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STUDY ON EXPOSURE TO ENDOCRINE DISRUPTORS AND MYCOTOXINS IN SUSCEPTIBLE POPULATION, THROUGH DIFFERENT COMPOUND-SPECIFIC CLEAN-UP METHODS AND APPLICATION OF GAS AND LIQUID CHROMATOGRAPHY COUPLED WITH FLUORESCENCE AND MASS SPECTROMETRY DETECTION

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ENGLISH ABSTRACT

Chemical risk related to food safety is linked to the occurrence of potentially toxic chemicals in the food. Due to the different nature of the chemicals that may be present in food, a proper assessment of toxicological risk is crucial. This implies the need to establish an acceptable daily intake (ADI) and a tolerable daily intake (TDI) for residues of contaminants. The legal limits for toxic chemicals in food are based on careful assessments of intake levels of these compounds. However, certain populations could show higher exposure levels than the rest of the population, due both to the lower body weight (babies and children) and to a greater consumption of contaminated products. In recent years, there has been an increasing interest in the exposure to endocrine disruptors, substances that can compete with steroid hormones, thus interfering with normal endocrine functions. Among these compounds, phthalates and bisphenols are present in epoxy resins used in the internal coating of aluminum containers, as well as additive in plastics intended to come into contact with food. Foodstuffs, therefore, can be contaminated by these compounds through a migration from the container to the product. As regards natural toxicants, mycotoxins play a fundamental role in terms of chemical risk. In recent years, particular interest was shown in fumonisins, secondary metabolites of some fungal species belonging to the genus Fusarium, that can attack a variety of plants, including corn. The B_1, B_2 and B_3 forms of these toxins have been classified by IARC as probably carcinogenic. Foods intend for people affected by celiac disease contain mainly corn, so the consumption of these products could pose a health concern in terms of daily exposure. Therefore objectives of this PhD work was the estimation of exposure to endocrine disruptors (di-ethylhexyl phthalate (DEHP), dibutyl phthalate (DBP) and bisphenol A (BPA)) of newborns, through the infants formulas consumption, and children from 3 to 10 years, through the consumption of soft drinks and detection of fumonisins in gluten-free foods, in order to estimate the exposure of children affected by celiac disease to such compounds. The infant formula samples showed a median level of total contamination equal to 1.136 µg / g dry weight (d.w.) for DEHP, 0.244 µg / g d.w. for DBP and 0.008 µg / g d.w. for BPA. The estimation of exposure to these contaminants showed that the effect on TDI issued by EFSA amounted up to 93% for DEHP, 175% for DBP and 25% for BPA. About children's exposure to BPA through the consumption of soft drinks, the exposure of the high consumers, was found to be equal to 44% of the EFSA TDI. The median total contamination levels were 0.79 µg / L. During this thesis an alternate clean-up method was evaluated for detection of bisphenols through cartridges based on Molecularly Imprinted Polymers (MIP), which led to satisfactory results in terms of recovery rates. Finally, exposure to fumonisins through consumption of gluten-free food could, in some cases, exceed by 30% the TDI issued by EFSA. In conclusion, canned sugary drinks can be a not negligible source of BPA. About infant formulas, the results showed a widespread contamination by DEHP, DBP and BPA. Such contamination seems to be due to environment contamination, rather than be related to a migration from the container. Due to matrix interferences that may occur during the analysis, the purification through MIP cartridges, even though more expensive, showed a great specificity for the bisphenols, with high recovery percentages, facilitating the determination of such compounds. As regards fumonisins, finally, further studies need to be carried out in order to evaluate also the exposure contribution of masked fumonisins.

ITALIAN ABSTRACT

Nell'ambito delle preparazioni alimentari il rischio chimico è legato alla presenza di sostanze chimiche potenzialmente tossiche presenti nell'alimento. A causa della diversa natura delle sostanze chimiche che possono essere presenti in un alimento, assume fondamentale importanza un'adeguata valutazione del rischio tossicologico. Ciò comporta la necessità di definire una dose giornaliera ammissibile (ADI) ed una dose giornaliera tollerabile (TDI) per i residui di contaminanti. I limiti di legge dei contaminanti chimici negli alimenti, sono basati su attente valutazioni dei livelli di assunzione di tali composti tossici. Tuttavia, alcune popolazioni possono presentare livelli di esposizione maggiori rispetto al resto della popolazione sia a causa del peso corporeo ridotto (neonati e bambini) sia per un consumo maggiore di prodotti contaminati. Negli ultimi anni, ha destato particolare interesse la valutazione dell'esposizione ai distruttori endocrini, sostanze in grado di competere con gli ormoni steroidei, interferendo quindi con le normali funzioni endocrine. Tra questi composti, gli ftalati e i bisfenoli sono presenti nelle resine epossidiche usate nei rivestimenti interni dei contenitori di alluminio, nonché come additivo nelle plastiche destinate al contatto con gli alimenti. I cibi, pertanto, possono essere contaminati da questi composti attraverso una migrazione dal contenitore al prodotto. Per quanto riguarda le sostanze tossiche naturali, le micotossine rivestono un ruolo fondamentale in termini di rischio chimico. Negli ultimi anni particolare interesse è stato rivolto alle le fumonisine, metaboliti secondari di alcune specie fungine appartenenti al genere Fusarium, in grado di attaccare vari vegetali, tra cui il mais. Le forme B_1, B_2 and B_3 di queste tossine sono state classificate dallo IARC come probabili cancerogeni. Gli alimenti destinati ai celiaci contengono in prevalenza mais, pertanto il consumo di tali prodotti potrebbe rappresentare un pericolo in termini di esposizione giornaliera. Pertanto obiettivi di questo lavoro di dottorato sono stati la stima dell'esposizione ai distruttori endocrini (di-etilesilftalato (DEHP), dibutil ftalato (DBP) e bisfenolo A (BPA)) di neonati, attraverso il consumo di formule lattee, e bambini da 3 a 10 anni, attraverso il consumo di bibite e la ricerca e dosaggio di fumonisine all'interno di prodotti alimentari gluten-free, al fine di stimare l'esposizione dei bambini celiaci a tali composti. I campioni di formule lattee hanno presentato un livello di contaminazione totale mediano pari a 1,136 μg/g sostanza secca (s.s.) per il DEHP, 0,244 μg/g s.s. per il DBP e 0,008 μg/g s.s. per il BPA. La stima dell'esposizione a tali contaminanti ha portato ad un'incidenza sui TDI emanati dal'EFSA fino al 93% per il DEHP, 175% per il DBP e 25% per il BPA. Per quanto riguarda l'esposizione dei bambini al BPA attraverso il consumo di bibite, l'esposizione, per la fascia di consumatori maggiori, è risultata essere pari al 44% del TDI EFSA. I livelli di contaminazione mediani totali sono stati pari a 0,79 μg/L. Durante il presente lavoro di tesi è stato valutato un metodo alternativo di purificazione per il dosaggio dei Bisfenoli e l'utilizzo di cartucce basate su Molecularly Imprinted Polymers (MIP) ha dato risultati soddisfacenti in termini di percentuali di recupero. Infine, per quanto riguarda la ricerca e dosaggio di fumonisine in prodotti destinati a bambini celiaci l'esposizione può, in alcuni casi, superare del 30% il TDI emanato dall'EFSA. In conclusione, dall'analisi delle bibite zuccherine emerge che le bevande in lattina possono rappresentare una fonte di assunzione di BPA non trascurabile. Per quanto riguarda le formule lattee, i risultati hanno mostrato una diffusa contaminazione da parte di DEHP, DBP e BPA. Tale contaminazione sembra essere di natura ambientale o di processo, piuttosto che essere correlata ad una migrazione dal contenitore. A causa delle interferenze di matrice che possono verificarsi in fase di analisi, la purificazione attraverso le colonne basate su fase MIP, pur presentando costi maggiori mostrano un'ottima specificità per i bisfenoli, con percentuali di recupero più che soddisfacenti, agevolando il successivo dosaggio dell'analita anche con metodi meno specifici quali la fluorescenza. Per quanto riguarda le fumonisine, infine, valori di esposizione andrebbero approfonditi con ulteriori studi, valutando anche le concentrazioni di fumonisine legate, non valutabili con i metodi normalmente utilizzati.

0 PREFACE

Food is known as one of the key factors for a healthy life for humans. The relationship between health and food is related to the quantity and quality of food ingested by individuals, according to their age and health conditions. The amount of ingested food should be adequate in relation to the requirements of nutrients (proteins, lipids, glucides, mineral salts, vitamins. An unbalanced diet could cause obesity or other diseases related to a poor diet. However, in addition to carbohydrates, lipids, proteins, minerals, vitamins, foodstuff could contain a large number of other chemicals, that can exert harmful effects to humans and animals and can lead to a **chemical risk** (Tennant 1997)**.**

0.1 Chemical risk and toxicological risk assessment

Chemical contamination of food refers to the presence of different kinds of chemicals harmful for human health and it is strictly bound to term **chemical risk**, which indicates the risk arising from a food product related to the presence of potentially toxic compounds. Frequently, health concerns linked to the exposure to toxic chemicals is considered out of control by media and people, instilling a common sense of insecurity and a total lack of confidence about these topics. Despite this, in the most advanced countries food safety is assured through a consolidated legislation and regulations that set control and limits for a lot of chemicals and controls are performed at each stage of production and distribution flows. Monitoring could pertain crop protection, antibiotic therapy in livestock, safety of food additives, environmental contaminants, occurrence of mycotoxins, etc.

Recent scientific findings show that the overall chemicals contamination could have synergistic effect in terms of toxicity, as humans could be exposed to a widespread chemical pollution. In fact, intake of toxic compounds, could be below the levels of toxicity, whereas they could, go beyond these thresholds considering the overall contribution. The procedures of toxicological risk assessment, led to the definition of an Acceptable Daily Intake for chemicals and a Tolerable daily intake for residues of contaminants.

0.1.1 Acceptable Daily Intake (ADI) and Tolerable Daily Intake (TDI)

ADI is usually expressed as mg / kg bw (body weight) and indicates the levels of toxicant, that can be ingested over a lifetime without appreciable health risk for individuals (EFSA 2013). Generally, ADI is derived from the **No Observed Effect Level** (NOEL) that is the lowest dose level without observable effect. NOEL is set on the basis of toxicological tests and, in order to obtain ADI, NOEL is divided by an appropriate safety and uncertainty factors, according to the results of tests on limited and homogeneous population of test subjects (Herrman & Younes 1999; Pease & Gentry 2015). The evaluation of ADI and persistence of the molecule in target individuals and in the environment should lead to the definition of Maximum Residue Limits that are essential for the risk management. The respect of the MRLs guarantees, that the total intake of chemicals through diet does not exceed ADI, also taking into account possible short-term peaks of exposure to chemicals. Besides, ADI values must ensure adequate protection not only in relation of a hypothetical average population, but also of any sub-population groups that may be particularly susceptible to that specific molecule (eg. infants and children are known to be susceptible to molecules that exert different effects on the immune, endocrine and nervous systems). The **Tolerable Daily Intake** (TDI) is essentially similar to ADI and it is also expressed on body weight basis (mg/kg bw). As stated above, ADI and TDI are based upon levels well below the concentrations that could exert harmful effects to humans. Despite this, susceptible individuals could anyhow exceed these thresholds, because of their dietary habits.

0.2 Sources of chemical contamination

Toxic compounds that could be detected in foodstuff may be due to different causes. As shown in Figure 1, chemicals could naturally occur in food or derive from anthropic activities (heavy metals, pesticides, fertilizers). In the latter case, the contamination can occur at any stage of the whole production flow, during process, storage and distribution of the product. Finally, toxic compounds occurrence could depend upon chemical reactions that take place during food-processes such as thermal treatments (acrylamide, polycyclic aromatic hydrocarbons, etc).

0.2.1 Natural occurring toxicants

As stated above, many toxic chemicals could be natural constituents of some foods and under certain conditions, can exert negative effects for humans. Among these compounds there are:

- anti-nutritional substances
- allergenic substances
- other compounds naturally occurring in food

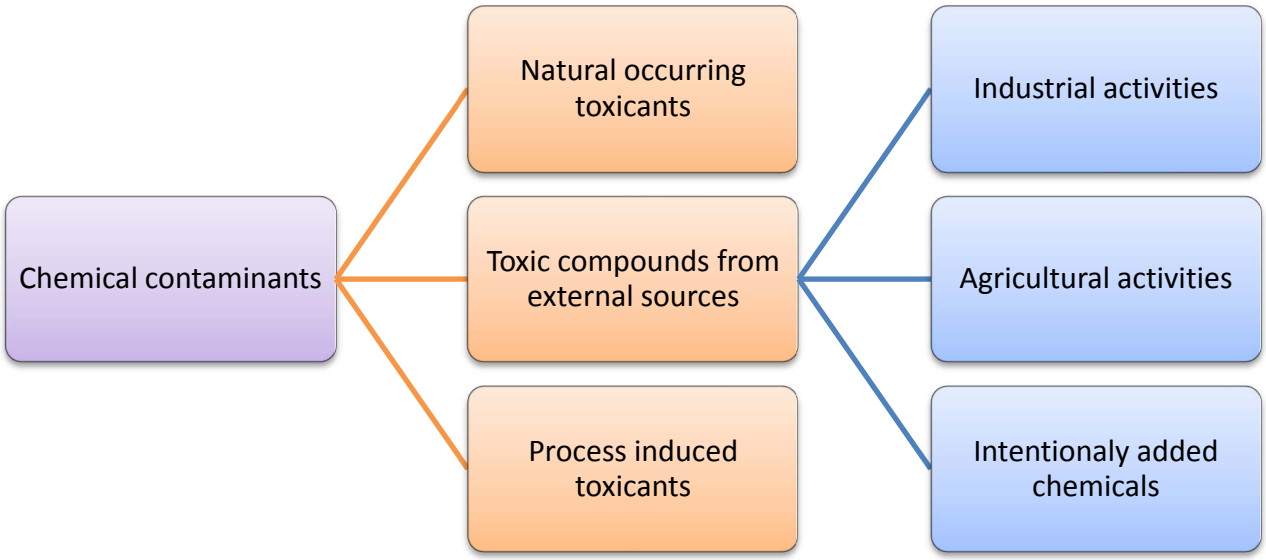

Figure 1 - Chemical contaminations scheme according to the source

0.2.1.1 Anti-nutritional substances

Some foods could naturally contain compounds that can cause nutritional deficiencies because of interference in the absorption of nutrients. These chemicals can act at different stages of the digestive process, inhibiting enzymes such as carbohydrases and proteases or interfering with the absorption or metabolic utilization of mineral and vitamins.

0.2.1.2 Allergenic substances

Some chemicals could cause reactions in susceptible individuals that could divided into allergies and food intolerances. A food-borne allergy is a disease linked to a reaction against molecules, called allergens, not recognized by immune system. Foods that often contribute the onset of allergic reactions could be wheat, soybeans, milk and dairy products, peanuts and tree nuts, eggs, fish, shellfish, strawberries and citrus fruits. Even though may cause symptoms similar to allergies, food intolerances do not involve the immune system and are typically caused by the difficulty of digesting a food (for example due to deficiency of some digestive enzymes). An example of a common food intolerance is lactose intolerance and is related to ingestion of milk in susceptible individuals unable to digest lactose, because of lacking of β-galactosidase enzyme. An allergic-like reaction can also be caused by sulphites in wine and foods.

0.2.1.3 Other compounds naturally occurring in food

According to their origin, natural toxic substances could be divided into:

- Toxic substances of plant origin
- Toxic substances of animal origin
- Toxic substances of fungal origin

One of the topics of this thesis is related to toxic substances of fungal origin known as **mycotoxins**, namely secondary metabolites of some fungi species like: Aspergillus, Penicillium, Fusarium and Alternaria. Hence, occurrence of mycotoxins in foodstuff is strictly related to the contamination by fungi species of plant origin food before or after harvesting, during storage. Foods exposed to direct contamination are cereals (corn, wheat, rice, barley, rye, etc.), oilseeds (peanut, sunflower, cotton seed, etc.), nuts and dried fruit, pulses, spices, coffee and cocoa. In addition, mycotoxins can be found as residues or toxic metabolites in products food deriving from animals fed with contaminated feed. According to fungal species, most commonly recurring mycotoxins are (Streit et al. 2012; Wan et al. 2013; Zhang et al. 2013; Snini et al. 2015):

- Aflatoxins (produced by Aspergillus species)
- Patulin (produced by Penicillium expansum)
- Ochratoxins (produced by Aspergillus and Penicillium species)
- Zearalenone, Fumonisins and Trichothecenes (produced by several Fusarium species)

Due to their detrimental action on cell functions, toxins of fungal origin may exert some harmful effects as:

- nephrotoxicity (ochratoxins)
- hepatotoxicity (aflatoxins)
- immunotoxicity (aflatoxins, ochratoxins)
- mutagenicity (aflatoxins)
- teratogenicity (ochratoxins)
- carcinogenicity (aflatoxins, ochratoxins, fumonisins).

The diversity of biological effects reflects the different chemical structures of various mycotoxins capable of reacting with DNA, RNA, functional proteins, enzyme cofactors and constituents of membrane. They are often resistant to heat and therefore they are not completely destroyed by normal cooking process. The impact of mycotoxins on health depends on the amount of chemical ingested through food, on the toxicity of the compound, on body weight of the individual and on the presence of other mycotoxins. Currently, in Europe there are regulations setting maximum amounts of mycotoxins levels and occurrence of these compounds in products intended for human consumption (EC 2007).

0.2.2 Toxic substances from external sources

Chemical compounds deriving from anthropic activity could be dispersed in the environment and come in contact with food during its production. These compounds could derive from:

- Industrial activities
- Agricultural activities
- Intentionally added chemicals

0.2.2.1 Industrial activities

These compounds are also known as xenobiotics (xenos = foreign and bios = life). Most common compounds arising from human activities could be:

- Polycyclic aromatic hydrocarbons (PAHs): they are a large group of organic compounds consisting of two or more aromatic rings fused together. PAHs are typically found in mixtures, which may consist of hundreds of compounds. These chemicals are formed mainly during incomplete combustion or pyrolysis of organic matter and during various industrial processes. The main sources of production is due to the combustion of fossil fuels and industrial processes such as production and processing of graphite, treatment of coal. Other sources could derive from forest fires, home boilers, fireplaces and tobacco smoke. Humans are exposed to PAHs through various pathways, especially by ingestion of food contaminated by environmental sources and wrong cooking procedures.
- Polychlorinated biphenyls (PCBs): they are a widespread class of persistent chemicals that build up in the environment and they are associated with a broad spectrum of effects on human health (Cirillo et al. 2008). PCBs had many industrial applications until they were banned in most countries in the 70s. Although the production and use of PCBs have been suspended, large amounts remain in electrical equipment, plastic products and building materials. PCBs can be released into the environment and people are exposed to PCBs mainly through food, with the exception of specific cases of accidental or occupational exposure.
- Dioxins: the term "dioxins" refers to a group of chemical polychlorinated aromatic compounds, containing chlorine, present in nature in an appreciable quantity even in different sedimentary rocks, such as clay or as pollutants from industrial heat treatments. Some of these chemicals are considered toxicologically relevant (Cirillo et al. 2014). These compounds are odorless, heat stable, insoluble in water and highly soluble in fat. They are not biodegradable so they tend to bioaccumulate and persist concentrating in the fat tissues of humans and animals. The Dioxins have technological applications and other uses, but are generated in various processes thermal and industrial as unwanted and often unavoidable.
- Heavy metals: Among the heavy metals the most recurring and harmful for human health are: mercury, cadmium, lead and arsenic. These are natural components of the earth crust, where could be found in trace amounts. However, many industrial processes, and the massive use of some consumer goods, could significantly increase their presence in the environment. These compounds show toxic effects directly on organs such as liver, kidney and brain and could be harmful to reproduction and could, finally, exert carcinogenic effects.
- Radioactive elements: About 200 radioactive elements are known so far and can contaminate the environment through fallout or as a result of nuclear explosions or accidents in nuclear power plants. Vegetables are mainly contaminated by deposition of radioactive waste on the outer surface and for absorption through the roots.

Effects on humans could be immediate (injuries to the skin) and delayed (cataracts, congenital malformations, cancer).

0.2.2.2 Agricultural activities

The production of crops requires the use of pesticides in order to control pest species. These substances could exert acute but especially chronic effect such as carcinogenicity and endocrine disruption that can occur even at low-dose exposure during lifetime. Dichlorodiphenyltrichloroethane (DDT) was one of the first pest control chemical to be used on crops and recognized as endocrine disruptor.

0.2.2.3 Intentionally added substances

Chemicals intentionally added to food are known as **food additives**, namely any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value, the intentional addition of which to food for a technological purpose (Council of the European Union 1989). The use of a food additive is allowed only if it does not pose a safety concern to the health of consumers; appropriate list of chemicals to be used in food is regulated by Commission Regulation (EU) No 1130/2011 which amends Annex III to Regulation (EC) No 1333/2008 (European Commission 2011).

0.2.3 Process induced toxicants

Frequently, specific production process (especially heat treatments), could induce the formation of toxic compounds. Common reactions that can take place in food during process phase can lead to the formation of:

- 3-monochloropropane-1,2-diol (3-MCPD)
- Polycyclic aromatic hydrocarbons (PAHs)
- Heterocyclic amines
- Acrylamide

Besides, contamination of foodstuff by xenobiotics could occur also during storage or after packaging of product. There are undoubtedly many aspects related to food packaging issues, both from the technological point of view (the choice of suitable material to preserve food) and for issues related to food safety in relation to microbiological, toxicological and chemicals compounds and related effects on human health.

0.2.3.1 Migration from packaging

Plastics are made of polymers, whose chemical inertia should be a basic requirement for use in the food industry. According to the chemical nature of the constituent compounds a lot of plastic materials are currently available:

- Polyethylene terephthalate (PET)
- Polypropylene (PP)
- Polyvinyl chloride (PVC)
- Polystyrene (PS)
- Low Density Polyethylene (LDPE)
- High-density polyethylene (HDPE)

As stated above, therefore, a fundamental requirement of these materials is health safety, and migration of harmful substances into food cannot exceed legal limits. The constituents of the plastic materials and articles intended to come into contact with food, but especially additives that are usually added to plastic in order to give flexibility or other technological characteristics, may migrate into any food matrices, hence it is necessary, also from a regulatory standpoint, thoroughly analyze the allowed limits according to the mechanisms of migration of these substances. In particular, beyond chemical inertia that a plastic polymer must ensure, as defined also by the current regulations, it is crucial that any additive in the plastics manufacturing process, doesn't diffuse into the food content above allowed limits. Among the various compounds used in the laminating process, phthalate esters are most common in use.

Other kind of packaging are aluminum cans and tinplate cans. This kind of package is often used for beverages and preserves. Metallic alloy these cans are made of, ensures good preservation of food. However, the inner surface of cans are often lacquered with protective coating that could contain chemicals like Bisphenol A and its derivatives thereof.

Bisphenols as well as phthalate esters are known to act as endocrine disruptors and they will be one of the topics discussed in this thesis.

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1 STATE OF THE ART

As stated in the preface section, the overall chemical concerns related to foodstuff can have multiple pathways according to the source of contamination that could be summarized as:

- Anthropic source
- Natural source

Recently, among the chemical contaminants of anthropic source, endocrine disruptors are certainly a major area of interest within the field of occurrence of xenobiotics in food and feed. A growing body of literature that recognizes the importance of these compounds focused on the role that endocrine disruptors could have because of their long-term toxic effects on individuals. This thesis focused on two toxic contaminants: Endocrine disruptors (anthropic source) and Fumonisins (natural soruce), in order to evaluate exposure of susceptible population to toxicants, despite legal limits or Tolerable Daily Intakes issued by European Authorities.

1.1 Endocrine disruptors

Endocrine Disruptors (ED) can be defined as exogenous substances that alter the function of the endocrine system and consequently causes adverse effects in an intact organism or its progeny (Amaral Mendes 2002). Specifically, they are chemicals that show androgen- and estrogen-like effects and able to interfere with normal functions of endocrine apparatus, altering synthesis, secretion, transport and other activities of hormones in human and animals. ED could interfere with normal endocrine-signaling pathways, causing adverse health effects such as neurological and immune effects, reproductive disorders, cancers, lowered fertility and increased incidence of endometriosis (Cobellis et al. 2003; Jenkins et al. 2009; Habert et al. 2009; Latini et al. 2006a; Latini et al. 2010). According to some authors, since 1940s a huge amount of chemicals has been released in the environment. One of the first compound recognized as endocrine disruptor was the dichlorodiphenyltrichloroethane (DDT) a chemical broadly employed as insecticide and pesticide. Since 1940, because of extreme use of DDT on crops, this compound and its metabolites, dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD) started to accumulate in environment. Other environmental pollutants as polychlorinated biphenyls (PCBs) were also among first toxic chemicals identified in the environment. These compounds are also known as Persistent Organochlorine Pollutant (POP) and even though their use was banned more than 30 years ago, they still could be detected in environment at low concentrations. Several issues have been identified firstly in animals that show high exposures to POPs including (DDT) and its metabolites, polychlorinated biphenyls (PCBs) and dioxins (Safe 2000). For this reason, most of pollutant are **ubiquitary**, still persistent in water, soil and air, also because the use of great part of them is currently allowed. During last 50 years there has been an increase in the incidence of breast cancer probably due to exposure to DDT and its metabolites (Soto & Sonnenschein 2015). According to Cohn et al. that conducted a prospective study, it was hypothesized that in utero DDT exposure could lead to development of breast cancer in elder (Cohn et al. 2015). Another study investigated early and long-term effects on population of 2,3,7,8-tetrachlorodibenzo-p-dioxin after an accident that occurred in 1976 in a chemical plant near Seveso, Italy. This study set out that among children born between 1977 and 1984 a possible modifications of the hormonal balance or an effect on genes controlling gender occurred (Bertazzi et al. 1998).

Several compounds with different purposes are known to exert endocrine adverse effects. These chemicals typically are non-steroidal organic compounds released into the environment through anthropic activity and include organochlorine, pesticides, fungicides, insecticides, nematocides, organic solvents, plasticizers, parabens and polychlorinated biphenyls and recent studies have ascertained the probable estrogenic interference capability by heavy metals such as Arsenic, Cadmium, Lead, Mercury (Colborn et al. 1993; Dyer 2007) (Table 1). Recently, a considerable literature has grown up around the theme of endocrine disruptors and their effects on human health, especially during prenatal and early childhood, when apparatus are forming (Jenkins et al. 2009; Habert et al. 2009). Researchers have shown an increased interest in the probable relationships between exposures to environmental contaminants and diseases, especially if they occur early in life (Koniecki et al. 2011; Sathyanarayana et al. 2013).

Table 1 - Ubiquitary chemical compounds recognized as endocrine disruptors (Colborn et al. 1993; Costas et al. 2015)

Category	Compound
Pesticides	2,4-D
	2,4,5-T
	Alachlor
	Amitrole
	Atrazine
	Metribuzin
	Nitrofen
	Trifluralin
Fungicides	Benomyl
	Hexachlorobenzene
	Mancozeb
	Maneb
	Metiram-complex
	Tributyl tin
	Zineb
	Ziram
Insecticides	B-HCH
	Carbaryl
	Chlordane
	Dicofol
	Dieldrin
	DDT and metabolites
	Endosulfan
	Heptachlor and H-epoxide
	Lindane
	Methomyl
	Methoxychlor
	Mirex
	Oxychlordane
	Parathion
	Synthetic pyrethroids
	Toxaphene
	Transnonachlor
Nematocides	Aldicarb
	DBCP
Heavy metals	Lead
	Arsenic
	Mercury
	Cadmium
Plasiticizers	Phthalates
	Bisphenols
	Alkylphenols
Organic solvents	Ethylene glycol ethers
	Styrene
	Perchloroethylene
	Toluene/xylene
	Trichloroethylene
Other pollutants	Parabens
	Dioxin (2,3,7,8-TCDD)
	PBBs
	PCBs
	Pentachlorophenol (PCP)

Recently, there has been renewed interest in plasticizers such Phthalate esters and Bisphenol A, most common in the manufacture of plastics intended to come into contact with food. Thus, the first topic of thesis focused on these categories of endocrine disruptors.

1.1.1 Phthalate esters

Phthalate esters (PAEs), also known as Phthalates, are diesters of phthalic acid (benzene-1,2-dicarboxylic acid), obtained through reaction of phthalic anhydride with a suitable alcohol. Phthalic anhydride could be obtained starting from Naphthalene or o-Xylene, according to the following equations (Figure 2):

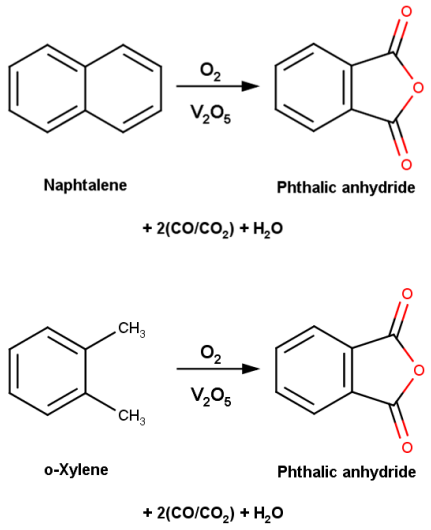


Figure 2 - Phthalic anhydride synthesis starting from Naphthalene and o-Xylene (Graham 1973)

Next esterification reaction is shown below (Figure 3):

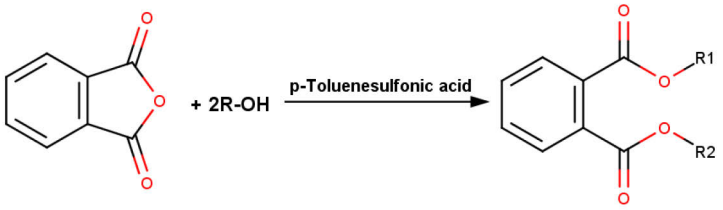


Figure 3 - Phthalic ester synthesis obtained from Phthalic anhydride (Graham 1973)

Currently, several type of phthalic esters are available and their use depend upon chemical and physical characteristics according to the length and the structure of side chains (Figure 4 and Table 2).

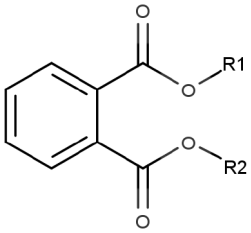


Figure 4 - Phthalic acid ester structure

PAEs were produced since 1920s and in 1933 they laid the groundwork to the industrial production of poly(vinyl chloride) (PVC) (Graham 1973). At present, Phthalates are widely used in industrial products. Among PAEs, those with

high molecular weight (Bis(2-ethylhexyl) phthalate, Diisononyl phthalate and Diisodecyl phthalate) are used to give flexibility to plastic materials, food packaging and medical devices. The compounds with low molecular weight (Dimethyl phthalate, Diethyl phthalate and Dibutyl phthalate) are used in cosmetics, solvents and plasticizers in cellulose acetate, hairsprays, construction materials, wood finishers, floorings, varnishes, inks and as glazing agents in pills of prolonged-release drugs (Hauser & Calafat 2005; Fromme et al. 2007; Frederiksen et al. 2007; Cirillo et al. 2013). Other scopes of application are related to manufacture of cleansers, lubricants, waxes, adhesives, insecticides, dentures, children's toys, glow sticks etc. (Schettler 2006).

This study focused on two phthalate esters: dibutyl phthalate (DBP) and di (2-ethylhexyl) phthalate (DEHP) as currently they are the most common in use in the world and their incidence on total exposure by diet has been estimated to range from 40% to up 95% for DBP and its isomers and from 50% to 98% for DEHP (Balafas et al. 1999; Wormuth et al. 2006).

Table 2 - Phthalic acid esters commonly used in industry

Chemical name	Acronym
Dimethyl phthalate	DMP
Diethyl phthalate	DEP
Dibutyl phthalate	DBP
Bis(2-ethylhexyl) phthalate	DEHP
Di-n-octyl phthalate	DnOP
Diisononyl phthalate	DiNP
Diisodecyl phthalate	DiDP

1.1.1.1 Dibutyl phthalate (DBP)

DBP is a clear colorless oily liquid, produced by the reaction of n-butanol with phthalic anhydride, slightly soluble in water, but soluble in most organic solvents (ethyl alcohol, benzene, ether, acetone, n-hexane) . Its chemical-physical characteristics are shown in Table 3 (EFSA 2005a; Pubchem 2016):

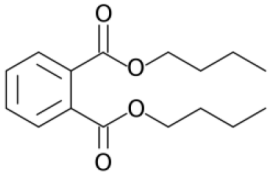
DBP can be used as a plasticizer (but is no longer used in the PVC) and also in the following products (ATSDR 2001; Jahnke et al. 2005):

- Polyvinyl acetate
- Insecticides
- Paints
- Perfumes and cosmetics
- Lubricants for the textile fibers

It is very common in the air, as it easily separates from the products in which is contained: from paints and adhesives passes easily in the gas phase; as it is not chemically bound to the other components of plastic polymers, it separates easily from plastic polymers (for this reason the migration from container to food matrices is facilitated).

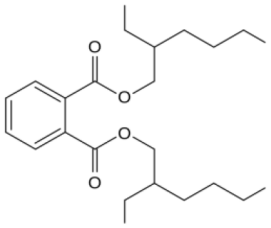
In the air, it is rapidly degraded under aerobic conditions, while in anaerobic conditions the degradation process takes longer, hence DBP can also contaminate the soil and groundwater, through the rain or the wind that carries dust particles and its degradation rate ranges from some days to some months, depending on the aerobic conditions and temperature (ATSDR 2001).

Table 3 - Chemical and physical properties of Dibutyl phthalate (Pubchem)

	Dibutyl phthalate *(DBP)*
CAS number	84-74-2
Molecular formula	$C_{16}H_{22}O_4$
Molecular weight	278.34 g mol^{-1}
Melting point	-35 °C
Boiling point	340 °C
Flashpoint	157 °C
Density (a 25 °C)	1.046 g ml^{-1}
Solubility in H_2O (at 20 °C)	11.20 mg l^{-1}
2D structure	

1.1.1.2 Bis(2-ethylhexyl) phthalate

Table 4 - Chemical and physical properties of Bis(2-ethylhexyl) phthalate (Pubchem)

	Bis(2-ethylhexyl) phthalate *(DEHP)*
CAS number	117-81-7
Molecular formula	$C_{24}H_{38}O_4$
Molecular weight	390.56 g mol^{-1}
Melting point	-50 °C
Boiling point	385 °C
Flashpoint	215 °C
Density (a 25 °C)	0.981 g ml^{-1}
Solubility in H_2O (at 20 °C)	0.27 mg l^{-1}
2D structure	

1.1.2 Toxic effects of PAEs

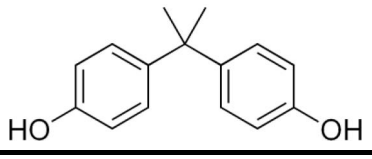
Human exposure to Phthalates can occur through ingestion, inhalation and dermal contact during the whole lifetime, including intrauterine life, but exposure in children exceeds more than in adults. PAEs determine toxic effects in laboratory animals, especially on reproductive systems. Once ingested, dibutyl phthalate (DBP) and DEHP are metabolized to the corresponding monoesters (monobutyl phthalate and mono-2-ethylhexyl phthalate) and can be excreted through urine or further undergo glucuronidation, probably in liver, prior to be excreted through urines (Silva et al. 2003). Human studies correlated phthalate exposure with adverse health effects on organs such as liver, kidney

and lung. Besides, sexual developmental abnormalities are also reported and finally phthalate esters may alter the methylation status of DNA and consequently the DNA sequence itself, thus transmitting these effects to future generations (Cobellis et al. 2003; Lovekamp-Swan & Davis 2003; Latini et al. 2006b; Singh & Li 2012). In humans, PAEs are rapidly metabolized and excreted with urine and faeces and they have been also detected in, amniotic fluids and breast milk (Fasano et al. 2012). Because of their toxicological profiles the European Food Safety Authority (EFSA) established Tolerable Daily Intakes (TDIs) of 0.01 mg/kg body weight (bw) and 0.05 mg/kg bw, respectively for DBP and DEHP (EFSA 2005a; EFSA 2005b)

1.1.3 Bisphenol A (BPA)

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl) propane, is a monomer used for the synthesis of polycarbonate and epoxy resins and polysulfonate, commonly used worldwide (Table 5). Polycarbonate is used for a lot of products like toys, sport equipments, medical and dental devices and baby bottles, even though in 2011 the EU banned its use for the production of polycarbonate baby bottles and prohibited its import and sale (EC 2011). Besides, internal coatings of many food and beverage cans consist of epoxy resins that could contain BPA and derivatives thereof (Vandenberg et al. 2007; Jenkins et al. 2009; Latini et al. 2010). In fact, internal surface of cans, tanks and metal lids intended to come into contact with food is lacquered with epoxy-resins which contain BPA or its derivative bisphenol A diglycidyl ether (BADGE). This coating has the purpose to protect from corrosion the metal surfaces in contact with the foods. In addition to bisphenol A and BADGE, there are other analogues of bisphenol. These include bisphenol F (BPF), bisphenol-F-diglycidyl ether (BFDGE), bisphenol S (BPS) and bisphenol B (BPB) (Lane et al. 2015). BPA is also used as an antioxidant in some plastics, and as a polymerization inhibitor of polyvinyl chloride (PVC) (Geens et al. 2011). It can be found in many materials intended to come into contact with food, such as reusable plastic bottles, plates, cups, tableware, food trays and in the protective linings of cans used for beverages and foodstuff (EFSA 2006).

Table 5 - Chemical and physical properties of Bisphenol A (Pubchem)

	Bisphenol A (BPA)
CAS number	80-05-7
Molecular formula	$C_{15}H_{16}O_2$
Molecular weight	228.29 g mol^{-1}
Melting point	153 °C
Boiling point	361 °C
Flashpoint	227 °C
Density (a 25 °C)	1.995 g ml^{-1}
Solubility in H_2O (at 20 °C)	300 mg l^{-1}
2D structure	

1.1.4 Toxic effects of Bisphenol A

BPA is recognized as an endocrine disruptor and related exposure during the perinatal period could interfere with prostate and mammary gland development, making these organs more susceptible to the development of cancer conditions and could also cause changes in anxiety and in brain biochemical signalling in animals (Moriyama et al. 2002; Zoeller et al. 2005; FAO/WHO 2011). BPA can alter receptors or protein expression in different potentially significant brain regions. A number of new studies report similar changes that indicate the effects of BPA on neurogenesis, neuroendocrine effects, on the morphology of certain brain regions, etc.). After ingestion, BPA is rapidly and completely absorbed from the gastrointestinal tract and metabolized in the liver by glucuronidation and excreted through the urine. Urinary excretion was chosen as markers of human exposure to BPA (Völkel et al. 2002; Calafat et al. 2008). Besides, high BPA exposure was linked to diabetes, heart diseases, high levels of liver enzymes and alterations of the thyroid function (Rubin 2011; Belcher et al. 2012; Sriphrapradang et al. 2013). Particular attention has to be addressed also to some *in vivo* studies showing that even lower concentrations of BPA, could exert adverse effect on mammary, reproductive and nervous systems, and hence a low dose effect exists for BPA (Richter et al. 2007;

Chapin et al. 2008; Vandenberg et al. 2013). For these reasons, baby bottles made of Polycarbonate (BPA polymer) were banned in Europe since March 2011 (EC 2011) and European Food Safety Authority established Total Daily Intakes (TDI) equal to 4 μg/kg bw per day (EFSA 2015).

1.1.5 Main exposure pathways to PAEs and Bisphenol A

According to biomonitoring studies it was demonstrated that the main sources of exposure of general population are ingestion, inhalation and dermal contact (Schettler 2006; Koch et al. 2012). In particular, ingestion seems to be a primary pathway of exposure to some PAEs especially those contained in food packaging (Kueseng et al. 2007). The ingestion of commonly used drugs may also be an important PAE's source, as many pills are coated with films that could contain phthalates (Hauser & Calafat 2005). Hospital patients undergoing medical procedures could be exposed to PAEs leached from PVC medical devices. Empirical data, demonstrated a positive association between the magnitude of exposure and the use of plastic tubing, catheters, and gloves (Tsumura et al. 2001; Tsumura et al. 2003). As stated above, PAEs are frequently used as additives in food packaging materials, from which they may migrate into the food because they are not chemically bound to the polymers (Dickson-Spillmann et al. 2009; Navarro et al. 2010).

As for PAEs, main source of exposure to BPA for humans are ingestion, inhalation and dermal contact (Fasano et al. 2015; Healy et al. 2015). Bisphenol A is found at low levels in surface water as well as soil and air. BPA shows a rapid degradation, but despite this it is a persistent pollutant in the environment due to continuous introduction (Flint et al. 2012). Accidental release of BPA in the environment could occur during production of plastic polymers (Chapin et al. 2008). Soil contamination by BPA is related to human densities as its occurrence in environment could be due to domestic and/or industrial wastes (Kawahata et al. 2004). According to Rudel et al. (2001) concentrations of BPA in air samples varied between 2 and 208 ng/m^3. However, inhalation could be considered a negligible pathway (Rudel et al. 2001; Kang et al. 2006). BPA could be also detected in receipts made of thermal papers, hence occupational dermal contact (as among cashiers) could not be considered a negligible pathway of exposure, as some studies have confirmed that BPA is promptly absorbed through this route (Zalko et al. 2011; Geens et al. 2012). Main exposure pathway of Bisphenol A is food, through ingestion of products packed. As stated above, occurrence of Bisphenol A as well as PAEs and derivatives thereof is mainly due to migration from packaging and this topic will be discussed in the following paragraph.

1.1.6 Migration from packaging

Foods may often come into direct contact with the materials constituting the packaging, leading to migration of chemicals contained in the package. According to Farhoodi et al. (2008), the migration mechanism of DEHP in acetic acid at 3%, as simulant used during a study on the yoghurt stored in PET bottles, is given by Fick's law according to the following equation:

$$J = -D \frac{\partial C}{\partial x}$$

J indicates the flow along the x direction, C the concentration of the chemical that diffuses in steady state conditions, and D is the diffusion coefficient, dependent on the environment in which the diffusing molecule is immersed. The experimental conditions observed in this study took advantage of two different temperatures: 25 ° C and 45 ° C. From the results obtained, it is clear that the diffusion of DEHP in food simulant, is in accordance with the Arrhenius law as follows:

$$D = D_0 \ e^{-\frac{E}{RT}}$$

This demonstrates that the process of diffusion of DEHP in a food matrix is temperature-dependent and the diffusion coefficients increase with higher storage temperatures (Farhoodi et al. 2008).

A relationship between the presence of PAE plasticizers in packaging and migration into food was hypothesized in a study on butter packaging (Page and Lacroix, 1995). Foodstuffs may be contaminated by PAEs during each step of production and, mainly, from packaging (Wormuth et al. 2006; Kueseng et al. 2007; Sathyanarayana et al. 2008; Dickson-Spillmann et al. 2009). Tsumura and colleagues demonstrated an increase in concentrantion of DEHP in chicken, from 80 μ g/kg before cooking to 13,100 μ g/kg after cooking in a Teflon coated pan, and further to 16,900 μ g/kg after packing (Tsumura et al. 2001). In fact, packed ready to eat meals, may be also highly contaminated, since heating food in ready-to-eat packages facilitates phthalates migration from packaging into food. Some studies also showed the presence of DEHP and other plasticizers in PVC films intended for domestic use, bags for honey and wrapping for candies at around 20%–35% w/w, while DEHP in plastic closure seals for oils and other fatty food varied from 18% to 33% w/w (Freire et al. 2006). Cirillo and colleagues analyzed packed meals intended for children before

and after packing in polyethylene coated Aluminium (PE/AL) dishes. Before packaging the DEHP median concentrations were 111.4-154.8 ng/g wet weight (ww) and DBP 32.5 ng/g-59.5 ng/g ww, while after packing the DEHP median values were 127.0-253.3 ng/g ww and 44.1-80.5 ng/g ww for DBP. Analogue trend was demonstrated in another study by same authors on ready to eat meals intended for consumption by hospital patients (Cirillo et al. 2011; Cirillo et al. 2013). Casajuana and Lacorte (2004) focused on migration of five phthalate esters (dimethylphthalate (DMP), diethylphthalate (DEP), di-n-butylphthalate (DBP), butylbenzylphthalate (BBP) and di(2-ethylhexyl)phthalate (DEHP)) in milk packed in two types of containers: Tetra Brik and high-density polyethylene (HDPE) and DEP, DBP and DEHP were the phthalates that showed higher levels suggesting the probable influence of the packaging on PAEs levels in milk (Casajuana & Lacorte 2004). Besides, plastic is not the only material that could lead to migration of PAEs. aluminium container, often used in place of plastics, during production processes may require lubrication through suitable mineral oils and other release agents that may contain PAEs (Tateo & Bononi 2006).

Foodstuff contamination by BPA could occur mainly during contact with the plastic linings of cans and the metal lids of glass jars and bottles as well as through migration from polycarbonate packaging and migration from containers into foods, is increased at higher temperature. (Cao et al. 2009; Cao et al. 2011; Jenkins et al. 2009; Oldring et al. 2014)

As migration from packaging and cookware could be a not negligible source of contamination by PAEs, Bisphenol A and other compounds, authorization of materials intended to come in contact with food in the EU is based upon the assessment of the migration levels into food matrices through the use of food simulants, (3% acetic acid, 10%-20%-50% ethanol, vegetable oils and Tenax™,. The EU Regulation 10/2011 sets a maximum level for total specific leaching (LMST) at 60 mg/kg, expressed as sum of PAE compounds from packaging into food and a specific leaching (LMS) for DEHP at 1.5 mg/kg and at 0.3 mg/kg for DBP, determined as overall migration from the polymer into food stimulants. However, the use of food simulants could lead to an underestimation of actual migration extent into food, as foodstuffs are more complex matrices that under certain conditions can lead to a higher release of chemicals (Grob 2008).

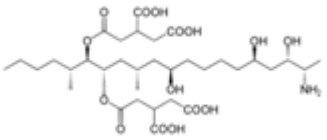
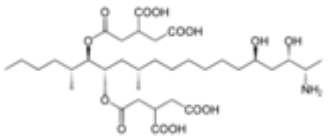
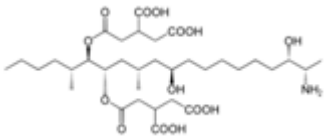
1.1.7 Exposure through Bisphenol A and dertivatives thereof through consumption of sugary drinks

Sugary drinks are commonly consumed by children and adolescents. In Italy, almost the 50% of children between 8-9 years old consumed sugary beverages at least once a day (Spinelli et al. 2012). This kind of drink are usually bottled in aluminium cans and as stated above, this kind of packaging could contain BPA and its derivatives like BADGE, BPF, BFDGE, BPB and BPS. Data on contamination of sugary drinks by BPA are scarce. Available data show concentrations ranging between 0.019 and 7 μg/L (Cao et al. 2009). In Europe, BPA contamination of sugary beverages was investigated in Belgium and BPA was detected at concentrations below 1 μg/L in 75% of 45 analyzed canned beverages(Geens et al. 2010). Therefore, consumption of sugary drinks may represent a non-negligible source of Bisphenol A and derivatives thereof.

1.2 Fumonisins

As stated above, the second topic of this work focused on Fumonisins, namely secondary metabolites produced by some fungi species belonging to Fusarium genus that usually affects maize crops, both before harvesting and during storage of foodstuffs (Escobar et al. 2013; Oliveira et al. 2015). These mycotoxins are chemically characterized by 19-20 carbon aminopolyhydroxy-alkyl chain esterified with two molecules of tricarballylic acid (TCA) (Dall'Asta et al. 2008). Currently, 28 analogues of fumonisin are known so far, divided in series A, B, C and P, but the most frequently occurring is the B type, in particular fumonisin B_1, B_2 and B_3 (Table 6) (Fromme et al. 2011). These compounds are known for their toxic and possible carcinogenic effects on humans and animals (Rheeder et al. 1992; IARC 2002; Domijan 2012). Occurrence of oesophageal cancer in human was related to ingestion of food contaminated by fumonisins (Myburg et al., 2002). According to their toxicocinetic mechanism, primary effect of fumonisins after ingestion is inhibition of ceramide synthase that leads to an increase of sphinganine concentration in cells and consequently to a modification of sphinganine/sphingosine (Sa/So) ratio. Hence, evaluation of Sa/So ratio in urine was proposed as a reliable biomarker as a measure of dietary exposure to fumonisins (Riley et al., 1994). Because of their recognized toxic effects, maximum amount of fumonisin in maize food is regulated by Commission Regulation No 1126/2007 setting maximum levels for fumonisins in foodstuffs (EC 2007). In recent years, an increasing amount of literature addressed the possible safety concern due to modified, bound and hidden forms of fumonisin, not detectable through standard methods, that could contaminate foodstuff (Dall'Asta et al. 2008; EFSA 2014). Because of their occurrence in corn-based food, it is commonly assumed that diet is the main source of exposure to these mycotoxins, especially in some countries where consumption of maize is a daily habit, or due to dietary restrictions as in celiac patients (Brera et al. 2014).

Table 6 - Chemical and physical properties of Fumonisins B_1, B_2 and B_3 (Pubchem)

	Fumonisin B_1	Fumonisin B_2	Fumonisin B_3
CAS number	116355-83-0	116355-84-1	136379-59-4
Molecular formula	$C_{34}H_{59}NO_{15}$	$C_{34}H_{59}NO_{14}$	$C_{34}H_{59}NO_{14}$
Molecular weight	721.83 g mol^{-1}	705.83 g mol^{-1}	705.83 g mol^{-1}
2D structure			

1.2.1 Exposure to fumonisins and celiac disease

Celiac disease is a chronic widespread and immuno-mediated disease induced in genetically predisposed individuals by the ingestion of food that contains gluten. It affects about 1% of population and it is one of the most common chronic disease among children, even though some evidence suggests that the prevalence of celiac disease among children could be greater than 1% (Catassi et al. 1999; Ivarsson et al. 2013; Schuppan & Zimmer 2013). Many studies have highlighted that the introduction of gluten in the diet of infants might act as a trigger in the development of disease as early as three months of age. Thus, timing of introduction of cereals containing gluten into the diet of babies should be delayed, especially for those individuals whose immediate family members are affected by celiac disease (Norris et al. 2005; Lionetti et al. 2014).

As celiac disease is a chronic and a permanent disorder, patients throughout their lifetime cannot eat any food that contains even little amount of wheat, oat, rye, barley and products thereof. Whereas corn and rice as well as potatoes and few other cereals can be safely employed as carbohydrate source in the diet of celiac patients, since these products do not contain gluten (Saturni et al. 2010). Therefore, children affected by celiac disease, must replace with maize or rice based products every source of carbohydrates like pasta, bread, biscuits, savory snacks and other similar products, that healthy people normally consume according to their personal taste and preference. Nowadays, a lot of gluten-free products, containing variable quantities of maize flour (up to 100%) are available on the market. For the reasons outlined above, daily ingestion of corn-based food in celiac children could be much greater than healthy peers and consequently their intake of fumonisins could be considerable, in the light of the Provisional Maximum Tolerable Daily Intake (PMTDI) equal to 2000 ng/kg body weight/ day (EFSA 2014).

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2 AIMS OF THE STUDY

Main purpose of this thesis was to demonstrate that, despite legal limits or suggested daily intakes, susceptible sub-population, especially infants, toddlers, children, adolescents or people following restrictive diet, could show a higher exposure than the rest of population to toxic chemicals, whose main pathway of exposure is diet.

2.1 Exposure to endocrine disruptors in infants

Infants' diet is made exclusively of breast milk or retail infant formulas that could be a not negligible source of phthalates and BPA, since these compounds have been found in breast milk. Besides, about infant formulas, the occurrence of phthalates and bisphenol A could be linked to a package-related migration mechanism (Cirillo et al. 2011; Cirillo et al. 2013; Latini et al. 2004; Latini et al. 2009). Therefore, first objective of this work was to evaluate the presence of DBP, DEHP and BPA in infant formulas in order to ascertain neonatal exposure to these endocrine disruptors.

2.2 Exposure to Bisphenol A in children and adolescents

In Italy, no data are available about BPA contamination of sugary beverages, but according to a survey by Istituto Superiore di Sanità (ISS), the consumption of such beverages among children increased to almost 50% in 2012 (ISS 2012). Therefore, as consumption of sugary drinks could be a not negligible pathway of exposure to Bisphenol A, another aim was to assess BPA concentrations in beverages usually consumed by children and evaluation of related daily intake. Besides, according to EFSA (2013) 68% of adolescents between 10 and 18 years of age consumes energy drinks. Among them 12% are usual drinkers with an average consumption of 7 liters per month (EFSA 2013). On this basis, objectives of this thesis was also the evaluation of levels of BPA and its derivatives through the application of an alternative clean-up method, as solid phase extraction are usually a bottleneck during the analysis of such compounds in complex matrices.

2.3 Exposure to fumonisins in children affected by celiac disease

Despite legal limits for occurrence of fumonisins in maize-based products, daily ingestion of such food by children affected by celiac disease could be higher than healthy peers and consequently their intake of fumonisins could exceed EFSA TDI (EFSA 2014). Thus, the last purpose of this thesis was the evaluation of the amount of fumonisins B_1, B_2 and B_3 in maize-based products, in order to ascertain the exposure of celiac children to such compounds, due to the consumption of gluten-free food.

2.4 References

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3 RESULTS AND DISCUSSION

3.1 Exposure to Endocrine disruptors in infants

Evaluation of exposure to DBP, DEHP in infants was performed through GC/FID detection method, whereas BPA determination was carried on through HPLC-RF method.

3.2 Materials and methods

3.2.1 Sampling

50 infant formula samples were collected at different neonatal nurseries of Campania region. Samples were made of 22 liquid ready to use formulas and 28 powder milk samples. Liquid samples were bottled in polyethylene terephthalate (PET) or Tetrapak™, milk powders packaging were made of aluminum (Al). The samples were collected in a glass vial. For DEHP and DBP analysis, 15 mL of liquid milk were lyophilized and stored at -18°C until analyses were performed, whereas 1 g of powder samples were stored in the dark. For BPA determination, 2 g of powder samples were used, dissolved in HPLC water (15 mL) and stored at -18°C until analyses.

3.2.2 DBP and DEHP

3.2.2.1 Chemical reagents

Acetonitrile, n-hexane, acetone (GC grade) and anhydrous sodium sulphate were supplied by Merck (Darmstadt, Germany). Florisil (60/100 mesh) by Supelco (Bellefonte, PA, USA) and Bondesil (PSA 40UM) by Varian (Palo Alto, CA, USA). Standard solutions of DBP and DEHP were purchased from Sigma Aldrich (St. Louis, MO, USA).

3.2.2.2 Instrumental parameters

The analyses of PAEs were performed through Shimadzu GC-17 (Shimadzu, Kyoto, Japan) capillary gas chromatography with a Flame Ionization Detector (GC-FID) and an HP-5 (Crosslinked 5% PHME Siloxane, 30 m length, 0.32 i.d., 0.25 μm film thickness) glass-capillary column. Carrier was Helium and a hydrogen/air mixture was used for FID. The volume of injection was 1 μl in splitless mode, the injector and detector temperatures were 260°C and 310°C respectively. The temperature program was 100°C for 1 min, an increase of 15°C/min up to 280°C, holding for 10 min.

3.2.2.3 DBP and DEHP detection

Because of PAEs ubiquity, all the glassware was thoroughly washed, rinsed twice with acetone and n-hexane and heated at 250°C for 2 h before use. According to Cirillo and colleagues (Cirillo et al. 2013), the lyophilized samples underwent three times extraction with 15 mL of acetonitrile in an ultrasound bath for 15 min, centrifugation at 3000 rpm for 10 min and the acetonitrile layers pooled and put in a separatory funnel. Finally, 10 mL of n-hexane saturated with acetonitrile were added and the funnel was vigorously shaken for 5 min. The acetonitrile phase was transferred into a flask and dried under vacuum at 50°C. The extracts were dissolved in 5 mL of n-hexane and a clean-up was performed through column packed with 2 g of Florisil, 0.5 g of Bondesil and 1 g of anhydrous sodium sulphate. The column was eluted three times with 10 mL of n-hexane/acetone mixture (95:5 v/v). The eluates were pooled and evaporated under vacuum at 40°C and dissolved in 1 mL of n-hexane for GC-FID analysis. The calibration curves were obtained using standard solutions at 0.625, 1.250 and 2.500, 5.00 and 10.00 μg/mL for DEHP, and at 0.312, 0.625, 1.250, 2.500 and 5.00 μg/mL for DBP. Limits of Detection (LODs) and Quantification (LOQs) were evaluated as follows:

- LOD = average concentration of blanks + 3 standard deviations
- LOQ = average concentration of blanks + 9 standard deviations

LOD and LOQ were equal to 5.0 ng/g and 15.0 ng/g for DEHP and to 7.5 ng/g and 22.5 ng/g for DBP respectively. The intra-day repeatability ranged from 7.0 to 9.0% for DEHP and from 5.0 to 8.0% for DBP. Inter-day repeatability varied from 6.0 to 8.0% for DEHP and from 4.5 to 6.5% for DBP. Samples with DEHP and DBP concentrations lower than LOD were used for recovery tests. Three liquid and three powder milk samples (each in triplicate) were spiked with standard solutions at concentration 2.0, 4.0 and 8.0 μg/mL for DEHP and 1.0, 2.0 and 4.0 μg/mL for DBP, and then processed as milk samples. Recoveries were equal to 99 ± 10 % for DEHP and 98 ± 9 % for DBP.

3.2.3 Bisphenol A

3.2.3.1 Chemical reagents

Acetonitrile, methanol and water (HPLC grade) were supplied by Merck (Darmstadt, Germany). Solid phase extraction cartridges (SPE) (Bond Elut C18 SPE, 1g/6mL) were purchased from Agilent Technologies (Palo Alto, CA, USA). A BPA standard (purity ≥ 99%) was supplied by Sigma Aldrich (St. 127 Louis, MO, USA).

3.2.3.2 Instrumental parameters

BPA detection was performed through a HPLC (LC-10AT VP Shimadzu, Kyoto, Japan) equipped with a fluorescence detector (Shimadzu RF-10A XL) and a reversed-phase column (Ascentis C18. L × I.D.: 15 cm × 4.6 mm; particle size: 5 µm, Supelco, Bellefonte, PA). Oven temperature was 40°C. The mobile phase consisted of 60% of acidified water (1% of glacial acetic acid), 35% of acetonitrile and 5% of methanol. The flow rate of mobile phase was set at 0.950 mL/min (isocratic run). The fluorimetric detection was carried out at: 275 nm (excitation) and 305 nm (emission).

3.2.3.3 BPA detection

BPA detection was carried on by adapting the procedure by Sun and colleagues (Sun et al. 2006). 5 mL of each sample was poured into a glass round-bottom flask and 20 mL of acetonitrile were added. Flasks were shaken for 25 minutes. Then sample was filtered through a filter paper and transferred into a separatory funnel. Then, 35mL of n-hexane were also added to the separatory funnel and the mixture was shaken for 25 minutes. The hexane layer was rinsed twice with acetonitrile (10 mL) and put in a round-bottom flask. Extract was dried through rotavapor, and the residue dissolved in 3 mL of a methanol: water (95:5 v/v) before clean-up. SPE cartridges were firstly conditioned with 5 mL of methanol and then with 5 mL of water. Then the sample was loaded, and the elution was carried out at a flow rate of 3-4 mL/min using a vacuum manifold. The cartridges were then washed with 2 mL of a methanol:water solution (30:70 v/v) and dried under vacuum for 1 min. Finally, the BPA was eluted with 3 mL of a methanol:water (80:20 v/v) mixture. The collected eluate was dried through rotavapor and dissolved in 1 mL of methanol and stored in an amber vial before the HPLC analysis. A calibration curve was obtained by injecting standard solutions of BPA at concentrations of 10.0, 20.0, 30.0, 40.0 and 50.0 µg/L. Instrumental LOD was calculated using the standard deviation of the response (σ) and the slope of the calibration curve (S) according to the formula:

$$LOD = 3.3\,\frac{\sigma}{S}$$

LOD was equal to 0.003 µg/g dry weight (dw).

Similarly, a LOQ was calculated as:

$$LOQ = 10\,\frac{\sigma}{S}$$

LOQ was equal to 0.009 µg/g dw.

Recoveries were assessed on six samples (3 liquid and 3 powder milk samples with BPA level below the LOD) by spiking each sample with BPA solutions in methanol at following concentrations: 50.0, 100.0 and 1000.0 µg/L. The recoveries were equal to 87 ± 3%. BPA quantification was performed comparing the peak areas obtained in the samples with the BPA standard calibration curve.

For each batch, a blank sample was processed according to the procedures mentioned before and a total of 15 blanks were analyzed. No blank sample showed BPA level above LOD value.

3.2.3.4 BPA confirmation by LC MS/MS

Since BPA detection may be affected by matrix related interferences, a confirmation by LC MS/MS was performed in accordance with method by Shao and colleagues (Shao et al. 2005).

3.2.3.5 Instrumental parameters

BPA detection was performed through alliance 2695 (Waters, USA) liquid chromatography equipped with a Quattro Ultima Pt (Micromass, UK) tandem mass spectrometer and a symmetry C-18 column (150mm×2.1mm i.d., 3.5m). The temperature of the oven was set at 40 °C, the flow rate was 0.2 mL/min and the injection volume was 10 µL. Mobile phases consisted of methanol and water with 0.1% ammonia, through following gradient: methanol increased from 10 to 55% in 10 min, then increased to 85% in 10 min and held for 8 min, finally decreased to 10% and held for 15 min before injection. The mass spectrometer operated in negative mode and ESI (Electro-Spray Ionization) in multiple-

reaction monitoring (MRM) mode. The capillary voltage was 3.5 kV, the cone voltage was 70V and the multiplier voltage was 650V. Nitrogen was used as nebulizing, desolvation and cone gas. UHP argon was used as the collision gas for the tandem mass spectrometric analysis and the pressure in the collision chamber was kept at 0.0028 mbar. A calibration curve in the concentration range 1 to 100 ng/g was obtained by linear regression of the normalized standard solution areas against BPA concentrations. The intra- and inter-day repeatability of the method were evaluated by injecting standard solutions at three different concentration levels (10, 50 and 100 ng/g) five times during a day (intra-day) and during five consecutive days (inter-day). The intra-day reproducibility ranged from 4.0 to 6.0%, whereas inter-day reproducibility varied from 4.5 to 6.0%.

3.2.4 Statistical analysis

In order to determine the appropriate sample size for this study, a power calculation was performed. A two-sided test power calculation was performed through software G*Power 3.1.9. This power calculation suggested that 11 samples in each group would be necessary to detect a 15% of difference in the DEHP concentration with a power of 80% at a 5% level of significance. Data distribution was assessed with the Shapiro Wilk's test and one-way ANOVA was performed through SPSS 20.0 software (IBM) in order to assess the differences between DEHP, DBP and BPA concentrations in liquid and powder milks ($p < 0.05$). The concentrations below LOD were assumed to be equal to LOD.

3.2.5 Dietary intake assessment for Italian infants (age 0–4 months)

In order to evaluate exposure of young children DEHP, DBP and BPA, daily intake was estimated as:

$$Intake = \frac{C\ (concentration) \times V\ (volume\ of\ milk\ per\ day)}{BW\ (body\ weight)}$$

Dietary exposure was calculated according to budget method (BM) model (FAO/WHO 2005) and weight growth charts by WHO (WHO 2006). Two scenarios were considered:

- Median levels of investigated compounds, infants at the 50th percentile of body weight or at the 95-97th percentile whose daily ingestion of milk is medium (medium case);
- Maximum levels of investigated compounds, children at the 50th percentile of body weight or at the 95-97th percentile whose daily ingestion of milk is higher (worst case).

3.3 Results and discussion

Most milk samples showed detectable levels of DEHP (80%, 86% of liquid and 96% of powder milks), DBP (90%, 82% of liquid milks and 96% of powder milks) and BPA (60%, 43% of liquids milks and 67% of powder milks) (Table 7). The mean levels of concentration of DEHP in all milk samples was equal to 1.327 ± 0.724 µg/g dw, (1.112 ± 0.716 µg/g dw in liquid milks and 1.496 ± 0.729 µg/g dw in powder milks). For DBP, the mean concentration in all milk samples was 0.354 ± 0.305 µg/g dw, (0.384 ± 0.385 µg/g dw in liquid milks and 0.330 ± 0.229 µg/g dw in powder milks). The mean concentration of BPA in all milk samples was 0.021 ± 0.022 µg/g dw, (0.019 ± 0.037 µg/g dw in liquid milks and 0.023 ± 0.028 µg/g dw in powder milks) (Table 7). DEHP concentrations ranged between 0.092 and 3.552 µg/g (median=1.136 µg/g), DBP concentrations from 0.008 to 1.624 µg/g (median=0.244 µg/g) and BPA concentrations from 0.003 to 0.169 µg/g (median=0.008 µg/g) (Table 7).

Table 7 - Bisphenol A (BPA), Dibutyl phthalate (DBP) and Bis(2-ethylhexyl) phthalate DEHP concentrations in µg/g dry weight (mean ± sd, median and range)

Sample	DEHP		DBP		BPA	
	POS (%)	**Mean ± sd Median** *(Range)*	**POS (%)**	**Mean ± sd Median** *(Range)*	**POS (%)**	**Mean ± sd Median** *(Range)*
Liquid Milk	86	1.112 ± 0.716 0.926 *0.092 – 2.919*	82	0.384 ± 0.385 0.280 *0.008 – 1.624*	43	0.019 ±0.037 0.003 *0.003 – 0.169*
Powder Milk	96	1.496 ± 0.729 1.159 *0.702 – 3.552*	96	0.330 ± 0.229 0.212 *0.101 – 0.812*	67	0.023 ± 0.028 0.011 *0.003 - 0.108*
Total	80	1.327 ± 0.724 1.136 *0.092 – 3.552*	90	0.354 ±0.305 0.244 *0.008 – 1.624*	60	0.021 ±0.022 0.008 *0.003-0.169*

Similar concentrations of the three compounds were detected in liquid and powder milks, even if packages were made of different materials (Figure 5).

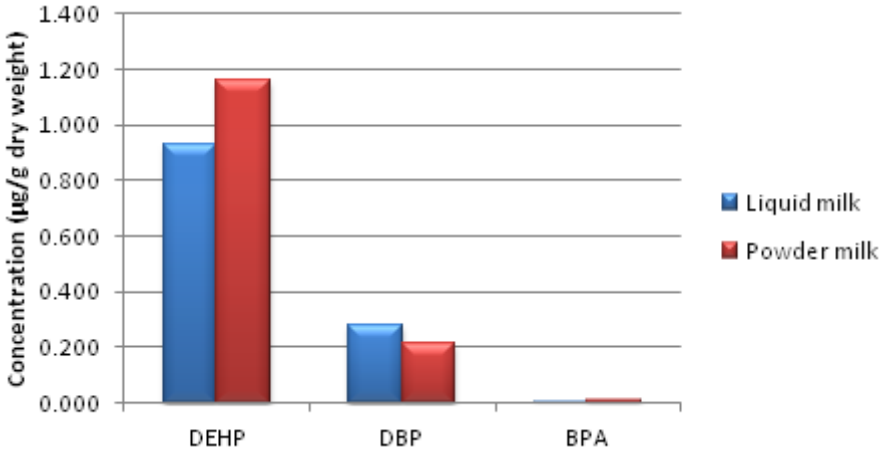

Figure 5 - Median concentrations of DEHP, DnBP and BPA in liquid and powder milk samples

The concentrations of DEHP, DBP and BPA in liquid and powder formulas and the type of packaging are shown in Table 8 and Table 9 respectively.

Table 8 - Concentrations of di(2- ethylhexyl)phthalate (DEHP), dibutyl phthalate (DBP) and Bisphenol A (BPA) in powder milk samples and type of packaging

Product	Type	Packaging	DEHP (µg/g dry weight)	DBP (µg/g dry weight)	BPA (µg/g dry weight)
C1	Infant formula	Tetrapak™	0.696	0.075	0.003
C2	Infant formula	PET	0.092	0.082	0.030
C3	Infant formula	Tetrapak™	1.831	0.067	0.003
C4	Infant formula	Tetrapak™	0.219	0.084	0.020
C6	Infant formula	Tetrapak™	0.633	0.142	0.009
C7	Infant formula	Tetrapak™	2.067	0.0075	0.003
C9	Infant formula	PET	1.456	0.287	0.003
C10	Infant formula	PET	0.301	0.067	0.003
C11	Infant formula	PET	2.099	0.624	0.003
C12	Infant formula	PET	0.784	0.482	0.058
C13	Infant formula	PET	0.606	0.216	0.014
C17	Infant formula	PET	1.877	0.787	0.003
C18	Infant formula	PET	1.202	0.351	0.003
C19	Infant formula	PET	0.923	0.899	0.017
C20	Infant formula	PET	0.256	0.14	0.018
C21	Infant formula	PET	0.929	0.088	0.003
C22	Infant formula	PET	2.919	1.624	0.169
C23	Infant formula	PET	0.852	0.423	0.030
C24	Infant formula	PET	1.428	0.384	0.003
C25	Infant formula	PET	0.796	0.807	0.003
C26	Infant formula	PET	1.137	0.272	0.003
C27	Infant formula	PET	1.371	0.548	0.003

Table 9 - Concentrations of di(2-ethylhexyl)phthalate (DEHP), dibutyl phthalate (DBP) and Bisphenol A (BPA) in powder milk samples and type of packaging

Product	Type	Packaging	DEHP (µg/g dry weight)	DBP (µg/g dry weight)	BPA (µg/g dry weight)
C5	Infant formula	Alluminium	1.408	0.321	0.003
C8	Infant formula	Alluminium	1.134	0.199	0.003
C14	Premature formula	Alluminium	0.997	0.201	0.003
C15	Infant formula	Alluminium	0.702	0.155	0.003
C16	Infant formula	Alluminium	0.871	0.212	0.028
C28	Infant formula	Alluminium	1.274	0.161	0.008
C29	Infant formula	Alluminium	0.883	0.137	0.003
C30	Infant formula	Alluminium	3.552	0.809	0.011
C31	Infant formula	Alluminium	2.909	0.765	0.100
C32	Infant formula	Alluminium	1.023	0.101	0.009
C33	Infant formula	Alluminium	1.142	0.356	0.022
C34	Infant formula	Alluminium	0.981	0.392	0.003
C35	Infant formula	Alluminium	1.024	0.161	0.003
C36	Infant formula	Alluminium	0.922	0.337	0.043
C37	Infant formula	Alluminium	1.052	0.709	0.054
C38	Infant formula	Alluminium	1.018	0.575	0.026
C39	Infant formula	Alluminium	2.341	0.187	0.012
C40	Infant formula	Alluminium	0.982	0.123	0.003
C41	Infant formula	Alluminium	1.723	0.118	0.016
C42	Infant formula	Alluminium	1.899	0.704	0.003
C43	Infant formula	Alluminium	1.175	0.148	0.035
C44	Infant formula	Alluminium	1.213	0.301	0.003
C45	Infant formula	Alluminium	1.897	0.812	0.108
C46	Rice milk formula	Alluminium	1.723	0.211	0.003
C47	Rice milk formula	Alluminium	2.871	0.321	0.003
C48	Rice milk formula	Alluminium	0.951	0.184	0.046
C49	Infant formula	Alluminium	1.821	0.201	0.018
C50	Infant formula	Alluminium	2.409	0.349	0.041

Data on dietary exposure to investigated compounds are shown in Table 10 and Table 11. The daily intake of DEHP in the medium case ranged between 19.84-24.85 µg/kg bw day at 50th percentile and between 17.63-21.14 µg/kg bw day at 97th percentile. In the worst case, DEHP intake varied between 42.57-54.68 µg/kg bw day at the 50th percentile and between 38.80-46.52 µg/kg bw day at the 97th percentile (Table 10 and Table 11). Data on dietary exposure to DBP in the medium case ranged between 4.15 µg/kg bw day and 5.34 µg/kg bw day at the 50th percentile and beteween 3.77 µg/kg bw day and 4.54 µg/kg bw day at the 97th percentile. In the worst case, the DBP intake varied from 13.62 to 17.50 µg/kg bw day at the 50th percentile and between 12.41-14.89 µg/kg bw day at the 97th percentile (Table 10 and Table 11). In the medium case, values ranged between 0.14 and 0.17 µg/kg bw day at the 50th percentile and between 0.12 and 0.15 µg/kg bw day at the 97th percentile. In the worst case, the BPA intake ranged between 0.99-1.27 µg/kg bw day at the 50th percentile and between 0.90-1.08 µg/kg bw day at the 97th percentile. For DEHP, DBP and BPA, both in the medium and the worst case the highest intake occurred at the 30th day of life, because the amount of consumed milk starts increasing while baby's weight is still low (Table 10 and Table 11).

Table 10 - Medium case, estimated daily dietary intake of di(2-ethylhexyl)phthalate (DEHP), dibutylphthalate (DBP) and Bisphenol A (BPA) in newborns fed with liquid or powder formulae according to the 50th and 97th of infant weight growth curve by WHO (WHO 2006)

Age (days)	Infant's average weight (kg)		Milk consumption (g dry weight/ day)		DEHP intake (µg/kg bw day)		DBP intake (µg/kg bw day)		BPA intake (µg/kg bw^2 day)	
	50th pctl[1]	97th pctl	50th pctl	97th Pctl	50th pctl	97th pctl	50th pctl	97th pctl	50th pctl	97th pctl
15	3.70	4.75	67.61	76.06	20.78	18.21	4.46	3.91	0.15	0.13
30	4.25	5.45	92.96	101.41	24.85	21.14	5.34	4.54	0.17	0.15
45	4.76	6.20	101.41	109.86	24.23	20.15	5.20	4.33	0.17	0.14
60	5.41	6.84	98.59	105.63	20.72	17.54	4.45	3.77	0.15	0.12
75	5.76	7.26	105.63	112.68	20.83	17.64	4.47	3.79	0.15	0.12
90	6.10	7.65	112.68	126.76	20.98	18.82	4.51	4.04	0.15	0.13
120	6.70	8.35	114.08	129.58	19.34	17.63	4.15	3.79	0.14	0.12

[1]pctl = percentile

[2]kg bw = kg body weight

Table 11 - Worst case, estimated daily dietary intake of di(2-ethylhexyl)phthalate (DEHP), dibutylphthalate (DnBP) and Bisphenol A (BPA) in newborns fed with liquid or powder formulae, according to the 50th and 97th of infant weight growth curve WHO (WHO 2006)

Age (days)	Infant's average weight (kg)		Milk consumption (g dry weight/ day)		DEHP intake (µg/kg bw day)		DBP intake (µg/kg bw day)		BPA intake (µg/kg bw^2 day)	
	50th pctl[1]	97th pctl	50th pctl	97th Pctl	50th pctl	97th pctl	50th pctl	97th pctl	50th pctl	97th pctl
15	3.70	4.75	67.61	76.06	45.74	40.07	14.64	12.82	1.06	0.93
30	4.25	5.45	92.96	101.41	54.68	46.52	17.50	14.89	1.27	1.08
45	4.76	6.20	101.41	109.86	53.32	44.33	17.06	14.19	1.24	1.03
60	5.41	6.84	98.59	105.63	45.60	38.61	14.59	12.35	1.06	0.90
75	5.76	7.26	105.63	112.68	45.85	38.83	14.67	12.42	1.06	0.90
90	6.10	7.65	112.68	126.76	46.18	41.43	14.78	13.26	1.07	0.96
120	6.70	8.35	114.08	129.58	42.57	38.80	13.62	12.41	0.99	0.90

[1]pctl = percentile

[2]kg bw = kg body weight

Data relevant to all contaminants showed a high variability but no significant differences were found in concentration of the investigated contaminants between liquid and powder milks, even though samples were packed in different types of containers. This finding would suggest that DEHP, DBP and BPA contamination could depend on raw materials or manufacturing processes rather than packaging. PAEs may contaminate milk during the production or preparation of infant formulas. Some studies related the migration of DEHP from the PVC tubing of the harvesting devices used in dairy farms (Castle et al. 1990; Feng et al. 2005). PVC tubing contains up to 40% DEHP by weight. A Norwegian study

showed a clear difference in DEHP concentration between raw milk collected by hand and machine milking through PVC distribution tubes (30 μg/kg in milking chamber and 50 μg/kg in collection tank) (Feng et al. 2005).

3.3.1 Dietary intake assessment for Italian infants (age 0–4 months)

In order to assess post-natal exposure to DEHP, DBP and BPA, the estimation of daily dietary intake of these contaminants was carried out in 0-4 month old children. For this sub-population, four possible diet scenarios are possible: infant powder, liquid formula, breast milk or a combination of these, but in this study only artificial nutrition was considered, assuming liquid or powder formulas (or both). The European Food Safety Authority (EFSA) established a Tolerable Daily Intake (TDI) equal to 50 μg/kg bw for DEHP and 10 μg/kg bw for DBP (EFSA, 2005a; 2005b). The highest intakes of DEHP and DBP occurred among infants with growth at the 50th percentile, who have a lower body weight than those at the 97th percentile. Daily intake of DEHP in the medium case varied from 20 to 25% and from 18 to 21% of TDI at 50th and 97th percentile respectively. In the worst case, intake was lower than TDI, except for the 50th percentile infants aged 30 and 45 days (Table 11). Daily intake of DBP in the medium case varied between 42-53% and 38-45% of TDI at 50th and 97th percentile respectively. In the worst case, instead, intake always exceeded TDI, up to 175%. Muller and colleagues estimated for 0-6 month old Danish infants a daily intake through infant formulas equal to 9.8 μg/kg bw/day for DEHP and to 16.4 μg/kg bw/day for DBP (Müller et al. 2003). These values for DEHP intake were higher than Muller's both in the medium and worst case, while DBP intake levels were lower in the medium case and comparable in the worst case. Assessment of DBP and DEHP daily intake was higher than that reported by MAFF (1998) for infants 0-3 months old (13 μg/kg bw/day for DEHP and 2.4 μg/kg bw/day for DBP). Our estimated BPA daily intakes were well below the temporary Tolerable Daily Intake (TDI) established by EFSA in 2014 (4.0 μg/kg bw) (MAFF 1998; EFSA 2015). In the medium case, our intakes ranged between 2.8-3.4% and between 2.4-3.0% of EFSA TDI for the 50th and 97th percentile respectively, and increased in the worst case to 20-25% and 18-22% of TDI for the 50th and 97th percentile respectively. Before 2011, infants could introduce BPA from polycarbonate baby bottles, especially when bottles were heated and reused multiple times (Jenkins et al. 2009; Wang et al. 2014). The EU Regulation No. 321/2011 forbade the use of BPA in the manufacture of baby bottles, thus reducing exposure. In 2008, a report of the U.S. National Toxicology Program (NTP) provided daily exposure estimates for infants, children and adults based on realistic scenarios (NTP 2008). The highest daily exposure to BPA was estimated to occur in infants and children. Estimated daily intake of infants fed with formulas (0-6 months of age) had a daily intakes between 1 and 11 μg/kg bw. In a report FAO/WHO identified 0-6 month infants fed with liquid formulas in polycarbonate bottles as the sub-population with the highest dietary exposure to BPA (2.4 μg/kg bw per day (average) and 4.5 μg BPA/kg bw per day (95th percentile)) (FAO/WHO 2011). In 2012, a Canadian surveys suggested that daily exposure to BPA in children ranged from 0.083 μg/kg bw (0-1 month old) to 0.164 μg/kg bw (children 4-7 months of age) (Health Canada 2012). Our data are similar to those of Health Canada but lower than NTP and FAO/WHO, probably because the BPA migration from baby bottles in Europe has been solved after 2011. The different packages (Tetrapack™, PET and aluminum) represent a probable bias of the present study. However, the investigated contaminants can be found not only in Tetrapack™ and PET but also in aluminum packages, as these are often internally coated with plastic derivatives.

3.4 Conclusions

Data obtained in this study showed a widespread contamination of infant formulas by DBP, DEHP and BPA, of environmental or process origin. Our findings suggest that infant formulas could be a main source of exposure to DEHP, DBP and BPA in infants. This risk is particularly relevant for DEHP and DBP because intake from formulated milk could exceed in the worst case the TDI from EFSA. In conclusion, potential hazards exist for infants fed with baby formulas, as these endocrine disruptors show the highest toxicity in infant population. EFSA established TDIs for the three investigated contaminants only referring to an adult population. Specific TDIs for children would help the protection of the susceptible population from endocrine disruptors effects.

3.5 References

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3.6 Exposure to Bisphenol A in children through consumption of sugary drinks

3.7 Materials and methods

3.7.1 Sampling

72 sugary drinks bottled in aluminium cans, polyethylene terephthalate (PET) bottles, high-density polyethylene (HDPE), polystyrene (PS), Tetra Pak™ and glass bottles were purchased from local retail markets, in Campania region (Italy), grouped into carbonated, non-carbonated and milk-based beverages. The most common brands among children and the most widespread types of packaging were selected from different production batches. All drinks were stored at room temperature before analysis.

3.7.2 Reagents

Methanol, acetonitrile and water (HPLC grade); anhydrous sodium sulphate (RG grade) and sodium chloride (for analysis) were purchased from Merck KGaA, Darmstadt, Germany. Bond Elut C18 SPE 1g/6mL and 0.5g/3mL cartridges were purchased from Agilent Technologies Inc., Palo Alto, CA, USA. BPA standard (purity ≥ 99%) (Sigma-Aldrich Co. LLC, St. Louis, MO, USA).

3.7.3 Beverage analyses

The analysis was performed by adapting the method used by Cao and colleagues (Cao et al. 2009). Carbonated drinks were degassed through vacuum pump for 30 min. Aliquots of 10 mL were stored at –18°C until analysis. Each aliquot was poured into a 50 mL glass tube and 10 mL of methanol:water (40:60 v/v) mixture were added and the tube was placed for 5 min in an ultrasonic bath. Then, half a spatula of both anhydrous sodium sulphate and sodium chloride were added and the tubes were centrifuged for 40 min at 5000 rpm. The supernatant was collected and filtered. Finally, the supernatant was dried up to 10 mL through rotavapor at 40°C. The samples were processed through solid-phase extraction (SPE) on Bond Elut C18 SPE 1 g/6 mL cartridges conditioned with 13 mL of methanol and with 13 mL of water. The sample was then loaded and elution was performed by gravity flow. The cartridge was washed with 6.5 mL of water and 13 mL of a methanol:water (30:70 v/v) mixture and the eluates were discarded. The BPA was then eluted drop wise adding 6.5 mL of an acetonitrile:water (50:50 v/v) mixture. Then, the cartridge was dried for 10 min through a SPE vacuum manifold. The eluate was finally dried using a rotavapor at 40° C and dissolved in 1 mL of methanol, filtered with 0.20 µm Sartorius cellulose filter and collected in an amber vial, before HPLC detection.

3.7.4 Milk-based drink analysis

The chocolate flavoured milk beverages were analysed according to the method by Sun and colleagues (Sun et al. 2006). Every sample were divided into aliquots of 10 mL and stored at –18°C until analysis. An aliquot (5 mL) of each sample was placed in a 250 mL glass round-bottom flask and 20 mL of acetonitrile were added. The flasks were then placed on a orbital shaker for 25 min. The sample was filtered using filter paper and transferred to a separatory funnel. The hexane layer was rinsed twice with acetonitrile (10 mL) and put in a round-bottom flask. Extract was dried through rotavapor, and the residue dissolved in 3 mL of a methanol:water (95:5 v/v) before clean-up. SPE cartridges were firstly conditioned with 5 mL of methanol and then with 5 mL of water. Then the sample was loaded, and the elution was carried out at a flow rate of 3-4 mL/min using a vacuum manifold. The cartridges were then washed with 2 mL of a methanol:water solution (30:70 v/v) and dried under vacuum for 1 min. Finally, the BPA was eluted with 3 mL of a methanol:water (80:20 v/v) mixture. The collected eluate was dried through rotavapor and dissolved in 1 mL of methanol and stored in an amber vial before the HPLC analysis.

3.7.5 HPLC analysis

BPA detection was performed through a HPLC (LC-10AT VP Shimadzu, Kyoto, Japan) equipped with a fluorescence detector (Shimadzu RF-10A XL) and a reversed-phase column (Ascentis C18. L × I.D.: 15 cm × 4.6 mm; particle size: 5 µm, Supelco, Bellefonte, PA). Oven temperature was 40°C. The mobile phase consisted of 60% of acidified water (1% of glacial acetic acid), 35% of acetonitrile and 5% of methanol. The flow rate of mobile phase was set at 0.950 mL/min (isocratic run). The fluorimetric detection was carried out at: 275 nm (excitation) and 305 nm (emission). At these conditions, the BPA retention time was 5.6 min.

3.7.6 Analytical performance

A calibration curve was obtained by injecting standard solutions of BPA at concentrations from 5.0 to 50.0 µg/L. Instrumental LOD was calculated using the standard deviation of the response (σ) and the slope of the calibration curve (S) according to the formula:

$$LOD = 3.3\,\frac{\sigma}{S}$$

LOD was equal to 0,18 µg/L dry weight (dw). Similarly, a LOQ was calculated as:

$$LOQ = 10\,\frac{\sigma}{S}$$

LOQ was equal to 0,54 µg/L dw.

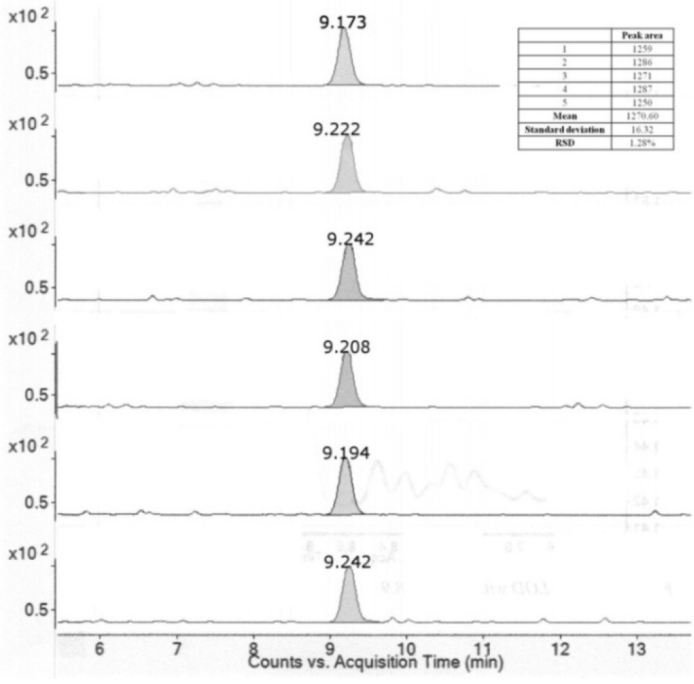
Recovery percentages were assessed on the basis of six samples (three carbonated drinks and three milk-based drinks), spiking each sample with 50 µL and 100 µL of a 1000 µg/L BPA standard methanol solution. The recoveries amounted to 92 ± 3% and 85 ± 3% for carbonated/non-carbonated drinks and milk beverages, respectively. BPA quantification was performed comparing the peak areas obtained in the samples with the BPA standard calibration curve. For each batch, a blank sample was processed according to the procedures mentioned before and a total of 15 blanks were analyzed. No blank sample showed BPA level above LOD value.

3.7.7 BPA confirmation by LC MS/MS

Since BPA detection can be affected by matrix related interferences, confirmation through LC-MS/MS was carried out according to Shao and colleagues (Shao et al. 2005). Confirmation regarded all chocolate milk samples and 50% of beverages from each category randomly selected.

3.7.8 Instrumental parameters

BPA detection was performed through alliance 2695 (Waters, USA) liquid chromatography equipped with a Quattro Ultima Pt (Micromass, UK) tandem mass spectrometer and a symmetry C-18 column (150mm×2.1mm i.d., 3.5m). The temperature of the oven was set at 40 °C, the flow rate was 0.2 mL/min and the injection volume was 10 µL. Mobile phases consisted of methanol and water with 0.1% ammonia, through following gradient: methanol increased from 10 to 55% in 10 min, then increased to 85% in 10 min and held for 8 min, finally brought back to 10% and held for 15 min before the following injection. The mass spectrometer operated in negative mode and ESI (Electro-Spray Ionization) in multiple-reaction monitoring (MRM) mode. The capillary voltage was 3.5 kV, the cone voltage was 70V and the multiplier voltage was 650V. Nitrogen was used as nebulizing, desolvation and cone gas. UHP argon was used as the collision gas for the tandem mass spectrometric analysis and the pressure in the collision chamber was kept at 0.0028 mbar. A calibration curve in the concentration range 1 to 100 ng/g was obtained by linear regression of the normalized standard solution areas against BPA concentrations. The intra- and inter-day repeatability of the method were evaluated by injecting six standard solutions at 100 ng/g. The inter-day and intra-day reproducibility always showed a RSD < 3% (Figure 6).



	Peak area
1	1259
2	1286
3	1271
4	1287
5	1250
Mean	1270.60
Standard deviation	16.32
RSD	1.28%

Figure 6 - Reproducibility of Bisphenol A (BPA) by injection of standard solution (100 µg/L)

3.7.9 MRM (Multiple Reaction Monitoring) optimization

The most abundant ion was selected and was used as a precursor ion. Table 12 shows optimized MRM transitions obtained using the Optimizer Software. The BPA precursor ion was at *m/z* 227 and quantitative ion was *m/z* 212 and qualitative ion was *m/z* 133.

Table 12 - Optimized Multiple Reaction Monitoring transitions

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Polarity
Bisphenol A	227.1	212	8	Negative
Bisphenol A	227.1	133	20	Negative
Bisphenol A	227.1	117	48	Negative
Bisphenol A	227.1	93	50	Negative

3.7.10 Statistical analysis

In order to assess the differences in BPA concentrations between canned and non-canned drinks, a statistical analysis was performed by two-sided t-test, using SPSS 19.0 software (IBM). The level of significance was set at $p < 0.05$. The BPA concentrations below LOD were evaluated as equal to LOD.

3.8 Results and discussion

The average pH values were equal to 3.0 ± 0.4 in carbonated drinks and to 3.3 ± 0.2 in non-carbonated drinks and 6.9 ± 0.1 in chocolate flavoured milk beverages. After degassing of sparkling drinks pH values were unchanged. Table 13, Table 14, Table 15 and Table 16 show the results of BPA detection, confirmed by LC-MS/MS.

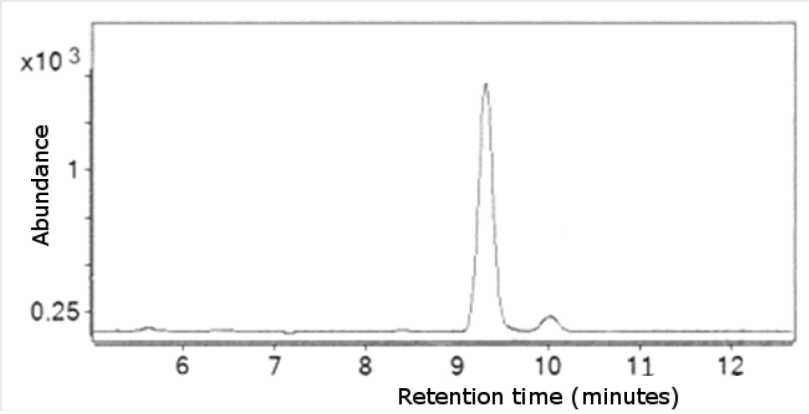


Figure 7 - LC-MS/MS chromatogram of a chocolate milk sample

BPA was found at detectable levels in 57% of the carbonated drinks, in 50% of the non-carbonated drinks, and in 100% of the milk based drinks (Figure 8), with mean concentrations ranging from 0.51 ± 0.56 µg/L (median value 0.18 µg/L) in carbonated drinks to 6.88 ± 5.95 µg/L (median value 3.60 µg/L) in the milk-based products (Table 13).

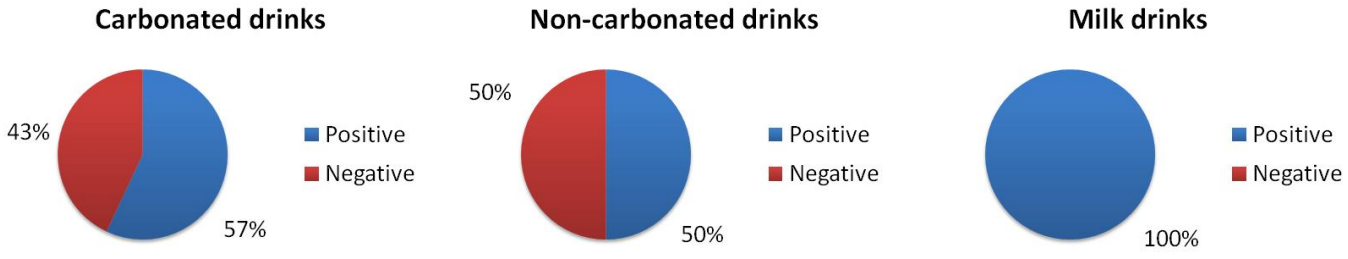


Figure 8 - Percentage of positive samples (Total n=72) (Bisphenol A levels above LOD)

Table 13 - Bisphenol A (BPA) concentrations in sugary drinks.

Drink	Packaging	BPA concentration (µg/L) Mean ± SD	BPA concentration (µg/L) Median *Range*
Carbonated	Can	1.46 ± 1.41	1.24 *(<LOD – 4.98)*
	Non-Can	0.51 ± 0.56	0.18 *(<LOD – 1.78)*
Non-carbonated	Can	1.04 ± 1.01	0.80 *(<LOD – 2.79)*
	Non-Can	0.80 ± 0.96	0.18 *(< LOD – 3.58)*
Milk based	Non-Can	6.88 ± 5.95	3.60 (1.00 – 17.65)
Total	Can / Non-Can	1.86 ± 3.14	0.79 (< *LOD* – 17.65)

In the carbonated drinks, the BPA levels ranged between <LOD and 4.98 µg/L. The highest concentrations occurred in Al canned beverages (Table 14).

Table 14 - Concentration of Bisphenol A (BPA) in carbonated samples and related values detected by LC-MS/MS confirmation.

Product	Packaging	BPA concentration (µg/L)	Concentration confirmed by LC-MS/MS
Cola A	Can	1.24	NC
Cola B	Can	<LOD	<LOD
Cola C	Can	1.75	NC
Cola D	Can	3.82	NC
Cola E	Can	0.74	NC
Cola F	Can	3.79	3.90
Cola G	Can	2.36	2.32
Cola H	PET	1.78	1.70
Cola I	PET	0.55	NC
Cola J	PET	1.51	NC
Cola K	PET	<LOD	<LOD
Cola L	Glass	<LOD	<LOD
Cola M	Glass	<LOD	NC
Cola N	Glass	<LOD	NC
Light Cola A	Can	2.98	3.04
Light Cola B	Can	0.66	NC
Light Cola C	Can	<LOD	NC
Light Cola D	Can	1.54	NC
Light Cola E	PET	<LOD	NC
Light Cola F	PET	<LOD	NC
Cola without caffeine A	Can	4.98	5.05
Cola without caffeine B	PET	0.72	0.77
Cola without caffeine C	Can	1.60	1.69
Soda, orange A	Can	<LOD	NC
Soda, orange B	Can	<LOD	NC
Soda, orange C	Can	<LOD	NC
Soda, orange D	Can	<LOD	NC
Soda, orange E	Can	2.13	2.10
Soda A	Can	0.54	NC
Soda B	Can	<LOD	<LOD
Soda C	PET	<LOD	<LOD
Soda, grapefruit A	Can	2.61	2.77
"Chinotto" drink A	Can	<LOD	<LOD
"Chinotto" drink B	Can	1.43	1.32
"Chinotto" drink C	PET	0.54	NC

NC = Not Confirmed

In the non-carbonated drinks, the BPA concentrations ranged between <LOD-3.58 µg/L, with the highest BPA concentration (3.58 µg/L) in a peach tea packed in polystyrene (PS) (Table 15). In glass-packed drinks, BPA was always below LOD (Table 14 and Table 15).

Table 15 - Concentration of Bisphenol A (BPA) in non-carbonated samples and related values detected by LC-MS/MS confirmation

Product	Packaging	BPA concentration (µg/L)	Concentration detected by LC-MS/MS
Pineapple juice	Tetrapak	0.75	0.81
Orange, carrot and lemon juice A	Tetrapak	<LOD	<LOD
Orange, carrot and lemon juice B	Tetrapak	0.82	NC
Blood orange juice A	Tetrapak	<LOD	<LOD
Blood orange juice B	Tetrapak	1.92	1.90
Pear and banana juice	Tetrapak	1.41	1.35
Pear juice A	Tetrapak	<LOD	NC
Pear juice B	Tetrapak	<LOD	<LOD
Grapefruit juice	Glass	<LOD	<LOD
Lemon tea A	Can	0.93	NC
Lemon tea B	Can	1.68	1.72
Lemon tea C	Can	<LOD	<LOD
Lemon tea D	Can	0.81	NC
Lemon tea E	PS	0.93	NC
Lemon tea F	Can	0.79	NC
Lemon tea G	PS	<LOD	<LOD
Lemon tea H	Glass	<LOD	<LOD
Green tea A	Can	2.64	2.66
Green tea B	Can	<LOD	<LOD
Green tea C	PET	1.80	NC
Peach tea A	Can	<LOD	NC
Peach tea B	PET	<LOD	NC
Peach tea C	PS	3.58	3.62
Peach tea D	Can	2.79	2.70
Peach tea E	Can	<LOD	NC
Decaffeinated lemon tea A	PET	<LOD	NC

NC = Not Confirmed

Among the carbonated/non-carbonated drinks, the BPA concentrations in canned beverages were significantly higher than in those packed in PET/PS and in Tetra Pak™. The mean BPA concentrations in milk beverages were higher than those found in the other beverages (mean 6.88 ± 5.95), with a range between 1.00-17.65 µg/L (Table 16), probably because of the lipid content of the milk which may facilitate the migration of BPA from the packaging.

Table 16 - BPA concentrations (µg/L) in ten differently packed milk beverages and related values detected by LC-MS/MS confirmation

Beverage	**Packaging**	**BPA concentration (µg/L)**	**Concentration detected by LC-MS/MS**
Chocolate milk A	PET	15.25	14.98
Chocolate milk B	Tetrapak	2.99	2.93
Chocolate milk C	PET	10.04	10.39
Chocolate milk D	PET	10.00	9.88
Chocolate milk E	PET	2.61	2.66
Chocolate milk F	PET	3.69	3.78
Chocolate milk G	Tetrapak	17.65	18.09
Chocolate milk H	Tetrapak	2.06	1.99
Chocolate milk I	Tetrapak	1.00	1.04
Chocolate milk J	Tetrapak	3.50	3.42

Since no difference was found in the pH values between carbonated and non-carbonated drinks and between canned and non-canned drinks, pH does not seem to be a crucial factor in the migration of BPA. Bisphenol A levels showed a great variability even among the beverages packed in the same type of container. This is probably due to alternative sources of contamination, such as environment and thermal and mechanical stress of the packages during transport and storage. The results of this thesis on all drinks except milk showed that 51% of beverages had BPA levels of < 0.5 μg/L, 66% had BPA levels of <1 μg/L, and the mean concentration of BPA was equal to 1.03 μg/L. Cao and colleagues analyzed BPA in 72 soft drinks and it was detectable in almost all samples of soft drinks, except in two energy drinks. About 75% of the products showed levels of BPA <0.5 μg/L, 85% had levels of BPA <1 μg/L, and the average level of BPA in all products was about 0.57 μg/L (Cao et al. 2009). According to other studies BPA levels in canned powder infant formulas from Greek market were between <1.7 and 15.2 ng/g, and between 0.38 and 6.31 ng/mL, although the authors did not give detailed information about the type of packaging (Maragou et al. 2006; Molina-Garcìa et al. 2012). These corroborate the hypothesis that BPA could contaminate milk not only because of package-related migration, but also during production, due to environment contamination. The Ministry of Health, Labour and Welfare in Japan investigated 42 sugary drinks from 2008 to 2009, including 20 canned coffee samples, 10 canned tea samples, 10 canned juice and carbonated beverages, and 2 drink-type canned soups. BPA was detected in 10 of the 20 coffee samples at concentrations ranging from 1 to 4 μg/kg, and in 2 of the 10 tea samples at concentrations of 1 and 7 μg/kg. No BPA was detected in 10 canned juice/carbonated drinks and in two canned soups (MHLW 2010). The Ministry of Health in Canada examined 38 beverages , these products included 22 soft drinks, packed in cans, PET and glass bottles, and 16 beer samples. Low levels of BPA were detected in all investigated canned beer samples with levels ranging from 0.08 to 0.54 μg/L (Health Canada 2010). The results of this work on all drinks except milk were higher than those reported by Cao and colleagues in drinks bought from the Canadian market (mean 0.57 μg/L) and those found in canned soft drinks from the Canadian market (0.019-0.21 μg/L) and finally, concentration level found by Gallart-Ayala and colleagues in beverages from Spanish market (range: <0.05 – 0.61) (Cao et al. 2009; Health Canada 2010; Gallart-Ayala et al. 2011). BPA levels detected in this thesis were also similar to those reported by Geens and colleagues in canned beverages obtained from the Belgian market (mean 1.0 μg/L) (Geens et al. 2010) and these data are consistent with concentration detected in beverages samples from Portoguese market (range: <0.01 – 4.7 μg/L) (Cunha et al. 2011). Finally, BPA levels detected in this study were lower than those reported by the Ministry of Health, Labour and Welfare of Japan in 2008-2009 (ranging from 1 to 7 μg/kg) and those detected in beverages from the Australian market (ranging from 1 to 290 μg/kg) (MHLW 2010; FSANZ 2011).

3.8.1 Potential BPA daily intake by soft drink consumption in children

In order to evaluate children's daily intake of BPA through drink consumption, budget method (BM) model was applied (FAO/WHO 2011). This model considered mean and maximum physiological levels of daily liquid consumption, respectively, as 0.03 and 0.1 L/kg bw. It took into account three possible scenarios of exposure: 100% consumption of beverages (worst case); 50% consumption of beverages (medium case), and 25% consumption of beverages (best case). Hence, estimated drink consumption were in the worst case, 0.03 L/kg bw/day (median) and 0.1 L/kg bw/day (maximum); in the medium case, 0.02 L/kg bw/day (median) and 0.05 L/kg bw/day (maximum); in the best case, 0.01 L/kg bw/day (median) and 0.03 L/kg bw/day (maximum). BPA median and maximum concentrations found in the beverages analyzed in this study were multiplied by the above mentioned values, and median and maximum potential exposures to BPA (μg/kg bw/day) were obtained. According to three different BM scenarios, exposures ranged between 0.008-0.024 μg/kg bw/day; the maximum exposure values varied from 0.530 μg/kg bw/day to 1.765 μg/kg bw/day. The median exposure values for the "best" and "worst case" were, respectively, 0.20 % and 0.60 % of the TDI set by EFSA at 4 μg/kg bw/day, and 13.25% to 44.13% of the TDI considering maximum levels (Table 17) (EFSA 2015).

In the above mentioned FAO/WHO report, the mean BPA total dietary exposure values for young children and teenagers in different countries were estimated equal to 0.1–0.5 μg/kg bw/day (0.3–1.1 μg/kg bw/day at the 95th/97.5th percentiles) (FAO/WHO 2011). The median BPA exposure levels found in this study were greater than the 95th/97.5th percentiles FAO/WHO values.

Table 17- Potential dietary exposure to BPA for 3-6 and 6-10 years old children through consumption of beverages according to Budget Method (BM) and the effect in percentage on EFSA TDI.

Model	Dietary exposure estimate (μg/kg bw/day)		Effect on EFSA TDI (%)	
	Median	Max	Median	Max
BM scenario (100% drinks)	0.024	1.765	0.60	44.13
BM scenario (50% drinks)	0.016	0.883	0.40	22.08
BM scenario (25% drinks)	0.008	0.530	0.20	13.25

3.9 Conclusions

Widespread contamination by BPA in soft drinks was assessed. The LOD obtained in this study, although lower than obtained in other studies (Kawamura et al. 1999; Goodson et al. 2002; Thomson & Grounds 2005), was higher than that obtained by Cao and colleagues (Cao et al. 2009) and therefore levels in drinks could be underestimated. Besides, the absence of BPA in glass bottled beverages and its occurrence in all canned and plastic packed products confirms how packaging could facilitate the release of BPA into beverages. A high contamination was assessed in chocolate flavoured milk beverages, probably due to the lipophilic nature of milk, which facilitates the migration of BPA from the bottles as well as to milk contamination during the production process. Milk-based drink contamination can derive from harvesting and storage in containers, heat treatments, and packaging, or from other ingredients such as chocolate. Unlike other countries, no data on children in Italy are available in the scientific literature concerning BPA exposure, so far. Besides, data on BPA exposure through drinks are still scarce both in Europe and in other countries. The median levels of exposure of children found in this study were lower than the TDI established by EFSA, but it must be taken into account that only the influence of soft drinks in the diet was assessed in current study, whereas the TDI issued by EFSA also considers other sources of exposure such as solid food ingestion, air inhalation, dermal contact, etc. Therefore, habitual consumption of sugary beverages could constitute a risk to the health of children.

3.10 References

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3.11 Evaluation of BPA and derivatives thereof in energy drinks

Detection of BPA in sugary drinks and infant formulas was performed through methods that involved SPE clean-up procedures through C18 cartridges (Sun et al. 2006; Cao et al. 2009; Fasano et al. 2015). Analysis of Energy drink samples was performed in order to evaluate an alternative clean-up method for Bisphenol A and derivatives thereof, through the use of compound-specific SPE cartridges.

3.12 Materials and methods

3.12.1 Sampling

40 energy drinks bottled in can, belonging to different brands, were purchased at different retail markets of Campania region. For each sample analyzed, pH value was previously measured, in order to verify a possible correlation with the concentration of investigated compounds.

3.12.2 Reagents

Acetonitrile, methanol and water (HPLC grade) were supplied by Merck (Darmstadt, Germany). Solid phase extraction cartridges (SPE) AFFINIMIP for Bisphenol A were supplied by AFFINISEP (Val-De-Reuil, France)

3.12.3 Preparation

The beverages were degassed by ultrasonic bath for 30 minutes and then an aliquot of 5 mL underwent analytical protocol described below.

3.12.4 Analytical Protocol

The multiresidual method used for this thesis was developed and set up in collaboration with Dr. Pasquale Gallo (Department of Chemistry - Istituto Zooprofilattico del Mezzogiorno). The experimental protocol included a purification step through the use of SPE cartridges AFFINIMIP for Bisphenol A, separation by ultra-performance liquid chromatography (UPLC) and fluorescence revelation (FLD) for BPA, BADGE, BPB, BPF and BFDGE. AFFINIMIP cartridges are based on a specific binding mechanism for BPA by molecularly imprinted polymers (MIPs); the reactive sites of these polymers showed the capability to bind also specifically analogs of bisphenol A like BPF, BPB, BADGE and BFDGE.

3.12.5 Purification

5 ml of beverage in a 10 ml glass test tube and 1 mL of ammonium acetate 0.2 M was added 1 ml to buffer the pH at an optimum level of 5.0, as indicated by the manufacturer of SPE cartridges. Before proceeding with the purification, the SPE cartridges AFFINIMIP were conditioned using 3 ml of methanol:acetic acid solution (98:2 v/v), 3 ml of acetonitrile and 3 ml of HPLC water. After conditioning the cartridges, the sample was loaded. The column was then washed by loading 9 ml of water and subsequently 6 ml of an acetonitrile:water mixture (40:60 v/v). Then cartridges were dried applying a vacuum for 30 seconds through vacuum manifold, the elution was carried out with 3 ml of methanol, and 3 ml of acetonitrile. The eluates were pooled and completely dried under gentle nitrogen flow; the dried residue was dissolved in 1 ml of methanol and transferred in glass vials before for UPLC-FLD analysis.

3.12.6 UPLC-FLD analysis

The quantification of the bisphenols was carried out through Acquity UPLC H-Class (Waters) equipped with fluorimetric (FLD). The chromatographic separation was obtained by reversed phase column RP-Amide Ascentis® Express 2.7 pm, 75 × 4.6 mm, with the injection volume of 5.0 µl, using a linear gradient elution as follow (Table 18):

Table 18 - Gradient elution parameters

Flow (ml min^{-1})	Time (min)	% H_2O	% Acetonitrile
0.5	0.0	50.0	50.0
0.5	0.5	50.0	50.0
0.5	6.0	5.0	95.0
0.5	9.0	5.0	95.0
0.5	11.0	50.0	50.0
0.5	15.0	50.0	50.0

Fluorescence detector wavelengths were set at 275 nm (excitation) and 305 nm (emission). Limit of detection (LOD) and the limit of quantification (LOQ) were calculated as follows:

- LOD = average concentration of blanks + 3 standard deviations
- LOQ = average concentration of blanks + 9 standard deviations

The LOD and LOQ values were respectively equal to 0.07 and 0.20 µg/L.

The quantification of bisphenols was made by comparison with the calibration curve obtained considering five points corresponding to standard solutions of 1.0 - 5.0 - 10.0 - 25.0 - 50.0 µg / L. The method has been previously validated by choosing two spiking levels (2.0 µg/L and 10.0 µ/L) performing six replicates for each level. Besides, for each batch a recovery test was carried out at 10.0 µg/L in order to verify the efficiency and the accuracy of the protocol. The results obtained from the quantitative analysis were corrected according to recovery percentage. The recovery percentages after the validation phase are shown in the following table (Table 19):

Table 19 - Recovery percentages of investigated analytes

	BPF	**BPA**	**BFDGE**	**BPB**	**BADGE**
Mean recovery % (2.0 µg/L)	52 ± 5	78 ± 6	89 ± 8	94 ± 8	91 ± 6
Mean recovery % (10.0 µg/L)	58 ± 3	93 ± 9	82 ± 5	88 ± 6	87 ± 4

3.13 Results and discussion

The percentages of positive samples were: 58% for BPA, 35% for BADGE, 33% for BPF and 20% for BFDGE. BPB and BPS were not detectable in any sample (data not shown). Mean levels of BPA were 0.943 ± 0.830 µg/L (median: 0.670 µg/L and range: 0.100 – 3.310 µg/L). BADGE showed mean levels equal to 3.153 ± 5.593 µg/L (median: 0.620 µg/L and range: 0.320 – 19.420 µg/L). In this case, higher maximum levels occurred in two samples (greater than 10 µg/L) led to higher means and standard deviations. Finally BPF showed contamination levels equal to 0.534 ± 0.370 µg/L (median: 0.420 µg/L and range: .110 – 1.290µg/L) and BFDGE mean levels were equal to 0.400 ± 0.150 µg/L (median: 0.415 µg/L and range: 0.120 – 0.570µg/L) (Table 20).

Table 20 - Bisphenol A (BPA), Bisphenol A diglycidyl ether (BADGE), Bisphenol F (BPF) and Bisphenol F diglycidyl ether concentrations in µg/L (mean ± sd, median and range).

Sample	**BPA(µg/L)**	**BADGE(µg/L)**	**BPF(µg/L)**	**BFDGE(µg/L)**
	Mean ± s.d. Median *Range*	Mean ± s.d. Median *Range*	Mean ± s.d. Median *Range*	Mean ± s.d. Median *Range*
Energy Drinks	0.943 ± 0.830 0.670 *0.100 - 3.310*	3.153 ± 5.593 0.620 *0.320 – 19.420*	0.534 ± 0.370 0.420 *0.110 – 1.290*	0.400 ± 0.150 0.415 *0.120 – 0.570*

Considering that the most common quantity of product per can available on the market is 0.250 L the following table shows the mean amount of investigated compounds per single-serve can (0.250 L) (Table 21):

Table 21 - Bisphenol A (BPA), Bisphenol A diglycidyl ether (BADGE), Bisphenol F (BPF) and Bisphenol F diglycidyl ether amount per single can.

Sample	**BPA(µg)**	**BADGE(µg)**	**BPF(µg)**	**BFDGE(µg)**
	Mean ± s.d. Median *Range*	Mean ± s.d. Median *Range*	Mean ± s.d. Median *Range*	Mean ± s.d. Median *Range*
Energy Drinks	0.241 ± 0.211 0.169 *0.025 - 0.828*	0.788 ± 1.451 0.155 *0.080 - 4.855*	0.133 ± 0.093 0.105 *0.028 - 0.323*	0.100 ± 0.040 0.104 *0.030 - 0.143*

An exposure assessment was performed considering adolescents 10-18 years of age as target sub-population. Daily intake was calculated considering that habitual consumers of energy drinks could assume up to a 0.250 L can per day (EFSA 2013). Daily intake data are reported in the following table (Table 22):

Table 22 – Daily intake to Bisphenol A (BPA), Bisphenol A diglycidyl ether (BADGE), Bisphenol F (BPF) and Bisphenol F diglycidyl ether through consumption of 0.250 L of Energy drink.

	BPA (µg/kg body weight)	BADGE (µg/kg body weight)	BPF (µg/kg body weight)	BFDGE (µg/kg body weight)
Medium case	0.0032	0.0029	0.0020	0.0020
Worst case	0.0157	0.0923	0.0061	0.0027

As EFSA issued only a TDI for BPA the following table shows the effect in percentage of Intake in the medium and worst case, evaluated in this study (Table 23):

Table 23 - Potential dietary exposure to BPA for 10-18 years old adolescents through consumption of energy drinks and related effect in percentage on EFSA TDI.

	BPA(µg/kg p.c)	Effect on EFSA TDI (%)
Energy Drinks (medium case)	0.0032	0.08
Energy Drinks (worst case)	0.0157	0.39

Exposure assessment was performed considering a body weight of 10-18 years old people which averages out at 52.6 kg, according to Leclercq and colleagues (Leclercq et al. 2009).

3.14 Conclusions

As demonstrated for sugary beverages, a widespread contamination of BPA and derivatives thereof occurred in more than 50% of samples and median levels of BPA in energy drinks were comparable to concentrations detected in sugary beverages. Due to matrix interference that may occur during the analysis, the purification through the columns based on Molecularly Imprinted Polymers cartridges, even though more expensive than the SPE by C18 columns, showed a great specificity for bisphenols (especially BPA and BADGE), with high recovery percentages, facilitating the detection phase even with less specific methods such as fluorescence.

3.15 References

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3.16 Exposure to Fumonisins in children affected by celiac disease

Last topic of this thesis was the evaluation of fumonisins B_1, B_2 and B_3 levels in gluten-free products, in order to ascertain the exposure of celiac children to such compounds.

3.17 Materials and methods

3.17.1 Sampling

80 maize-based products intended to celiac people were bought at dedicated shops and supermarkets in Campania region. Samples consisted of: bread, pasta, cookies, corn-flakes, savoury snacks (hardtacks, extruded products), sweet snacks (cereal bars and cakes). Every sample was made of a variable amount of maize. Samples were grinded into flour with an average particle size of about 500 μm, homogenized and finally stored at 20° C before analysis.

3.17.2 Reagents and equipments

Water, methanol, acetonitrile (all HPLC grade), sodium dihydrogen phosphate; disodium tetraborate solution; sodium chloride; disodium hydrogen phosphate; potassium dihydrogen phosphate; potassium chloride were purchased from Merck KGaA, (Darmstadt, Germany); acetic acid (purity > 99%) chloridric acid; o-phosphoric acid; o-phthaldialdehyde (OPA) and 2-mercaptoethanol (purity > 99%) were both purchased from Sigma Aldrich (St. Louis, MO, USA); immunoaffinity columns purchased from Romer Labs Diagnostic GmbH, Tulln, Austria; syringe filters (pore-size 0.45 μm) purchased from Sartorius (Goettingen, Germany); HPLC system (Shimadzu LC-10AT VP) equipped with Ascentis C18 reversed-phase column (octadecylsilane phase; Length × I.D.: 15 cm × 4.6 mm; particle size: 5 μm) and fluorescence detector Shimadzu RF-10XL.

3.17.3 Analyitical method

Detection of fumonisins B_1 and B_2 in corn-based food was performed according to EN 14352:2005 method, as described below (CEN 2004). 20 g of sample were put in a 250 mL centrifuge bottle and 50 mL of acetonitrile:methanol:water solution (25:25:50 v/v). Bottles were shaken in an orbital shaker for 30 minutes at 300 rev/min and finally centrifuged for 10 minutes at 4000 rpm. Then, supernatant was collected and filtered through filter paper. 10 mL of filtered extract were put in a 100 mL flask and 40 mL of Phosphate Buffer Saline (PBS) were added. PBS was made of sodium chloride (8.0 g), disodium hydrogen phosphate (1.2 g); potassium dihydrogen phosphate (0.2 g), potassium chloride (0.2 g) and deionized water to have a final volume of 1 L and pH of this solution was adjusted to 7.0 with 0.1 M HCl. 10 mL of solution made of 10 mL of filtered supernatant and 40 mL of PBS, obtained as described above, were loaded onto an immunoaffinity column. Then, 10 mL of PBS were added onto the column and eluates were discarded. Finally, fumonisins were collected in an amber vial, loading 3 mL of a methanol:acetic acid (98:2 v/v) onto the column. The collected eluates were dried under a gentle stream of nitrogen at 50° C and vials were stored at 4° C until detection through HPLC equipped with a fluorescence detector.

3.17.4 HPLC detection

Excitation and emission wavelenghts of fluorimetric detection were set at 335 nm and 440 nm, respectively. The column was kept at a constant temperature of 40° C. The mobile phase consisted of 77% of methanol and 23% of a sodium dihydrogen phosphate solution 0.1 M and pH of mobile phase was adjusted to 3.3 with o-phosphoric acid before use. The flow rate of run was set at 0.950 mL/min (isocratic run). 0.200 μL of acetonitrile:water solution (50:50 v/v) were added into the vial stored at 4° C and gently vortexed for 20 seconds in order to reconstitute the sample that was previously dried. Before injection a pre-column derivatization was performed mixing 25 μL of reconstituted sample to 225 μL of derivatization solution made of 50 μL of 2-mercaptoethanol, 1 mL methanol, 40 mg of OPA and 5 mL of tetraborate solution 0.1 M.

3.17.5 Analytical performance

A calibration curve was obtained by injecting standard solutions of fumonisins mix (B_1+B_2+B_3) at following concentrations:

- 1000 + 1000 + 1000 ng/mL;
- 500 + 500 + 500 ng/mL;
- 250 + 250 + 250 ng/mL;
- 125 + 125 + 125 ng/mL;
- 63 + 63 + 63 ng/mL.

Recovery test was performed spiking 5 g aliquots of pasta and maize flour with FB_1+FB_2+FB_3 fumnisins mix in order to obtain spiked samples at following contamination levels: 1600, 800, 400 ng/g. Recoveries were > 95% for eeach sample. Limit of detection (LOD) and Limit of Quantification (LOQ) were calculated through spiked blanks samples injections, evaluating a signal/noise ratio equal to 3:1 for LOD and 10:1 for LOQ. On this basis, LOD and LOQ were equal to:

5.0 ng/g for FB_1

2.5 ng/g for FB_2 ed FB_3

and:

12.5 ng/g for FB_1

10.0 ng/g for FB_2

7.5 ng/g for FB_3

3.17.6 Exposure assessment

Evaluation of the daily exposure of children between 3 and 10 years through diet was performed taking into account median and maximum values of concentration detected in the samples and the daily ingestion of food as reported by Leclercq and colleagues in the daily consumption tables (Leclercq et al. 2009), assuming that every source of carbohydrates was replaced by a consistent gluten-free substitute. In this way the intake of fumonisins (as sum of B_1 and B_2 type) was calculated according to the following formula:

$$I = \frac{C \times Q}{W}$$

I = Daily Intake of fumonisins (ng/kg body weight day)

C = Concentration of fumonisins detected in samples, expressed as sum of Fumonisin B_1 + Fumonisin B_2 (ng/g)

Q = individual food daily consumption of children from 3 to 10 years according to Leclercq et al. (2009) (Leclercq et al. 2009) (g/day)

W = average body weight of children from 3 to 10 years old, according to the growth curves by World Health Organization (kg body weight)(WHO 2006).

Concentration levels (C) that were used for the calculation of intake were the median and the maximum values detected in samples, whereas the individual food daily consumptions considered (Q) were the median values (average consumers) and the values at 95th percentile (high consumers). Finally, the product C x Q was divided by the mean body weight (W) of 3-10 years children and the body weight of individuals at 95th percentile according to growth curves. Therefore, two cases were considered for each consumer: a medium-case and a worst-case and for total of four exposure categories.

3.18 Results and discussion

A widespread contamination emerged from the analysis of food, even though no sample showed a contamination above limit of Commission Regulation No 1126/2007. These data are corroborated by the high percentage of positive samples (Figure 9).

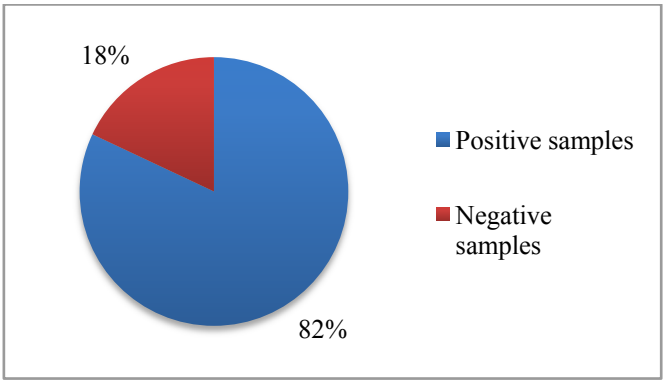

Figure 9 - Percentage of samples (n = 90) where fumonisin levels were detectable

Table 24 shows an overview of the fumonisin levels detected in samples, according to the following food categories: bread, pasta, breakfast cereals, cookies, savoury snacks and sweet snacks.

Table 24 - Concentrations expressed as Σ FB1+FB2+FB3 detected in samples

Food category	Median (ng/g)	Range (ng/g)	Positivity (%)
Bread	32.1	12.5-89.5	80
Pasta	32.4	12.5-87.4	100
Rice	12.5	12.5-12.5	30
Breakfast cereals	145.9	26.3-281.5	67
Cookies	50.6	12.5-386.6	95
Sweet snacks	12.5	12.5-29.4	30
Savoury snacks	107.5	12.5-400.9	89

For each category median, minimum and maximum concentration levels were reported. Median values ranged from 12.5 to 145.9 ng/g. A comparison of other literature data shows firstly that contamination levels could be very variable and diffuse. Unlike other studies by Dall'Asta and colleagues and Brera and colleagues, who likewise investigated the fumonisins occurrence in gluten-free products, the median and maximum concentrations emerged in this study were lower, comparing similar food categories, except for savoury snacks that showed higher median values (Dall'Asta et al. 2009; Brera et al. 2014). Despite this, the percentage of positive samples was mainly above the occurrence reported in the above mentioned studies (Table 25).

Table 25 - Comparison of median and maximum concentrations of fumonisins detected in gluten-free food samples, expressed as sum of FB_1 (fumonisin B_1) FB_2 (fumonisin B_2) anf FB_3 (fumonisin B_3) and percentage of positive samples.

Food	This study $FB_1+FB_2+FB_3$			Dall'Asta et al. (2009) $FB_1+FB_2+FB_3$			Brera et al. (2014) FB_1+FB_2		
	Median (ng/g)	Maximum (ng/g)	Positivity (%)	Median (ng/g)	Maximum (ng/g)	Positivity (%)	Mean[2] (ng/g)	Maximum (ng/g)	Positivity (%)
Bread	32	90	80	66[1]	513[1]	88[1]	30	205	5
Pasta	32	87	100				113	421	59
Cereals	146	282	67	NA	NA	NA	NA	NA	NA
Cookies	51	387	95	NA	NA	NA	NA	NA	NA
Sweet snack	12.5	29	30	354	554	78	NA	NA	NA
Savory snack	108	401	89	39	2250	57	48	761	9

[1]Authors reported merged data for pasta and bread categories
[2]Brera et al., (2014) reported data as mean instead of median values.
NA = data not available

Turning now to the experimental evidence on exposure assessment, as explained earlier, the median and maximum contamination levels were used to perform the evaluation of intake of fumonisins, considering a medium-case and a worst-case. Data about exposure assessment are shown in Table 26 and Table 27.

Table 26 - Daily intake for medium consumers.

Food category	Median intake (ng/kg p.c/day)	Maximum intake (ng/kg p.c/day)
Bread	90.5	252.38
Pasta	72.24	194.89
Rice	6.03	6.03
Breakfast cereals	21.24	40.98
Cookies	35.86	274.02
Savoury snacks	24.3	90.62
Sweet snacks	13.4	13.4
Total intake	263.73	872.32

Table 27 - Daily intake for 95th percentile consumers

Food category	Median intake (ng/kg p.c/day)	Maximum intake (ng/kg p.c/day)
Bread	221.37	617.24
Pasta	130.2	348.26
Rice	37.02	37.02
Breakfast cereals	125.77	242.67
Cookies	118.84	907.99
Savoury snacks	123.56	460.8
Sweet snacks	45.25	45.25
Total intake	802.01	2656.23

A similar approach for exposure evaluation has been attempted by Brera and colleagues taking into account a lower and an upper bound limit for medium consumer and consumer at 95th percentile and who reported an exposure of 348 ng/kg body weight/day and 582 ng/kg body weight/day for lower and upper bounds in the average consumer and 792 ng/kg body weight/day and 1385 ng/kg body weight/day for lower and upper bounds in the consumer at 95th percentile (Brera et al. 2014). On the basis of total intake was calculated the effect on PMTDI set by EFSA (2000 ng / kg of body weight per day), by dividing the total intake with TDI and multiplying the result by 100 (EFSA 2014). Incidence on EFSA PMTDI for medium consumer was equal to 13.1% and 43.6% respectively for medium and worst case. Whereas, for consumers at 95th percentile, the incidence was found to be 40.1% and 132.8% for the medium and worst case, respectively. In spite of Brera's study, where exposure levels were below the PMTDI, our data reveal that in the worst case the exposure might exceed about 30% the PMTDI (Figure 10).

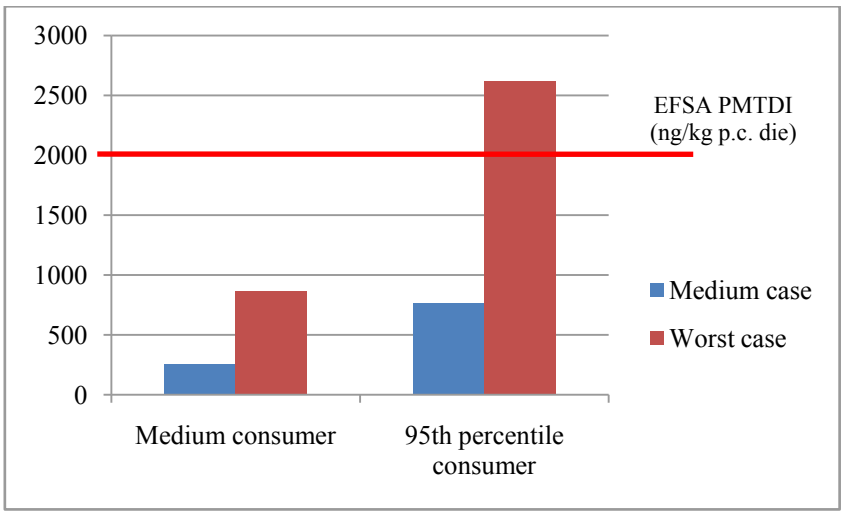

Figure 10 - Intake of medium and 95th percentile consumers and related comparison with PMTDI by EFSA (EFSA, 2014)

This result is somewhat unexpected as the concentrations of fumonisin detected in this study were lower than those by the same author. A possible explanation for this might be that this study took into account more gluten-free food categories like sweet snacks, cookies and breakfast cereals and bearing also in mind that this was a preliminary study with a limited number of samples allowing limited conclusions. Anyway, although results on people affected by celiac disease differ from the studies mentioned above, they appears consistent with those reported by European Food Safety Authority (EFSA) in 2014 in a scientific opinion on the risks for human related to the presence of masked forms of fumonisins. According to this EFSA report, exposure may remarkably exceed more than 3000 ng/kg bw/day in children at 95th percentile (EFSA 2014). Comparison with other authors that took into account also people not affected by celiac disease confirms that the higher consumption of maize-based foodstuff, the greater could be the exposure to fumonisins (Table 28).

Table 28 – Comparison of exposures to fumonisins between children affected by celiac disease and healthy peers.

Intake in children affected by celiac disease (ng/kg bw/day)		Intake in healthy children (ng/kg bw/day)	
Brera et al.,2014	348-1385	Sirot et al. (2013)	15-106
Dall'Asta et al.,2012	390-1940	Leblanc et al. (2005)	46-17
This study	256-2622	Bakker et al. (2009)	28
		Petersen and Thorup (2001)	400
		D'Arco et al. (2009)	2-720 (Spain) 5 (Italy)

3.19 Conclusions

Overall, even though this was a preliminary study including a small number of samples, there seems to be a clear evidence to indicate that exposure data, although below the PMTDI limit in the medium-case, could be not negligible for high consumers affected by celiac disease, whose intake of non-masked fumonisins could be greater than healthy people. Besides, also considering the amount of hidden and bound fumonisins, exposure could be much greater, especially in children. These findings have a number of important implications for future in-depth analysis and suggest that neglecting bound and hidden fumonisins could lead to an underestimation of total exposure even in healthy children. Therefore, further studies, which take these variables into account, will certainly need to be undertaken.

3.20 References

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APPENDIX 1 - PUBLICATIONS

Journal article #1. Microencapsulation of nisin in alginate beads by vibrating technology: Preliminary investigation

Maresca D., De Prisco A., La Storia A., Cirillo T., **Esposito F.** and Mauriello G.

LWT-Food Sci Technol (2016) 66:436-443

Abstract

Nisin is an antimicrobial peptide widely used as natural food preservative. Unfortunately, some factors lead to a reduction in its antimicrobial activity in food matrices. In this work nisin was microencapsulated in alginate matrix by vibrating technology with a high efficiency (75%) and microcapsules were characterized by having homogeneity in shape and surface morphology. Antimicrobial activity of microcapsules was evaluated against *Brochothrix thermosphacta* 7R1 immediately after microencapsulation process and during storage (24, 48, 72, 120, 144 and 168 h) under different conditions (i.e. 4 and 20° C, pH 2.5, 4.5 and 6.0). We proposed a new method to evaluate the residual activity of encapsulated antimicrobial substances measured in Active Microcapsule Units per ml (AMU/ml). The test revealed that during a storage at 4° C and pH 6.0, microcapsules retained their antimicrobial activity better than under other tested storage conditions. Furthermore, microcapsules efficiently protected nisin from the activity of protease. Resting cell experiments for the determination of antimicrobial activity of microcapsules during 120 h of storage under several conditions indicated greater activity at pH 6.0 with stirring. Nisin release from microcapsules determined by HPLC analysis showed that no trace of nisin was detected in the storage solution.

Journal article #2. Migration of monomers and plasticizers from packed foods and heated microwave foods using QuEChERS sample preparation and gas chromatography/mass spectrometry

Fasano E., Cirillo T., **Esposito F.** & Lacorte S.

LWT - Food Sci Technol (2015) 64(2):1015-1021.

Abstract

The objective of this study was to evaluate the migration of monomers and plastic additives in microwave heated homemade courses and in packed liquid food. Compounds studied were 3 phthalates, 4-tert-octylphenol (OP), 4-nonylphenol (NP), bisphenol A (BPA) and di(2-ethylhexyl)adipate (DEHA). A QuEChERS based method was optimized for the analysis of these compounds in solid or liquid packed and retailed food. Using gas chromatography coupled to mass spectrometry, recoveries ranged from 49 ± 16% (OP) to 130 ± 16% (BPA) and from 63 ± 22% (OP) to 127 ± 29% (NP) in solid and liquid foods, with limits of detection below 14.37 ng g^{-1} and 2.25 ng mL^{-1}, respectively. NP showed the highest mean level in homemade foods (1064 ± 363 ng g−1 wet weight) and reheating did not produce an increase in the levels detected. Phthalates and DEHA were detected at low concentrations. Among liquid foods, meat broth mean concentrations of NP were of 9.61 ± 1.93 ng mL^{-1} and of 9.68 ± 1.75 ng mL^{-1} for butylbenzylphthalate in fish broth, while in wine, the most abundant compound was di-n-butylphthalate. Overall, the developed QuEChERS method permitted to determine the presence of investigated chemicals in packed food allowing the evaluation of compounds that can affect food quality.

Journal article #3. Detection of polycyclic aromatic hydrocarbons in smoked buffalo mozzarella cheese produced in Campania Region, Italy

Fasano E., **Esposito F.**, Scognamiglio G., Cocchieri Amodio R. & Cirillo T.

J Sci Food Agr (2015) 96:1704-1708

Abstract

BACKGROUND

Smoked mozzarella is obtained through traditional smoking techniques or the use of liquid smoke. Polycyclic aromatic hydrocarbons (PAHs) may be produced during the organic matrix combustion. The aim of this study was to evaluate benzo[a]pyrene (B(a)P), benzo[a]anthracene (B(a)A), benzo[b]fluoranthene (B(b)FA), benzo[k]fluoranthene (B(k)FA), benzo[ghi]perylene (B(g,h,i)PE), chrysene (CHR), dibenz[a,h]anthracene (DB(a,h)A) and indeno[1,2,3-cd]pyrene (IP) in smoked buffalo mozzarella produced in Campania, evaluating also the influence of the different smoking techniques. Milk and mozzarella of the same batch, before and after smoking, were collected. The detection method was basic hydrolysis, clean-up with silica and detection by HPLC equipped with a fluorescence detector.

RESULTS

For milk, only 30% was contaminated. In non-smoked products the medians were >LODs only for B(a)A and CHR. In smoked mozzarellas the highest median was 0.37 ng g^{-1} wet weight (CHR).

CONCLUSION

It was found that the consumption of this typical food of Campania does not represent a risk for consumers, considering that the incidences on EFSA dietary intake were always lower than 1.5% for mozzarella cheese and lower than 3% for smoked mozzarella cheese.

Journal article #4. Exposure to di-2-ethylhexyl phthalate, di-n-butyl phthalate and Bisphenol A through infant formulae

Cirillo T., Latini G., Castaldi M. A., Dipaola L., Fasano E., **Esposito F.**, Scognamiglio G., Di Francesco F. and Cobellis L.

J Agr Food Chem (2015) 63 (12):3303-3310

Abstract

Phthalates and bisphenol A (BPA) are ubiquitous contaminants identified as endocrine disruptors. Phthalates are worldwide used as plasticizers, in particular to improve the mechanical properties of polymers such as polyvinyl chloride. Because they are not chemically bound to the polymer, they tend to leach out with time and use. Di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP) are the two most common phthalates. BPA is an estrogenic compound used to manufacture polycarbonate containers for food and drink, including baby bottles. It can migrate from container into foods, especially at elevated temperatures. Diet is a predominant source of exposure for phthalates and BPA, especially for infants. The aim of this study was to test the presence of DEHP, DnBP, and BPA in infant formulas. DEHP, DnBP, and BPA concentrations were measured in 22 liquid and 28 powder milks by gas chromatography with flame ionization detection and high performance liquid chromatography with fluorimetric detection, respectively. DEHP concentrations in our samples were between 0.005 and 5.088 μg/g (median 0.906 μg/g), DnBP concentrations were between 0.008 and 1.297 μg/g (median 0.053 μg/g), and BPA concentrations were between 0.003 and 0.375 μg/g (median 0.015 μg/g). Concentrations of the investigated contaminants in liquid and powder milks were not significantly different, even though samples were packed in different types of containers. These data point out potential hazards for infants fed with baby formulas. Contamination seems more related to the production of formulas than to a release from containers.

Journal article #5. Bisphenol A contamination in soft drinks as a risk for children's health in Italy.

Fasano E., **Esposito F.**, Scognamiglio G., Di Francesco F., Montuori P., Cocchieri Amodio R. and Cirillo T.

Food Addit Contam A (2015) 32(7):1207-1214.

Abstract

Bisphenol A (BPA) was determined in sugary carbonated, non-carbonated and milk-based beverages, through HLPC-fluorescence detection and confirmed by LC-MS/MS, in a selection of brands that are mostly consumed by Italian children. The daily intake was determined through the WHO budget method (BM). BPA was found at detectable levels in 57% of carbonated beverages, in 50% of non-carbonated and in 100% of milk-based beverages. The median concentrations were 1.24 μg l^{-1} (range = < LOD–4.98 μg l^{-1}) in canned carbonated beverages and 0.18 μg l^{-1} (< LOD–1.78 μg l^{-1}) in non-canned carbonated beverages. In non-carbonated beverages, median concentrations were 0.80 μg l^{-1} (< LOD–2.79 μg l^{-1}) and 0.18 μg l^{-1} (< LOD–3.58 μg l^{-1}), respectively, for canned and non-canned beverages; in milk-based products the BPA median concentration was 3.60 μg l^{-1} (1.00–17.65 μg l^{-1}). BPA daily intake from sugary drink consumption in children ranged from 0.008 to 1.765 μg kg^{-1} bw day^{-1}. The median exposure values for the 'best' and 'worst' cases were 0.16% and 0.47% respectively of the EFSA t-TDI for BPA (4 μg kg^{-1}bw day^{-1}), and 10.59% and 35.30% of the t-TDI when the maximum levels were considered.

Journal article #6. Occurrence of NDL-PCBs, DL-PCBs, PCDD/Fs, lead and cadmium in feed and in rainbow trout (Oncorhynchus mykiss) farmed in Italy

Cirillo T., Fasano E., **Esposito F.**, Amorena M. and Amodio Cocchieri R.

Food Addit Contam A (2014) 31(2):276-287

Abstract

The safety of farmed rainbow trout (*Oncorhynchus mykiss*) is correlated with the quality of the production process. Polychlorinated biphenyls (PCBs), dioxins (polychlorinated dibenzodioxins and furans – PCDD/Fs), and heavy metals such as lead and cadmium were investigated because they can represent a risk for the consumer. The levels of these compounds in water, feed and specimens of trout farmed with two different feeds (A and B) were assessed. Their accumulation in muscle of A and B trout was evaluated and their dependence on the levels of feed contamination was considered. The results showed a widespread contamination in feed and in the examined trout, although lower than the European Union limits. For all compounds, concentrations in the farming waters were always < LOQs. Mean concentrations of NDL-PCBs in the A feed were significantly higher than in the B feed, except for PCBs 52 and 28. DL-PCB and PCDD/F concentrations were significantly higher in A feed. Lead and cadmium mean concentrations in A feed were 0.26 ± 0.01 and 0.1013 ± 0.0009 μg g^{-1}, respectively; and in B feed were 0.10 ± 0.01 and 0.0855 ± 0.0078 μg g^{-1}, respectively. The results showed that intakes for ∑DL-PCB + ∑PCDD/F ranged from 4.4% to 12% of the TDI, and for Pb and Cd from 1.9% to 2.7% and from 0.3% to 0.4% of the TDI, respectively.

Journal article #7. Spatial distribution and partitioning of polychlorinated biphenyl and organochlorine pesticide in water and sediment from Sarno River and Estuary, Southern Italy

Montuori P., Cirillo T., Fasano E., Nardone A., **Esposito F.** and Triassi M.

Environ Sci Pollut Res (2013) 21(7):5023-5035

Abstract

The Sarno River is nicknamed "the most polluted river in Europe". The main goal of this study is to enhance our knowledge on the Sarno River water and sediment quality and on its environmental impact on the gulf of Naples (Tyrrhenian Sea, Central Mediterranean Sea) in order to become a useful assessment tool for the regional administrations. For these reasons, 32 selected polychlorinated biphenyls (PCBs) and aldrin, α-BHC, β-BHC, δ-BHC, γ-BHC (lindane), 4,4′-DDD, 4,4′-DDE, 4,4′-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulphate, endrin, endrin aldehyde, heptachlor, heptachlor epoxide (isomer B) and methoxychlor were determined in the water dissolved phase (DP), suspended particulate matter (SPM) and sediments. Total concentrations of PCBs ranged from 1.4 to 24.9 ng L^{-1} in water (sum of DP and SPM) and from 1.01 to 42.54 ng g^{-1}in sediment samples. The concentrations of total organochlorine pesticides (OCPs) obtained in water (sum of DP and SPM) ranged from 0.54 to 7.32 ng L^{-1} and from 0.08 to 5.99 ng g^{-1} in sediment samples. Contaminant discharges of PCBs and OCPs into the sea were calculated in about 1,247 g day^{-1} (948 g day^{-1} of PCBs and 326 g day^{-1} of OCPs), showing that this river should account as one of the main contribution sources of PCBs and OCPs to the Tyrrhenian Sea.

Abstract #1. Esposizione infantile al Bisfenolo A tramite il consumo di soft drinks

Cirillo T., Fasano E., **Esposito F.** & Amodio Cocchieri R.

46° Congresso Nazionale Siti - 17-20 ottobre 2013, At Giardini di Naxos - Taormina (ME) – Italy.

Abstract

Il Bisfenolo A (BPA), composto utilizzato nella produzione di materiali quali policarbonati, resine epossidiche, PVC, etc. destinati a contenere alimenti, è oggi al centro degli interessi delle organizzazioni scientifiche internazionali che si occupano di sicurezza alimentare quali EFSA, FDA e WHO. Infatti studi tossicologici hanno recentemente evidenziato che il BPA può indurre effetti avversi sul cervello e sulla ghiandola prostatica in feti, effetti sul comportamento in neonati e bambini e possibili danni anche alla ghiandola mammaria in femmine in età prepuberale. Gli alimenti sono considerati la principale via di esposizione al BPA a causa della contaminazione generata dal contatto con materiali da imballaggio. Allo stato attuale, anche se i dati effettivi di contaminazione da BPA in alimenti e bevande sono ancora piuttosto scarsi, tra i settori più studiati vi è quello delle bevande zuccherate e gassate (soft drinks), per lo più confezionate in lattina, il cui consumo va incrementandosi, in particolare tra bambini e adolescenti, anche in Italia, come evidenziato da una recente indagine del Ministero della Salute. E' stato stimato, infatti, che dal 2008 al 2010 la percentuale di bambini di 8-9 anni che consumano abitualmente tali bevande almeno una volta al giorno è passata dal 40,6 al 48,3 %. Obiettivi del presente studio sono stati la ricerca ed il dosaggio del BPA in bevande confezionate e la stima dell'asunzione al BPA in soggetti di età compresa tra 7 e 9 anni, attraverso il consumo di tali bevande. Materiali e Metodi: sono state campionate, tra Napoli e provincia, bevande gassate e non confezionate in lattina, PET, tetrapak e vetro. Le bevande gassate sono state preventivamente degassate mediante una pompa da vuoto. Aliquote di 5 ml delle bevande non contenenti latte sono state processate mediante estrazione con acetonitrile, purificazione degli estratti su cartucce SPE e dosaggio mediante HPLC con rivelatore a fluorescenza. Le bevande contenenti latte sono state sottoposte ad una estrazione preliminare dei grassi e quindi analizzate secondo il metodo sopra descritto. Risultati: I risultati ottenuti hanno mostrato livelli di BPA dosabili in circa il 70% dei campioni, con concentrazioni significativamente superiori nelle bibite in lattina rispetto a quelle confezionate in PET, tetrapak o vetro. Correlando le concentrazioni di BPA al volume di ciascuna confezione ed ipotizzandone un consumo pari ad una bibita al giorno, è stata effettuata una stima dell'assunzione giornaliera tramite tale veicolo che è risultata oscillare da un minimo di 0.16 ad un massimo di 1.42 μg/bibita. I risultati ottenuti in questo studio sono mediamente superiori a quelli riportati in analoghi studi effettuati in Giappone, Canada e Belgio. Da tale studio emerge che le bibite in lattina possono rappresentare una fonte di assunzione di BPA non trascurabile. Va sottolineata l'importanza della problematica in considerazione del fatto che la fascia di consumatori di soft drink è rappresentata prevalentemente da bambini e adolescenti che costituiscono le classi di popolazioni più sensibili agli effetti tossici del BPA.

Poster #1. Occurrence of Bisphenol A in sugary beverages and related intake assessment in children

Esposito F.

19th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, September 24th-26th, 2014. University of Bari, Bari - Italy.

Occurrence of Bisphenol A in sugary beverages and related intake assessment in children

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Tutor: Prof. Teresa Cirillo

INTRODUCTION

Dietary exposure to endocrine disruptors is increasingly recognized as a serious worldwide public health concern, because of their influence on endocrine functions. These harmful effects could be more dangerous if exposure occurs during neonatal, puberty and adolescence age (Moryiama *et al.*, 2006; Cirillo *et al.*, 2012). Among these chemicals, Bisphenol A (BPA) (Fig. 1) deserves special attention, as there is an unambiguous relationship between exposure to BPA and endocrine issues (Zoeller *et al.*, 2005). BPA is a compound used in the manufacture of polymers such as epoxy resins, employed in the internal coating layer of aluminum cans or as an additive in plastics for food and beverages and can migrate, under certain conditions, from the packaging to food matrix, such as beverage packed in can or plastic bottles (EFSA, 2006; Cao *et al.*, 2009). Therefore, according to PhD objectives, the first activities described in this poster regarded: detection of Bisphenol A in sugary drinks intended for children's consumption and related evaluation of BPA daily intake in children through consumption of sugary drinks.

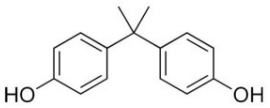
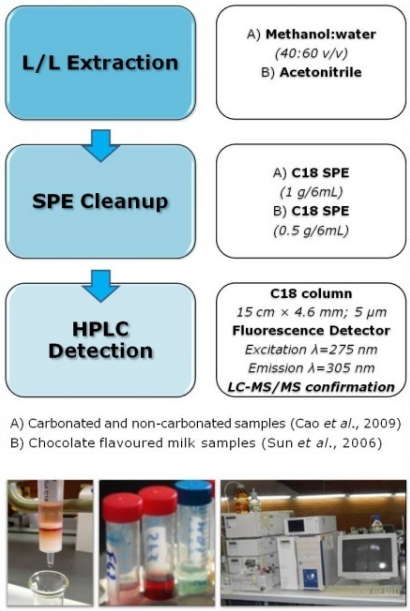


Fig. 1 - Skeletal formula of Bisphenol A

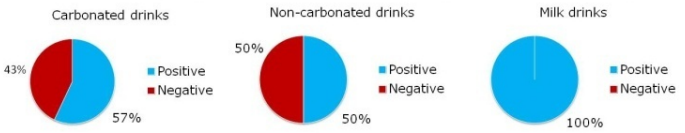
MATERIALS AND METHODS



A) Carbonated and non-carbonated samples (Cao *et al.*, 2009)
B) Chocolate flavoured milk samples (Sun *et al.*, 2006)

RESULTS

Fig. 2 - Percentage of positive samples (Total n=72) (Bisphenol A levels above LOD)



Tab.1 - Median BPA levels in analysed samples

Drink	Packaging	BPA concentration (µg/L) Median (Range)
Carbonated	Can	1.48 (<LOD – 4.98)
	Non-Can	0.18 (<LOD – 1.78)
Non-carbonated	Can	0.80 (<LOD – 2.79)
	Non-Can	0.18 (<LOD – 3.58)
Milk drinks	Non-Can	3.60 (1.00 – 17.65)

Tab.2 - Potential dietary exposure to BPA for children through consumption of sugary drinks and incidence on EFSA t-TDI (5 µg/kg bw/day) (EFSA, 2014).

Daily sugary beverage consumption	Dietary exposure estimate (µg/kg bw/day) Median	Dietary exposure estimate (µg/kg bw/day) Maximum	Incidence on EFSA t-TDI (%) Median	Incidence on EFSA t-TDI (%) Maximum
100% of daily liquid consumption[1]	0.024	1.765	0.47	35.30
50% of daily liquid consumption[1]	0.016	0.883	0.32	17.65
25% of daily liquid consumption[1]	0.008	0.530	0.16	10.59

[1] Recommended liquid intake per day equal to 0.03 L/kg body weight according to WHO (2009).

Canned drinks showed higher BPA concentrations than non-canned drinks (Tab.1). Milk drinks, packed in PET bottles, showed the highest BPA levels. The median levels of exposure in children are lower than the temporary Total Daily Intake established by EFSA of 5 µg/kg body weight/day (Tab.2).

DISCUSSION

The higher levels of BPA detected in canned samples could be due to the migration of BPA from the internal lining of aluminum cans as showed also by Cao *et al.* (2009). The unexpected result occurred in milk samples suggests that BPA could be an ubiquitous pollutant and could come in contact with food also during the production phases and not just after bottling and that fat content of milk could be a factor that facilitates migration of BPA. About exposure results, it must be taken into account that only the influence of sugary beverages in the diet was evaluated in this first activity, while the t-TDI issued by EFSA considers also other sources of exposure such as solid food ingestion. Hence, future works will be devoted to the evaluation of other food matrices, intended to susceptible population, in order to better ascertain the total daily intake through diet.

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XIX WORKSHOP
ON THE DEVELOPMENTS IN THE ITALIAN
PHD RESEARCH ON FOOD SCIENCE
TECHNOLOGY AND BIOTECHNOLOGY

Bari, September 24th-26th, 2014

Abstract #2. Presenza di distruttori endocrini quali Bisfenolo A e Ftalati nei latti formulati della prima infanzia ed esposizione neonatale a tali sostanze

Cirillo T., Fasano E., **Esposito F.** and Scognamiglio G.

47° Congresso Nazionale Siti - 1- 4 ottobre 2014, Palazzo dei Congressi - Riccione (RN) – Italy.

Abstract

INTRODUZIONE: La protezione dei bambini dall'esposizione involontaria ai contaminanti, in particolare ai distruttori endocrini è riconosciuta come un'importante priorità di sanità pubblica. Tra i distruttori endocrini il Bisfenolo A (BPA) e gli Ftalati sono quelli maggiormente investigati. Il BPA è un composto chimico estrogenico prodotto in tutto il mondo e impiegato nella fabbricazione di materiali come i policarbonati, le resine epossidiche anticorrosione, il PVC, destinati anche a contenere alimenti. Studi tossicologici hanno evidenziato che il BPA può indurre effetti avversi sul cervello, sul comportamento e sull'apparato riproduttore di feti, neonati, bambini e donne in età prepuberale. Gli ftalati sono un gruppo di sostanze multifunzionali utilizzate in una vasta gamma di prodotti come solventi, additivi e particolarmente plastificanti per conferire flessibilità e longevità alle materie plastiche ed in particolare al cloruro di polivinile. Tra questi i più utilizzati sono il di-2-(etilesil)ftalato (DEHP), il dibutilftalato (DBP) che sembrano possedere un potenziale effetto dannoso, in particolare sull'apparato riproduttivo, oltre ad effetti cancerogeni, genotossici, neurologici e sull'apparato respiratorio. Le potenziali vie di esposizione per tutti e tre i contaminanti considerati, sono l'inalazione, l'iniezione intravenosa, l'assorbimento attraverso la pelle e l'ingestione, che rappresenta la via principale. La presenza di BPA, DBP e DEHP negli alimenti trae origine prevalentemente dalla contaminazione ambientale, dal contatto con materiali che li contengono nelle fasi di trasformazione e dagli imballaggi. La "European Food Safety Autority" (EFSA) ha fissato per il BPA un temporary Tolerable Daily Intake (tTDI) di 5 µg/kg peso corporeo (pc)/giorno, mentre per il DBP un TDI di 0.01 mg/kg pc/giorno e per il DEHP di 0.05 mg/kg pc/giorno. Dai dati di letteratura emerge che la fascia di popolazione maggiormente esposta al BPA, al DBP ad al DEHP risulta essere quella infantile ed in particolare neonatale (0-3 mesi). L'obiettivo del nostro lavoro è stato quello di valutare la presenza di BPA, DBP e DEHP nei latti formulati per la prima infanzia e valutare l'eventuale esposizione dei bambini mediante la via alimentare. MATERIALI E METODI: Sono stati raccolti 50 campioni di latte artificiale, sia liquido che in polvere, destinato a lattanti. I latti sono stati campionati nel corso di 4 mesi in 2 diversi reparti di neonatologia di ospedali napoletani ed altri campioni sono stati acquistati presso farmacie della provincia di Napoli. Il latte campionato era confezionato in contenitori differenti che variavano da barattoli in alluminio, contenitori in polietilenterenftalato (PET) e Tetrapak™. Il metodo applicato per la ricerca del BPA era caratterizzato da una fase di estrazione della frazione lipidica mediante acetonitrile, lavaggio degli estratti con esano, purificazione del campione mediante cartucce per SPE (C18 da 1g/6 mL Blond Elut) e dosaggio tramite HPLC con rivelatore fluorimetrico. I campioni sono stati poi confermati mediante HPLC/MS MS. La metodica utilizzata per la ricerca del DBP e del DEHP ha previsto un'estrazione della fase lipidica mediante acetonitrile, purificazione su colonna cromatografica contenente Florisil, Bondesil e Na2SO4 e successivo dosaggio mediante GC-FID. RISULTATI: I risultati ottenuti hanno mostrato una diffusa contaminazione da BPA, DBP e DEHP nei campioni di latte in formula per lattanti. Il 58% dei campioni di latte hanno presentato valori rilevabili di BPA. Per quanto riguarda il DEHP e DBP, il 90% dei campioni di latte, hanno mostrato valori rilevabili di DBP e nel 92% dei campioni di latte DEHP è stato trovato a livelli rilevabili. I valori di concentrazione del BPA sono variati da 0.003 al peso secco 0.375 di µg/g (dw) (mediana 0.015 µ g/g dw), le concentrazioni di DBP sono variate da 0.008 a 1.297 µ g/g dw (mediana 0.053 µ g/g dw) mentre i valori di concentrazione di DEHP sono oscillati da un minimo di 0.005 ad un massimo di 5.088 µg/g dw (mediana 0.906 µg/g dw). Le stime dell'esposizione al BPA mediante la dieta mediamente hanno mostrato valori da 0.14 a 0.17 µg/kg peso corporeo (pc) giorno per neonati al 50° percentile e da 0.12 a 0.15 µg/kg pc giorno per neonati al 97° percentile. Per il DBP le stime sono state mediamente tra il 4.15 µg/kg di peso corporeo al giorno e 5.34 µg/kg pc giorno per neonati al 50° percentile e da 3.79 µg/kg di peso corporeo al giorno e 4.54 µg/kg pc giorno per neonati al 97° percentile. L'esposizione media al DEHP è andata da 19.84 a 24.85 µg / kg pc giorno per i bambini al 50° percentile e da 17.63 a 19.14 µg/kg pc al giorno al 97° percentile. I livelli di esposizione dei neonati a tutte e tre i contaminanti indagati mediamente non superano il TDI stabilito dall'EFSA né al 50° né al 97° percentile. CONCLUSIONI: I risultati ottenuti hanno mostrato una diffusa contaminazione da BPA, DBP e DEHP nei campioni di latte in formula per lattanti. Tale contaminazione sembra essere di natura ambientale o di processo, piuttosto che correlata ad una migrazione dal packaging. Per quanto riguarda l'esposizione ai tre contaminanti indagati, anche se mediamente i dati non superano i limiti imposti dall'EFSA, ma incidono anche al 50% sul TDI. Considerando che si tratta di una fascia di popolazione sensibile, su cui gli effetti tossici dei distruttori endocrini potrebbero rappresentare un rischio per tale popolazione, sarebbe necessario controllare la problematica al fine di aumentare la protezione della salute dei piccoli consumatori, in linea con quanto stabilito dall' Organizzazione mondiale della sanità (OMS). Infatti, l'OMS ha sottolineato la necessità di attuare impegni di protezione infantile a tali contaminanti evidenziando che, rispetto agli adulti, i bambini, per i loro aspetti fisiologici e di sviluppo, mostrano una maggiore suscettibilità e vulnerabilità agli effetti tossici degli inquinanti ambientali.

Abstract #3. Ricerca e dosaggio delle fumonisine in alimenti destinati a bambini affetti da morbo celiaco e valutazione della loro esposizione per via alimentare

Cirillo T., Scognamiglio G., **Esposito F.** and Fasano E. *48° Congresso Nazionale Siti - 14- 17 ottobre 2015, Palazzo dei Congressi - Milano – Italy.*

Abstract

Introduzione: Le fumonisine sono metaboliti secondari di alcune specie fungine appartenenti al genere Fusarium, in grado di attaccare vari vegetali, tra cui il mais. Le forme B1 e B2 di queste tossine sono state classificate dallo IARC come probabili cancerogeni. La normativa attuale, infatti, prevede limiti restrittivi circa la presenza di questi composti negli alimenti, il TDI fissato dall'EFSA è pari a 2000 ng/kg peso corporeo al giorno. Gli alimenti destinati ai celiaci contengono in prevalenza mais, pertanto il consumo di tali prodotti potrebbe rappresentare un pericolo in termini di esposizione giornaliera alle fumonisine. Pertanto, obiettivo del seguente studio è stato quello di valutare la quantità di fumonisine B1 e B2 all'interno di prodotti alimentari gluten-free, al fine di stimare l'esposizione dei bambini celiaci a tali composti, attraverso il consumo di tali prodotti.

Metodi: 80 prodotti destinati a consumatori celiaci sono stati analizzati in accordo col metodo ufficiale UNI EN 14352:2005, che prevede estrazione, purificazione attraverso colonne di immunoaffinità e dosaggio all'HPLC-FLD. È stata, infine, effettuata una stima dell'esposizione, considerando sia i consumatori medi che al 95° percentile e valutando l'esposizione con i valori mediani di concentrazione e con gli estremi superiori, distinguendo quindi un medium-case ed un worst-case.

Risultati: I risultati mostrano una diffusa contaminazione da fumonisine nei prodotti alimentari e l'esposizione nel consumatore medio e in quello al 95° percentile non supera il TDI, considerando valori di concentrazione mediana. Tuttavia, se si considera il worst-case al 95° percentile, l'esposizione può superare di circa il 20% il TDI EFSA. Conclusioni: Il superamento del TDI nel worst-case suggerisce che per le fumonisine potrebbe essere opportuno prevedere limiti più restrittivi per i prodotti destinati a celiaci.