti-androgenic phthalates (DBP, BBzP and DEHP) in Iranian children and adolescents.

In this study we estimated the daily phthalate intake of 56 children and adolescents aged 6 to18 years by extrapolating from their spot urinary levels of the phthalate metabolites MEHP, MEHHP, MEHOP, MEP, MBP, MBzP and MMP. We applied a calculation model based on the creatinine-adjusted urinary metabolite concentrations to obtain the EDIs for the respective phthalates.

The EDI values thus obtained were compared to available health based guidance values including TDI values to calculate the HQ values for single phthalates. Assuming additive effects, the HI was calculated for combined exposure using TDI values determined by the European Food Safety Authorities (EFSA) and Reference Doses for Anti-Androgenicity (RfD-AA) determined by Kortenkamp and Faust (Kortenkamp & Faust, 2010) as acceptable levels of exposure for DBP, BBzP, and DEHP based on anti-androgenicity as the critical effect.

EFSA TDI values for DBP and DEHP are based on loss of germ cell development and mammary gland changes in rats exposed from gestation day 15 to postnatal day 21 and testicular toxicity and developmental toxicity in a multigenerational reproduction study in rats, respectively (Lee et al., 2004; EFSA 2005a). The EFSA TDI value for BBP is based on reduction of anogenital distance in rats in the F1 and F2 generation after exposure via the diet which is a marker of impaired androgen action or production during the masculinization programming window (Tyl et al., 2004; Welsh et al., 2008).

The RfD-AA for DBP and BBP are based on suppression of testicular testosterone production in the rat at gestation day 18 after exposure via gavage on gestation days 8–18 (Howdeshell et al., 2008).

The RfD-AA for DEHP is based on nipple retention in rat male offspring of exposed dams (Christiansen et al., 2009). Nipple retention is an endpoint known to be related to decreased androgen action during fetal development in the extended one-generation reproductive toxicity study (OECD 2011).

In contrast to the EFSA TDI values and RfD-AA values defined by Kortenkamp and Faust, the U.S. U.S. EPA RfD values may be less adequate for assessing combined risk because U.S. EPA RfD values have been defined based on different endpoint than anti-androgenicity. The U.S. EPA RfD values for DBP is based on increased mortality in rats exposed via the diet for a year (Smith, 1953), while for DEHP it is based on increased relative liver weight in the guinea pig exposed via the diet for 1 year (Carpenter et al., 1953) and for BBP it is based on increased liver-to-body weight and liver-to-brain weight ratios in the rat following exposure via the diet for 6 months (U.S. EPA, 1993).

Furthermore, the Maximum Cumulative Ratio (MCR) was used to quantify the degree to which a single chemical drives the risk from combined exposure to the phthalates. The MCR of the individual's exposure to multiple substances is an index that can be calculated based on HQs of the individual substances and the cumulative HI by dividing the HI of the combined exposure to the maximum of the HQs of the individual substances (max HQi), which helps identify if one or multiple components are driving the risk estimate for a co-exposure (Price et al., 2014).

Six of the seven phthalates metabolites were detected in all the samples, with MBzP in 92.9%. The ranges of urinary phthalate metabolite concentrations were 0.13 to 11.6 µg/L for MEHP, 1.9 to 79.43 µg/L for MEOHP, 3.04 to 129.85µg/L for MEHHP, 3.5 to 62.1 µg/L for MEP, 10.6 to 72.3 µg/L for MBP, 3.1 to 43.4 µg/L for MMP and lower than the limit of quantification amounting to 5.1 µg/L for MBzP. Generally, boys exhibited slightly higher urinary levels for the majority of investigated phthalates except for DEP and BBP. The EDI values derived from these biomonitoring data amounted to 0.01 µg/kg body weight/day for BBP and 17.85 for DEHP µg/kg body weight/day. The results from the risk assessment suggested that not only the exposure to the single phthalates, but also the combined exposure would not raise a safety concern (HI values in the surveyed participants averaged 0.2). The range of MCR values in the 56 participants was 1.1 to 2.32 indicating that the combined exposures of concern mainly originated from one or two of the three anti-androgenic phthalates including specially DBP and DEHP.

The detection of all phthalate metabolites in about all of the samples indicated that Iranian children and adolescents are exposed to low levels of a mixture of these phthalates, but a subsequent risk assessment revealed that none of the surveyed participants had HI values that raised a concern. The phthalate exposure pattern for the study population of Iranian children appeared comparable to the

results reported for children and adolescents from other parts of the world with the greatest similarity being found for children from Taiwan, China, Brazil and Greece (Chen et al. 2017; Huang et al., 2015; Wu et al. 2016, Rocha et al., 2017).

Although, combined exposure to anti-androgenic phthalates did not exceed the acceptable level of exposure, people typically come into contact with also other chemicals with anti-androgenic properties, for example the multiple chemicals found in food, air, drinking water, and in household and consumer products and cosmetics which may result in a combined anti-androgenic effect (Howdeshell et al., 2017; Kortenkamp et al., 2010). Since, it is unavoidable that humans are exposed to more than one chemical at a time (Evans et al., 2016), combined exposure assessment to mixtures of chemicals with anti-androgenicity effects is becoming an indispensable feature of the chemical risk assessment landscape.

The result of the present thesis corroborated that drinking water, as a monitored source for external exposure assessment, has a very small contribution to total phthalate EDI values indicating that drinking water appears to be a minor source of phthalate exposure. As shown in Table 1 (data taken from Chapter 3 and 7), only 4% of total phthalate intake in children originates from phthalate exposure via drinking water. The calculated total phthalate intake from biomonitoring data (resulting in the direct exposure assessment) differs from the exposure assessment based on the levels detected in the bottled water and thus indicate that there are other far more important sources of exposure to phthalates than the bottled water.

Humans can be exposed to phthalates through multiple routes and pathways (Wang et al., 2018). Nevertheless, dietary intake is the predominant exposure route for phthalates (Giovanoulis et al., 2018, Larsson et al., 2017). Country specific use patterns for phthalates in relevant products, cultural diversity, differences in food consumption habits, and/or differences in socio-economic factors might be correlated with particular consumption patterns (EFSA 2011; Johns et al., 2015). In this regard, future local research may investigate the contribution profiles of different foodstuff to dietary intake of phthalates and contribute to identification of major sources of exposure thereby defining priorities for a further reduction of exposure.

Table 1. Overview of direct and indirect phthalates exposure assessment in Children

Population	Exposure assessment method	Media	Estimation method	∑ Phthalates Exposure estimate (μg/kg/day)	The portion of water in total exposure to phthalates
Children and adolescents	Indirect	water	Phthalates content × ingestion rate of water	0.3 (worst case scenario)	4%
	Direct	Urine	Total intake from urinary biomarkers	7.5 (mean)	

Future perspectives

Although the present thesis assessed the contribution of drinking water to the total daily intake of phthalates for Iranian populations, there is a paucity of available data about the contribution of other food categories to the total daily intake of phthalates. In general, knowledge gaps concerning phthalates in Iran include trends in phthalate exposure, importance of dietary and non-dietary sources (e.g. food, pharmaceuticals, personal care products), environmental distribution and fate, and methods to define internal and external exposure. Future investigations are needed to evaluate the recommended priority topics for phthalate exposure including identification of external exposure sources, defining internal exposure and converting biomarker levels from biomonitoring studies to external dose levels needed in risk assessment, and characterizing potential health effects among the population (Ministry of health Education of Iran, annual report 2016).
The following sections present some considerations on future topics of importance in this field.

1- Trends of contamination and resulting phthalate exposure in Iran

There is a lack of data regarding the phthalate sources and routes of exposure in Iran. For ubiquitous environmental pollutants with multiple exposure pathways like phthalates, exposure assessment based on environmental data requires quantitation of phthalate levels in multiple media (WHO 2015). To elucidate the extent of external exposure to phthalates in Iran, building research infrastructures that aim to increase knowledge of exposure to phthalates in order to make a comprehensive dataset for an accurate identification of phthalate exposure sources and routes, is required to facilitate a refined exposure assessment that goes beyond estimating the exposure from bottled water. Regarding food safety, an EU-wide project with the use of total diet studies (TDS-Exposure) provided representative and realistic data on food contamination and chronic exposure levels to chemicals of relevant populations (Turrini et al., 2018). TDSs are designed to cover the average diet or the most commonly consumed foods, based on data from dietary surveys, in a country or by a specific population group. The international organizations such as Food and Agriculture Organization (FAO), WHO, and EFSA have tried to harmonize the TDS methodology. In fact, EFSA chemical concentration data partly originated from TDS (EFSA 2011).

2- Setting up a national data base on parameters that enable adequate exposure assessment

One of the steps for performing an adequate risk and safety assessment is adequate exposure assessment. In this exposure assessment the relevant exposure factors have to be defined (ExpoFacts, 2007; EPA, 2011). Exposure factors as data on individual behavioural patterns

affecting exposure, include anthropometric data (e.g., body weights, skin-surface areas and life expectancy), behavioral data (e.g., activity/time use patterns, consumer product use), physiology (e.g., inhalation rates, dermal adherence factors), dietary ingestion, ingestion of drinking water, non-dietary ingestion, soil and dust ingestion, and environmental (housing characteristics) (Zaleski et al., 2016). These exposure factors may differ between population groups because they are influenced by age, gender, community, dissimilar geographical, time-activity patterns, cultural or social factors (Vuori et al., 2006). At a national level, a compilation of exposure data is not available in Iran. In absence of specific data, exposure/risk assessors usually use recommended values of exposure factors provided in other studies and from other countries (e.g., the European exposure factors (ExpoFacts) sourcebook for the E.U. population, U.S. EPA Exposure Factors Handbook for the U.S. population, RefXP Exposure Factors Database for the German population (GFEA 2014), The Dutch National Institute for Public Health and the Environment (RIVM) ConsExpo with data aimed at the Dutch population (Te Biesebeek et al., 2014), the Concise European Food Consumption Database containing information from individual dietary surveys from 22 EU Member States for a limited number of broad food categories (EFSA 2011), the "Korean Exposure Factors Handbook" providing information specific to the Korean population (Kim et al., 2006), the Japanese Exposure Factors Handbook" containing data for the Japanese population (Gamo et al., 2006) and Highlights of the Chinese Exposure Factors Handbook for the Chinese population (Duan, et al., 2015)). Using these values as default values to assess the exposure among Iranian population will result in calculated exposure (intake) values that are not fully representative for the investigated population. For example, using default values for parameters like body weight and drinking water consumption may overestimate or underestimate the risk of exposure to environmental contaminants for the Iranian population (ITRC, 2015). Hence, it is of paramount importance to provide a national guideline as a reference tool and primary source of various factors used in assessing exposure for the general population and various demographic groups based on national data. Globally, developing and maintaining exposure data resources are ongoing challenges given potential changes in lifestyle, new product formulations, imports and consumer use patterns. Improvements in technology, for example the ongoing development of sensors that can assess personal exposure, may help accrued exposure assessment (Zaleski et al., 2016).

3- *The trends of human exposure to phthalates*

At the current state of the art, there is a lack of human biomonitoring (HBM) data to track total phthalate exposure in the Iranian population. Indeed, information about internal phthalate exposure levels for different populations, such as different age groups, prenatal exposures, living area and income-depended lifestyle is rudimentary. Chapter 7 of this thesis described the use of HBM data from a small scale, local survey of urinary concentrations of phthalates in Iranian children to estimate their exposure. Similar studies in other age groups within the population would be of use to validate intake estimates based on different strategies. Implementation of comprehensive population-representative biomonitoring projects for measuring the concentrations of phthalates in human samples will be a useful tool for assessing consistent exposure trends for different populations in Iran and to validate exposure estimates based on other strategies. With this, drawing conclusions regarding the links between external and internal concentrations will also be possible (Bonnell et al., 2018). In addition, HBM data may provide useful insights into ongoing market changes in production and use of phthalates in consumer products (Calafat et al., 2015, Choi et al., 2015). In general, the lack of HBM data on phthalates in countries in the different parts of the world provides a topic for future research (WHO 2015; Choi et al., 2015).

4- Improve the chemical legislation in Iran

Phthalates are ubiquitous environmental contaminants and they can be present in food contact material as non-intentionally added substances (NIAS) (Muncke 2011; Muncke wt al., 2017). In the present thesis, the presence of phthalates in PET bottled water was evaluated as an end-use product. Although PET plastic bottle factories state that phthalates as plasticizer are not used in the manufacture of the PET plastic bottles, detectable levels of phthalates were found in the majority of samples indicating that phthalates are present in the end-use products as NIAS.

The food and drug administration of Iran does not regulate phthalate levels in bottled drinking water, and neither classifies them as a health hazard due to the fact that they are supposedly absent in PET plastic bottles.

There is a dearth of international information on production, import and usage as well as distribution of phthalates and fate in the environment. In this regard, the following actions are required to address chemical safety legislation in Iran at the level beyond only phthalates:

- Creating a local registration system and international harmonization of regulations, toxicological information, use and application of chemicals including phthalates.

In this order, combined efforts of legislators, academia and industry are required to control end products, manufacture, import and application of phthalates as plasticizer and/or NIAS in Iran.

REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) as the key European Union law on chemicals has significantly enhanced the protection of human health and the environment and promoted alternatives to animal testing and REACH is expanding rapidly to new markets in the rest of the world (EC 2018). Some non-EU countries established REACH-like regulation. The Ministry of Environmental Protection (MEP) of China released the regulation which is similar to EU REACH and is also known as "China REACH or MEP Order 7" in 2010. Korea enacted the Act on the Registration and Evaluation of Chemicals (AREC or K-REACH) on 01 January 2015. On 23 June 2017, the Ministry of Environment and Urbanization (MoEU) in Turkey published its REACH-like KKDIK regulation which came into force on 23 Dec 2017. Developing REACH-like regulation in Iran would bring various Iranian chemicals' legislation under one law to register all substances manufactured in Iran or imported into Iran. It will improve public health and the environment by providing information on chemical substances and products containing chemical substances.

Regarding existing and emerging risks associated with the food chain (food contaminants), coordination of national food legislation (food and drug administration of Iran) with international agencies such as WHO, EFSA, the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the U.S. FDA would be a step forward providing greater protection for the health and safety of the population.

These action should also include;

- Streamlining information sources globally, harmonize quality, information content, use of search engines

Several organizations in different fields throughout the world provide databases with information on chemicals, products, toxicokinetics, adverse health effects, or others. Acquaintance and connection to the international databases would enable all researchers and stakeholders to keep abreast of relevant developments.

- *Declarations of chemicals in materials and goods*

The obtained biomonitoring data based on measurements of urinary phthalate metabolites in chapter 7 of the present thesis demonstrated that children are exposed to multiple phthalates, which can be derived from either known or undefined sources. It is noted in this thesis in chapter 3, 4 and 5 that these exposures come partly from water, but there is a host of other exposure sources.

Therefore, declaration of chemicals in materials and goods, to better know which chemicals are being used and where, is an important issue all over the world. Such actions may identify the actual sources of the phthalates detected in the products and/or help to further reduce the levels in material and goods that appear to contribute to the overall exposure to a relatively larger extent. Overall,

supply chain insight is needed through which the understanding of potential exposure to single chemical and/or chemical mixtures could be significantly improved (Altenburger et al., 2018).

5- Use of epidemiological data in exposure and safety assessment

Epidemiological studies by initiating large prospective cohorts, combining existing cohorts (Flanagan et al., 2017) or building large administrative cohorts, can support the production of substantial knowledge looking for new associations between environmental exposures and health outcomes in human (Brook et al., 2018). A national data base could provide the opportunity to combine HBM studies and health studies in order to obtain more information from the study participants using the most cost-effective methods. In the present thesis, by applying a systematic review for addressing the role of phthalate exposure in development of autism, none of the few studies performed in Iran could be included in this review, because of inadequate data, different methodology and poor study quality. For instance, none of the existing few cohort and case-control studies in neurodevelopmental disorders in Iran considered phthalate levels in biological samples while they measured other chemicals with an overlap among these investigated chemicals across studies.

There is not any available database or harmonized Iranian nationwide program about the ongoing and/or previous HBM studies especially across cohort studies as it exists in Europe within the context of the HBM4EU project (Joas et al., 2017). Phthalates are known as a prioritized substance group according to the prioritization strategy of this HBM4EU project and DBP, BBP and DEHP are categorized as substances for which HBM data are sufficient to provide an overall picture of exposure levels across Europe, and for which interpretation of biomonitoring results in terms of health risks is possible (Kolossa-Gehring et al., 2017) whereas in Iran this categorization is impossible due to lack of sufficient biomonitoring data. In this regard, national data platforms with connecting and expanding national and international activities in biobanking, biomonitoring and cohort studies would offer an innovative approach to pinpoint gaps in current activities and to optimize use of existing data from both HBM and health studies for adequate assessment of the role of phthalate (and other) exposures in the etiology of disease (Bouwmeester et al., 2017). In addition, a national data platform on results from epidemiological studies would bring together scientific and other expertise from academia, government, non-governmental stakeholders and industries to focus on specific research priorities (Brook et al., 2018). Ultimately, such an effort should result in identification of modifiable risk factors leading to interventions that have benefits for human health.

6- Incorporating the exposome into traditional biomonitoring approaches

Another topic for future consideration is the role of the so-called exposome. This is a term to describe the totality of a person's environmental exposures. It includes aspects of space and time such as living surroundings, social interactions, lifestyle and the extent to which these affect the biological functions encoded by our genome (Dennis et al., 2017; Escher et al., 2017).

Although such data on both environmental and genetic causes of disease is augmenting as a consequence of large-scale epidemiological studies, exposure data (including diet lifestyle, environmental, socio-demographic and occupational factors) is often fragmentary (in time and depth), non-standardized, at crude resolution and often does not include estimates at the individual level (Wild 2005). In addition, current understanding suggests that the body of research is relatively large for impacts of single chemical exposure whereas there is still less research evaluating exposures to chemical mixtures (NRC 2009; EC 2012; Altenburger et al., 2013).

In our 2016 systematic review about association between phthalates exposure and ASD (Chapter 6), we expressed concern that phthalates as a group of endocrine disruptor chemicals might remain undiscovered among the chemicals known to be neurotoxic to human beings. Effects induced by exposure to endocrine disruptor chemicals especially during critical windows of neurodevelopment are more severe, long-term, and potentially irreversible and can be transgenerational (Sutton et al., 2019; Gore 2018). Owing to the fact that particularly important new evidence derives from prospective epidemiological birth cohort studies, implementation of birth cohort studies for assessing the contribution of prenatal exposure to phthalates along with other risk factors in the development of ASD and other neurodevelopmental disorders is warranted. (Grandjean et al., 2014). Moreover, it appeared that there was not one study aiming to assess prenatal exposure to phthalates and other chemicals along with other prenatal and/or post- natal confounders in association with ASD. In fact, data from the external environmental assessment could be combined with data regarding internal exposure plus other variables to build the exposome and to derive environment-wide associations between exposure and disease (Steckling et al., 2018). Biomonitoring data as a key tool to define exposure–disease risks given the biological significance of internal exposure measurements are central to the development and implementation of the concept of the exposome (Dennis et al., 2017; Aylward 2018).

The exposome concept was launched some years ago to draw attention to critical need for more complete environmental exposure assessment in epidemiological studies as a complement to the genome (Berglund et al., 2016). According to the Rappaport and Smith definition, the exposome takes into account that exposures are comprised of an external (chemicals entering the body from the environment) and an internal (compounds produced in the body by inflammation, (oxidative)

stress, lipid peroxidation, infections, the microbiome, etc.) chemical environment (Rappaport et al., 2010). In order to establishing a personalized picture of a specific individuals' exposures, measurements during critical life stages, including fetal development (cord blood analysis), early childhood, and puberty, are essential (Escher et al., 2017). It is expected that, incorporating the exposome into epidemiologic research will enable researchers to improve the ability to reveal the environmental contributors to health and disease from conception to death (Berglund et al., 2016; Stingone et al., 2017). Broad characterization and understanding of internal exposures and their consequences are achievable under the exposome paradigm through capitalizing on emerging technologies and seeking to characterize the early-life exposome and relate it to health end-points specifically in relation to neurodevelopmental disorders as neuroexposom (Dennis et al., 2017; Heffernan et al., 2018).

Within Europe and the United States, there are some ongoing exposome projects, the EXPOsOMICS, the Human Early-Life Exposome (HELIX) project and the Children's Health Exposure Analysis Resource (CHEAR), for example.

The EXPOsOMICS project is a European Union funded project that leverages existing long-term European cohorts, and their stored biospecimens, to integrate external exposures from personal exposure monitoring technologies and population-based measures of exposure with internal measures resulting from -omics technologies (Vineis et al., 2017). With a focus on air pollution and water contaminants, EXPOsOMICS will examine this segment of the exposome across studies of populations during critical periods of life (Vineis et al., 2017).

The HELIX project supplements existing data for 32,000 mother–child pairs from six European birth cohorts with new internal measures of exposure and biological response on a smaller subsample of 1,200 mother–child pairs on the base of existing study infrastructure and past data collection to facilitate the newer, exposome-related measurements, including personal exposure monitoring and analysis of molecular signatures within stored and new biological samples (Vrijheid et al., 2014).

CHEAR includes a network of laboratories with extensive analytic abilities for exposure assessment and measures of biologic response in a variety of biological samples aiming to implement the exposome concept in children’s health studies and create a public resource of children’s exposures across the country, as well as to develop novel statistical approaches for combining data across studies and analysis of high-dimensional exposure data (https://www.niehs.nih.gov/research/supported/exposure/chear/).

There is no registered exposome study in Iran and indeed the few existing birth cohort studies are not harmonized. Therefore, it seems essential to encourage researchers to embark on exposome

research and to further develop the essential approaches and analytical methods to ensure that potential exposure assessment strategies are rigorously evaluated, ultimately promoting the well-being of all populations.

7- Application of physiologically based kinetic (PBK) modeling

In the present thesis HBM data on urinary biomarkers were translated to oral dose levels providing estimated daily intakes (EDIs) using predefined relationships between biomarker levels and dose levels, as one of the simplest methods for the interpretation of HBM data. A more advanced way of making this translation would be via physiologically based kinetic (PBK) modelling. Computational PBK modeling based reverse dosimetry is an approach to define exposure to chemicals from levels of relevant metabolites in selected body fluids or tissues (Egeghy et al., 2016). HBM data can be used to calibrate pharmacokinetic models that predict concentrations in different body compartments (U.S. EPA, 2013) and, after validation of the model, one can use the model for reverse dosimetry to convert a urinary biomarker level to an oral dose. PBK models are computational tools, which simulate the absorption, distribution, metabolism, and excretion (ADME) characteristics of a compound and/or metabolites within an organism (Paini et al., 2017). The structure of the PBK model depends primarily on the aims for which the model is developed and on the available data. PBK modelling can be used to support the interpretation of HBM data from the perspective of exposure reconstruction and risk characterization, by relating a measured concentration of a chemical in a human tissue or fluid to an exposure level (Clewell, Tan et al. 2008). PBK models are seen as an advance in that they describe physiologically relevant compartments into which a chemical is taken up and eliminated on the basis of (i) physiological and anatomical parameters (e.g. cardiac output, tissue volumes and tissue blood flows), (ii) physico-chemical parameters (e.g. blood/ tissue partition coefficients) and (iii) kinetic parameters (e.g. kinetic constants for metabolic reactions) (Rietjens, et al. 2011).

The parent phthalates are rapidly metabolized to their corresponding metabolites, which are rapidly excreted in urine (Johns et al., 2015). This feature allows one to assume that the daily excretion of the metabolite is equal to the daily intake of the parent chemical and due to the fact phthalates are ubiquitous environmental contaminants human exposure to phthalates can occur daily leading to pseudo steady-state exposure conditions (Bui, Alves et al. 2017). Therefore, conversion of phthalate urinary biomonitoring data to daily exposure dose levels is possible based on multiple-compartment models for non-lipid-soluble chemicals at steady state (NRC 2006).

The phthalates on which pharmacokinetic data are most extensive are DBP and DEHP. Keys et al. (1999, 2000) first developed PBK models to evaluate the role of various transport processes in the

clearance of MBP and MEHP in the adult male rat in 1999 and 2000. The models accurately describe plasma MBP and MEHP kinetics after administration of the phthalates (Keys et al., 1999, 2000). In addition, another PBK model was developed for disposition of DBP in the adult, pregnant, and fetal rat (Clewell, Tan et al. 2008). The model provides a means of extrapolating rat fetal levels of different phthalate exposure biomarkers in various compartments or biologic matrices to actual maternal dose levels (Clewell, Tan et al. 2008). The PBK model for DBP has also been extrapolated for use in human by adjusting the physiological parameters and scaling chemical-specific parameters allometrically (Campbell et al. 2007). Results reported in an abstract (Campbell et al. 2007) indicated that the model was able to predict MBP concentrations in the urine of human adults given controlled doses of DBP without changing chemical- specific parameters; this suggested that the metabolism of DBP to MBP and of MBP to MBP-glucuronide is similar in the rat and human at human-relevant doses. More recently, an experimental PBK model has been developed to predict DiBP, DnBP and DEHP metabolite levels in urine and serum after oral doses of the parent compounds (Lorber et al. 2010, Lorber and Koch 2013). For DEHP Lober et al. developed a forward-based simulation model based on daily intake and predicts serum and urine concentrations (creatinine- and urine volume-based approaches) over time, and verified that it has merit for DEHP. Regarding DiBP and DnBP, the model predicted much lower urinary concentrations of the related metabolites (MiBp and MnBP), than were experimentally observed in all individuals (Lorber and Koch 2013).

Existing studies that used HBM data to define PBK models for phthalates are displayed in Table 2. Overall, urinary biomarkers can be successfully used to perform a more refined exposure assessment, in which PBK modelling may be used to improve the accuracy of biomarker based exposure estimates translating the biomarker levels to dose levels by reverse dosimetry.

Future studies also should be focused on developing mixture PBK models for combined exposure to chemicals with additive mode of action (dose addition) or by response addition/dissimilar mode of action.

Table 2. Overview of existing studies about human PBK modeling for phthalates.

Compound	Metabolites	available references PBK models
DEHP	MEHP	Marjory Moreau, et al (2017) Martinez et al. (2017) Koichiro Adachi et al. (2015) Zeman et al. (2013) Campbell et al. (2011) Lorber et al. (2010) Cahill et al. (2003)
	MEHHP	
	MEOHP	
DBP	MBP	Marjory Moreau et al. (2017) Martinez et al. (2017) Lorber et al. (2016) Lorber et al. (2013) Zeman et al. (2013) Campbell et al. (2011) Clewell et al. (2008) Cahill et al. (2003)
BBzP	MBzP	-
DEP	MEP	-
DMP	MMP	-

8- Risk assessment of mixtures

There are substantial differences between risk assessment approaches for single chemicals and chemical mixtures (Health Canada, 2015). Three potential approaches to quantify hazards associated with combined exposures to multiple chemicals include: dose (or concentration) addition, and response addition (or independent action) and Integrated addition (Health Canada, 2015). Dose addition is often stated to be applicable to mixtures composed of chemicals that have a similar or common mechanism of action, where the overall mixture toxicity equals the summation of the potency corrected exposure concentrations of individual chemicals (Kienzler et al., 2014). In contrast, independent action is widely assumed to be appropriate for mixtures of agents that have diverse or dissimilar mechanisms of action (same endpoint). Independent action is often held to be the default assessment concept when the similarity criteria of dose addition appear to be violated (Kienzler et al., 2014). With regard to the independent action approach, the mixture toxicity will not occur if the individual chemicals are all present at sub-toxic levels, whereas in dose addition based approaches all components contribute to the total toxicity depending on their concentration and potency (Heys et al., 2016). Integrated addition is used for those groups of chemicals that have both similar and dissimilar modes of action.

Based on the basic assumption of a dose-additive behaviour of the mixture, risk assessment should be carried in accordance with international cumulative risk assessment approaches, such as the HI approach the MOE approach the Relative Potency Factor (RPF) approach, or the groups ADI/TDI approach (Health Canada 2015; EFSA 2013; VKM 2008).

This thesis applied the HI approach to assess the cumulative risk of phthalates to the Iranian population, which is in alignment with the previous assessments (Kortenkamp and Faust 2010; Benson 2009; Chang et al. 2014; CHAP 2014). The Risk Assessment Committee of ECHA judged the use of the HI approach appropriate in the case of the DBP, BBP and DEHP (ECHA 2012, ECHA 2016).

The MOE approach does not encompass uncertainty factors for each individual chemical; as a result, the limitations of the database for each chemical are not quantified within the assessment of cumulative risk (Health Canada 2015).

Some compounds with similar structure and effects have been allocated a group ADI/TDI. EFSA considered a group ADI/TDI for the five phthalates BBP, DBP, DEHP, diisononyl phthalate (DINP) and diisodecyl phthalate (DIDP), based on their peroxisome proliferation potencies. Since peroxisome proliferation is considered not relevant to humans, a group-TDI was not allocated (EFSA 2005d).

Relative potency factors are applied in a dose addition formula and are developed for chemicals that have adequate evidence of toxicological similarity, e.g., chemicals that share a common adverse outcome pathway and are assumed not to elicit toxicological interactions (Hertzberg et al., 2018). In relation to phthalates, the challenge in using the RPF approach based on the current state of the art lies in the varying potencies for inducing the rat phthalate syndrome by the different phthalates (Health Canada 2015). The potency across phthalates is not the same for various effects leading to the different RPFs between phthalates depending on the chosen endpoint (Health Canada 2015). Due to this fact, accurate consideration of the representative endpoints is required in using the RPF approach. In addition, selection of the index chemical is challenging and requires an adequate toxicological database in the RPF approach (Varshavsky et al., 2016; Hertzberg et al., 2018). For instance, DBP seems to be the most potent phthalate regarding toxicity on testes based on a decreased number of spermatocytes. In contrast, regarding decreased AGD and nipple retention, the comparison of data suggests that DEHP would be the most potent (CLH report 2017). In the absence of a common effect that can be compared across the phthalates in the chemical group, the RPF approach was not recommended (Health Canada 2015). However, if the data becomes available for a common measure of effect across the assessment group, consideration can be given to the use of an RPF method for the estimation of cumulative risk.

The need to better manage chemical mixtures has been highlighted globally (Altenburger et al., 2018). EU policies have been successful in limiting pollution and adverse effects from single substances and single exposure routes. However, the challenge remains to reduce the risks from hazardous chemical mixtures and from the total exposure, in single media, across media and across

legislations or regulatory sectors (Kienzler et al., 2016). Currently, in EU legislation, there is no mechanism for a systematic and integrated assessment of mixture effects taking into account different routes of exposure and different product types (Kienzler et al., 2016).

9-*Evaluating other phthalates*

The risks of five individual phthalates (DBP, BBP, DEHP, DEP and DMP) were considered in the present study, and combined risks were considered for only three anti-androgenic phthalates (DBP, BBP, DEHP). Consideration is also needed to be given to other and newer phthalates that that may potentially contribute to a cumulative risk.

Based on the HBM4EU project report upon substance classification and prioritization, phthalates could be categorized in three groups as follows: substances where A) sufficient data are already available including DEHP, DnBP, DiBP, BBzP and DEP, B) only insufficient data are available including DINP, DIDP, di-is-octyl phthalate (DIOP), DMP, di-(2-propylheptyl)phthalate (DPHP), diheptyl phthalate (DHeP), and dihexyl phthalate (DHP), and C) no data are available/published, and/or no biomarkers have been established including diisopentylphthalate (DIPP), di-C7-11-(linear and branched)-alkyl phthalate (DHNUP), di-n-hexyl phthalate (DHEXP), di(methoxyethyl) phthalate (DEMP), di-n-pentyl phthalate (DPP) and dicyclohexyl Phthalate (DCHP) (HBM4EU 2018).

In case of group A) the existing toxicology data must be harmonized and exposure data should be made available on a worldwide scale, assessed in comparison to each other and in relation to their geographical origin and focus must be drawn to mixture exposure of these chemicals. They build a basis on which either direct conclusion for policy advice can be derived or research gaps can be identified. For some phthalates in the group B and C, there is a lack of information for assessing either the hazard or the exposure, or both. For these phthalates with no precise information available, the goals are to identify and prioritize knowledge and data gaps and related research needs and identify missing analytical methods and the potential health hazard to move a substance in group A (HBM4EU).

Among investigated phthalates in this thesis, the human health guidance values (i.e. TDI, RfD) were established for all phthalates with the exception of DMP. However, there are controversies between different agencies concerning the most protective reference values based on the considered endpoints. Toxicity data are also limited for several phthalates like DINP, DIOP, DIDP, DHP DHeP, and DPP. Future evaluation of the importance of phthalates other than the ones evaluated in the present thesis requires generation of toxicological and exposure data necessary to assess any potential risks.

It is important to note that some other phthalates like DIND, DHeP, DheP, DIOP, have been reported to induce anti-androgenic effects in animals (Boberg et al. 2011, Varshavsky et al., 2016, Conley et al., 2018), and dose additive effects have been observed for a mixture of such phthalates (Hannas et al., 2012), indicating that other phthalates than DBP, BBP, DEHP can contribute to the cumulative risk assessment for anti-androgenic effects.

Overall, new toxicological studies on mixtures of antiandrogens and other combinations of endocrine-disrupting chemicals, in combination with human biomonitoring studies, are needed to inform more accurate cumulative risk assessments to preserve human reproductive health (Howdeshell et al., 2017; Conley et al., 2018).

In conclusion, the present thesis, which applied indirect and direct exposure assessment approaches for phthalates exposure in different groups within the population in Iran, provided a first small step on the way to create a foundation for risk assessment of the phthalate exposure in Iran. It is concluded that the exposure via bottled drinking water does not raise a health concern and the contribution of bottled water as a source of phthalate exposure is negligible when compared with the total phthalate intake estimated based on results from biomonitoring data in children. Especially the comparison of indirect food-level based exposure assessment (chapter 3, 4 and 5) and biomarker based direct exposure assessments (Chapter 7) revealed that assessing other sources for phthalate exposure than drinking water exist and may be more important but need to be elucidated and further quantified. The results also indicted that overall exposure did not exceed currently established safety values, also not when total phthalate exposure was taken into account. Uncertainty remains with respect to the possible adverse health effects of combined exposure to all anti-androgenic compounds with modes of action similar to those of the phthalates. To enable such a combined and integrated risk assessment further development of novel methodologies are required, such as definition of the exposome and toxic equivalency methods.

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CHAPTER 9

Summary

Phthalates are diesters of phthalic acid that are widely used in industry and personal care products resulting in exposure via ingestion, inhalation and dermal routes. There is an interest in the safety evaluation of phthalate exposure because these compounds are ubiquitous environmental contaminants with endocrine-disrupting properties, suspected to interfere with developmental androgen action, possibly leading to adverse effects on reproductive function. Toxicological properties of phthalates, the presence of phthalates in polyethylene terephthalate (PET) bottles as impurities, the high and regular consumption of bottled water, and the uncertainty about the impact of storage conditions of PET bottled water on migration of phthalates into the water, initiated the interest in their presence in bottled water and the accompanying risk assessment. In this study, common Iranian brands of bottled water were screened for phthalates. The effect of storage temperature on selected target chemical concentrations was investigated. A toxicological risk assessment was conducted to determine the potential health risks associated with the consumption of the bottled water. Along with indirect exposure assessment, a human biomonitoring approach was applied to facilitate better human exposure assessment of individual phthalates and their mixtures providing important information for identifying exposure sources and the contribution of intake from bottled water to the total daily intake.

Chapter 1 of the thesis presents an introduction to the topic, the toxicological properties of phthalates, risk assessment strategies and the regulatory status of phthalates. **Chapter 2** of the thesis describes the development of a method to extract phthalates from bottled water by applying surface-functionalized magnetic particles (MPs) as the adsorbent used in Magnetic Solid-Phase Extraction (MSPE). Based on the results obtained, it was concluded that the MSPE-GC-MS method developed provides a new method for the determination of phthalates in water samples.

To extend the work to real samples **chapter 3** presents the occurrence and concentrations of common phthalates (dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and diethylhexyl phthalate (DEHP)) in PET bottled water locally produced in the Iranian market and stored under various common storage conditions. According to the results obtained, an increase in temperature and/or in the duration of storage increases phthalate migration. The highest concentrations of all phthalates were observed when bottled water samples were kept at 40 °C for 45 days. DEHP in bottled water was the most abundant phthalate under all storage conditions, although the observed level of DEHP in the worst-case scenario (40 °C for 45 days) was still much lower than the DEHP maximum concentration limit (MCL) in bottled water (MCL= 6 µg/L) set by the U.S. Food and Drug Administration (U.S. FDA). When comparing the concentrations of DBP, BBP and DEHP with initial levels in the bottled water, the results demonstrate that the release of phthalates was not

substantial under all storage conditions, and especially minimal at low temperatures (<25 °C) and under freezing conditions. Based on the measured concentrations of phthalates, an indirect exposure assessment through PET bottled water consumption was performed for children in Iran. The risk assessment indicated that non-carcinogenic risks for DEHP, DBP, and BBP were low, and that the carcinogenic risks for DEHP were negligible.

In **chapter 4**, concentrations of diethyl phthalate (DEP) were measured in bottled water kept under various storage conditions, similar as those used in chapter 3 for DEHP, DBP and BBP, and the resulting risks of consumption of this water for children but also for other age groups were evaluated. The results indicate that storage duration and storage temperature also influence the release of DEP from PET bottles into water. In comparison to the initial level of DEP in bottled water samples, the migration of DEP appeared not considerable under most storage conditions, especially at low temperatures (<25 °C) and freezing conditions. The level of exposure to DEP (expressed as mg/kg body weight/day) via consumption of bottled water in different age groups based on the worst-case scenario exposure assessment was as follows: pre-school children > children > lactating women > teenagers > adults > pregnant women. However, for all age groups, none of the individuals exceeds existing intake limit values for DEP.

Due to the anti-androgenic activity of some phthalates, in **chapter 5** the cumulative health risks in pregnant and lactating women posed by combined exposure to BBP, DBP, and DEHP via consumption of bottled water was estimated. To this end, hazard quotient (HQ) values, representing the margin between health based guidance values (EPA RfD values) and estimated exposures, and hazard index (HI) values, representing the sum of HQ values of individual phthalates, were determined. The results of the study showed that the HQ values for individual phthalate intake via bottled water consumption in pregnant and lactating women were much lower than 1, and cumulative risk assessment for combined phthalate exposure demonstrated that the HIs for anti-androgenic effects were also lower than 1 which implies that adverse effects are unlikely to occur.

In **chapter 6** of the thesis a systematic review method was used to investigate whether the phthalate exposure would be a factor contributing to the development of autism spectrum disorders (ASD). The results of this systematic review revealed that only a limited number of studies has addressed phthalates in relation to autism. A total of five studies met the inclusion criteria and were included in the review. Of the 5 studies, two studies were cohort studies both from the U.S.A. and three were case-control studies conducted in the U.S.A., Italy and Turkey. Because of the heterogeneity in the type of included studies, different methods of assessing exposure to phthalates and the use of different statistics for summarizing the results, a meta-analysis could not be performed to combine

the results of included studies. The review showed equivocal evidence for a possible connection between exposure to phthalates and ASD. Further comprehensive research is needed with appropriate attention to exposure assessment and relevant pre and post-natal confounders.

In the next step we set our goal to get better insight in the total phthalate exposure of Iranian children, and to assess the proportion of phthalate intake from bottled water to the total daily intake. This was done using biomonitoring based exposure assessment. **Chapter 7** of the thesis shows the data on the levels of phthalate metabolites in the spot urine samples of children and adolescents. We applied a calculation model based on the creatinine-adjusted urinary metabolite concentrations to obtain the EDIs for DEHP, DBP and BBP. The EDI values thus obtained were compared to available health-based guidance values (RfD and TDI values based on anti-androgenic effects). Assuming additive effects, the cumulative risk for combined exposure were estimated for three phthalates based on anti-androgenicity as the critical effect. The results from the risk assessment suggest that Iranian children and adolescents are exposed to low levels of a mixture of these phthalates. Risk assessment indicates that not only the exposure to the single phthalates, but also the combined exposure would not raise a safety concern. However, people typically come into contact with several chemicals with anti-androgenic properties in addition to the investigated phthalates in this study, which may also contribute to combined anti-androgenic effects. This indicates that a risk assessment of combined exposure including other anti-androgenic chemicals would be required to determine whether combined exposure to anti-androgenic chemicals is below acceptable levels.Comparison of the exposure values obtained to those obtained based on indirect estimates in earlier chapters of the thesis, revealed that bottled water provides only a limited contribution to total daily phthalates exposure in Iran.

Chapter 8 presents a discussion of the results obtained and also presents some perspectives for future research and risk management of exposure to phthalates in Iran.

APPENDIX

Acknowledgments

I would like to express my sincere gratitude and appreciation to my promotor, Prof. Dr I.M.C.M. Rietjens, for her dedicated supervision, constructive instruction, encouragement, constant help and support. She is a great role model for me and I have learned to be dedicated, critical, precise and efficient as a scientist.

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Maryam

About the Author

Maryam Zare Jeddi was born on the 7th of December 1985, in Tehran, Iran. She did her undergraduate degree in Nutrition science in 2007. After obtaining the Medical Council Registration Number, she started her career as a nutritionist and diet therapist at the hospitals. Her interest in exposure science began when she first got to know about food contaminants in the food chain and dietary exposure to food chemicals. Therefore, she shifted her career to exposure science with enrolling in a 2 year master program in food safety and hygiene at Tehran University of medical sciences, Tehran, Iran. Ever since she was a master student, her interest increased more and more and after graduation she continued to work on food safety, toxicology and human health risk assessment as a researcher at the Institute for Environmental Research (IER) at the Tehran University of Medical Sciences in Tehran, Iran. In February 2015 her work was awarded at the Festival of Education and research as a 'Best Master thesis research' in Iran and she was selected as a Distinguished Young Researcher within the Ministry of Health and Medical Education of Iran.

Maryam also has been active as a scientific consulter in the area of exposure to chemicals in consumer products at the ministry of health and medical education of Iran for risk assessment-based regulatory decision-making. She was actively involved in knowledge translation exchange (KTE) as a part of risk communication that is important for government and public health (health policy).

She conducted several research on dietary risk assessment and she set up a Human Early-Life Exposome project that aims to evaluate the genome and exposure to environmental chemicals, lifestyle socio-demographic and socioeconomic factors, and nutrition during the pregnancy period on pregnancy outcomes and neurodevelopmental disorders such as autism spectrum disorders (ASD) in children.

In 2017 she got involved in a PhD program at Wageningen University and research under the supervision of Prof. Dr. I.M.C.M. Rietjens and on the 18th of September 2018 she was selected for a trainee position at the European Food Safety Authority (EFSA), Parma, Italy, starting in November 2018. Also, in 2018 she became active within the European Chapter of the International Society of Exposure Science (ISES Europe) and is contributing to the development of a European Strategy on Exposure Science with a roadmap 2020-2025-2030.

Her research interests lie in the area of computational toxicology, ranging from theory to the design of computational methods and tools to the implementation within the risk assessment process. During her career she collaborated actively with international researchers in many other disciplines that are related to exposure science, in particularly toxicology and epidemiology.

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Book chapters

Maryam Zare Jeddi, Yuri Bruinen de Bruin, Sander van der Linden. **Information Resources in Toxicology, Testing Methods and Toxicity Assessment (Including Alternatives),** Fifth edition (2018).

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Overview of completed training activities

Courses

The Theory and Practise of Modelling Toxicokinetic and Toxicodynamics, European Commission Joint Research Centre (JRC), Ispra, Italy (2017)

Fit-For-Purpose Exposure Assessments for Risk-Based Decision Making, ICCA-LRI and JRC Workshop, Como, Italy (2017)

In Silico Method for Food Safety, Parma & European Food Safety Authority (EFSA) summer school, Parma, Italy (2017)

The JRC Summer School on Alternative Approaches for Risk Assessment European Commission Joint Research Centre (JRC), Ispra, Italy (2017)

Health Risk Assessment-Principles and Applications, IMM, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden (2017)

Toxicology and Environmental Health, Utrecht University/ the Netherlands (2015)

How to write an International Article, in Tehran/ Iran, ELSEVIER workshop at Tehran University of Medical Sciences (TUMS), Tehran, Iran (2014)

Systematic Review Course Tehran University of Medical Sciences (TUMS), Tehran, Iran (2013)

Electronic database searching- Advance, Tehran University of Medical Sciences (TUMS), Tehran, Iran (2013)

Appendix

Meetings

The International Society for Environmental Epidemiology (ISEE) annual meeting, Swiss Tropical and Public Health Institute | Swiss TPH, Basel, Switzerland (2015)

The International Society for Exposure Science (ISES) annual meeting, Utrecht, the Netherlands (2016)

1st International and 19th national conference on Environmental Health and sustainable Development, Tehran, Iran (2016)

The Joint Annual Meeting of the International Society of Exposure Science and the International Society for Environmental Epidemiology (ISES-ISEE) Ottawa, Canada (2018)

Optional

Mentorship for 2 PhD and 3 Master Science students during the thesis writing process (2014-2016)

Charing a scientific session during the meeting of the International Society of Exposure Science (ISES) in Utrecht, the Netherlands (2016)

Co-charing a scientific session during the ISES-Europe workshop in Dortmund, Germany (2018)

Organizing and leading a scientific session in the 1st International and 19th national conference on Environmental Health and sustainable Development, Tehran, Iran (2016)

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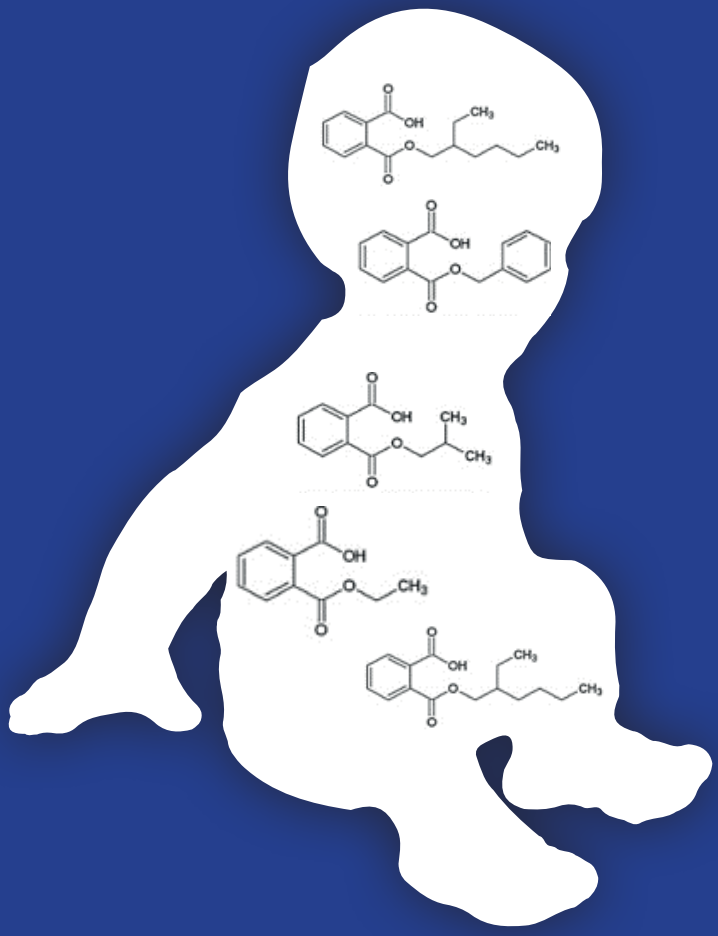
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Propositions

1. Current phthalate exposure of Iranian children via bottled water does not raise a health concern.
   (this thesis)
2. Bottled water consumption hardly contributes to total phthalate exposure of Iranian children.
   (this thesis)
3. Big data will accelerate the development of personalized medicines.
4. Only by developing exposome research will prevention and treatment of disease be improved.
5. Diagnosis of many rare and new diseases can be ameliorated by creating new symptom-driven algorithms in bioinformatics.
6. In future risk assessments of chemicals, environmental sustainability should be included.

Propositions belonging to the PhD thesis, entitled:

"Phthalates mixtures in bottled water in Iran: human health risk assessment using direct and indirect exposure assessment."

Maryam Zare Jeddi
Wageningen, 16 November 2018.