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DETERMINATION OF ENVIRONMENTAL CONTAMINANTS IN FOOD: MONITORING AND RISK EVALUATION FOR CONSUMERS' HEALTH

ILARIA DI MARCO PISCIOTTANO

PhD TUTOR

PROF. STEFANIA ALBRIZIO

PhD COORDINATOR:

PROF. ANGELO A. IZZO

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1. INTRODUCTION

1.1. Environmental contaminants

Environmental contaminants are chemicals produced naturally or by human activity which can adversely affect the environment, thus being toxic to the human, animal, and plant health. These contaminants include solid, liquid, and gaseous substances that are accidentally or intentionally released in the environment. The sources of environmental contamination are several and the pollutants include several different kind of compounds and materials. For example, among them we can count the plastics, wood, textile, preservatives, microelectronics, and refineries resulting from industrial activity or the fertilizers and pesticides deriving from agriculture activities. The mining activities and the waste disposal are other human sources of environmental pollution but, as said before, there are also naturally occurring contaminants that can result from volcanic eruptions, sea-salt sprays, forest fires, rock weathering and others natural events. Anyway, sometimes industrial activities can increase the mobility or the amount of these natural contaminants originating a synergistic effect that increases the levels and the widespread of all the pollutants, thus posing a health risk for all the living beings. In fact, these environmental contaminants can easily enter the food chain because of the food being grown, raised or processed in contaminated soil, water or air.

Some of the naturally occurring environmental contaminants whose levels can be increased by human activities are the heavy metals, the perchlorate, and the radionuclides, whereas the human-made chemicals most widespread into the environment are compounds like benzene, dioxins and polychlorinated biphenyls (PCBs), and Per- and Polyfluoroalkyl Substances (PFAS).

Furthermore, in the last decade, a lot of emerging environmental contaminants have been added to the list because of the development of new highly sensitive analytical techniques allowing to detect the chemicals even at very low levels (Puri et al., 2023; Richardson & Kimura, 2017). These emerging contaminants include also pharmacologically active substances, illicit drugs, antibacterial, hormones, microplastics, algal toxins and Endocrine Disrupting Chemicals (EDCs).

My PhD project had the aim to monitor the presence of some of the over 3000 environmental contaminants in food and to subsequently evaluate the possible risk for consumers' health. Three groups of environmental contaminants of both human and natural origin were chosen, that is EDCs, heavy metals and microcystins from cyanobacteria. The EDCs are a group of compounds quite ubiquitous, that have been found even in the indoor dust, air and in the freshwater (Caban & Stepnowski, 2020; Dueñas-Mas et al., 2019; Wu et al., 2018; J. Zhang et al., 2020). The attention paid to these emerging contaminants is always growing and because of the lack of contamination data, it hasn't been possible yet to perform a thorough evaluation of the risk for consumers health. Heavy metals, on the other hand, are well known contaminants, whose presence has been carefully investigated in a lot of different matrices through the years (Arjomandi & Shirkhanloo, 2019; Mohod et al., 2013; Rai et al., 2019; Soylak & Aydin, 2011; Suvarapu & Baek, 2016). However, only in the last years the monitoring of these compound in food supplements has been intensified, also focusing on the content of essential nutrients, such as cobalt, selenium, magnesium and zinc, that are toxic when ingested in great amount and in some chemical forms. Lastly, microcystins from cyanobacteria could represent an issue for human health when the cultivation method in natural water is not performed with appropriate quality control, thus leading to the presence of toxic cyanobacteria in widely sold food supplements (such as Spirulina) (Cong et al., 2006; Manali et al., 2016; Papadimitriou et al., 2021; Vichi et al., 2012). Since the last decades, the analytical technique of choice for the determination of the environmental contaminants has always been the liquid chromatography coupled to both low and high-resolution mass spectrometry (Moscoso-Ruiz et al. 2022; Sreedhashyam et al. 2022; Shaaban et al. 2022; Chen et al. 2022; He et al. 2022; Van Hassel et al. 2022). That is because of the high sensitivity and selectivity of the detector that allows to determine very low concentrations of these small molecules and in a very unambiguous way. However, there is still a lack of analytical methods and, as a consequence, of contamination data regarding some EDCs like the bisphenols into different commodities, since the past monitoring activities have been focused mainly on the bisphenol A. Furthermore, the analytical methods from the scientific literature for the

determination of the microcystins are limited to only some of the most toxic ones and water has always been the matrix of choice for the monitoring activities (Massey et al. 2020; Kumar et al. 2020).

During the first year of the PhD project, the following activities were carried out: optimization of the cleanup of heavy metals, microcystins and EDCs from different commodities and food supplements; development of analytical methods to determine these contaminants in the investigated matrices using liquid chromatography coupled to tandem mass spectrometry and high-resolution mass spectrometry, inductively coupled plasma mass spectrometry and direct mercury analysis; in-house validation of the analytical methods in accordance with the European Regulations in force. During the second and third years of the PhD trajectory, all the samples for the monitoring activity were collected and analyzed to assess the contamination levels of the above-mentioned environmental contaminants; the most relevant contamination data were then used to evaluate the health risk for the consumers due to the intake of the analyzed products. Furthermore, the six-month period abroad was spent at the Wageningen Food Safety Research in the Wageningen University (The Netherlands) to participate in a pioneering study for production and identification of plant metabolites produced by algal cultures of different kind of contaminants, such as veterinary drugs and pesticides.

The challenges related to the determination of the chosen contaminants in food and food supplements are indeed several, mostly due to the their widespread in the environment and the wide panel of matrices that can be investigated. The first challenge to face was to optimize the purification of the contaminants from matrices that were all different in ingredients and main composition. To that aim, different cleanup procedures were evaluated, in terms of percentage recovery of spiked samples, to select the one leading to the cleanest extract and the highest yield. In fact, if a cleaner extract means less matrix effect during the analysis of the samples, in particular when it comes to mass spectrometry detection using an electrospray (ESI) source, a high yield of the cleanup step allows to go down with the limits of quantification/detection of the method. In the frame of some environmental contaminants, such as EDCs and microcystins, it is desirable to also detect small concentrations because of the low-

dose effects and the high toxicity they have in living organisms. The second part of the PhD project was the monitoring of the contaminants in several commodities and also in the production cycle of buffalo milk as it was done for the EDCs. During this step, a specific attention was put in assuring and assessing the quality of the process during each working session, in terms of trueness, precision and selectivity of the analytical method and, as a consequence, the reliability of the results. Regarding the analysis of the EDCs in the buffalo farms, for example, no plastic materials were used during both the sampling and the analysis, to accurately avoid any contamination that was not deriving from the normal production conditions. Furthermore, all the laboratory glassware used for the determination of the heavy metals were cleaned with appropriate solvents, after every working session, to remove the possible traces thus preventing cross-contamination between samples. The work carried out during the three years of the PhD project was published in scientific articles led by the PhD candidate and reported in this manuscript; all the details about the PhD activities are thoroughly described in the following chapters.

1.2. Endocrine Disruptors

Bisphenol A (BPA) is an acknowledged EDC widely employed in industrial production, such as polycarbonate plastics, epoxy-resin linings of food and beverage metal cans, medical equipment, electronics, thermal paper, water pipes and toys. Its extensive use resulted in widespread occurrence of BPA both in the environment and in food and beverages (Almeida et al., 2018; Cirillo et al., 2019; Di Marco Pisciottano et al., 2020; Gallo et al., 2017, 2019; Manzoor et al., 2022), mainly through migration from packaging. Nevertheless, there are also human exposure routes for BPA and EDCs in general other than food, such as occupational exposure, inhalation, and dermal absorption from the environment (Bousoumah et al., 2021; S. S. Lee et al., 2022; Niu et al., 2021; Wu et al., 2018). The concern due to the consumers' exposure to BPA and to the possible adverse effects on human and animal health, led many countries to either restrict or ban its use. Overseas, both the Canadian Government and the Food and Drug Administration (FDA) banned the use of BPA in plastic

drinkware for babies and children (FDA (Food and Drug Administration), 2012; Government of Canada., 2010). The European Commission (EU) has restricted the use of BPA in plastics for infant feeding since 2011 (European Commission, 2011, 2018), and in 2015 France has forbidden BPA in all packaging, containers and utensils intended to be directly in contact with food (JORF, 2015). Also, in 2015 the European Food Safety Authority (EFSA) published a scientific opinion about the risk for human health due to BPA occurrence in foodstuffs (Bolognesi et al., 2015) stating that the levels of dietary exposure to BPA are not a concern for consumers. Nevertheless, EFSA reduced the temporary Tolerable Daily Intake (t-TDI) from 50 to 4 μg/kg bw/day, and recently the European Commission lowered the specific migration limit of BPA to 0.05 mg/kg food (European Commission, 2018). In 2016, the European Commission mandated EFSA to re-evaluate the risks to public health from the presence of BPA in foodstuffs and to establish a tolerable daily intake (TDI). Taking into consideration the evidence from animal data and support from human observational studies, the CEP panel identified the immune system as the most sensitive to BPA exposure. A reference point (RP) of 8.2 ng/kg bw per day, expressed as human equivalent dose, was identified for the critical effect and, after several evaluations, the CEP Panel applied an overall Uncertainty Factor of 50 to the RP, establishing a new TDI of 0.2 ng BPA/kg bw per day (Lambré et al., 2023), that is 20000 times lower than the t-TDI set in 2015. After a comparison of this new TDI with the dietary exposure estimates from the 2015 EFSA opinion, the CEP Panel concluded that there is a health concern from dietary BPA exposure. In fact, the comparison showed that both the mean and the 95th percentile dietary exposures in all age groups exceeded the new TDI by two to three orders of magnitude. Anyway, also before this EFSA opinion on the BPA and following the restrictions set by several Countries to the BPA use in the manufacturing, several other bisphenols have been proposed as BPA substitutes in the industrial production. These compounds, generally identified as bisphenols (BPs), share physico-chemical properties with BPA, presenting different substitution on the aromatic rings of the molecule. Among the wide number of BPA analogues, the most used in industrial production are bisphenol F (BPF), bisphenol S (BPS), bisphenol AF (BPAF), bisphenol AP (BPAP), bisphenol B (BPB), bisphenol Z

(BPZ) (Česen et al., 2018; Lian et al., 2020; H. Wang et al., 2020; J. Wang et al., 2020; R. Wang, Huang, et al., 2021). These compounds have already been worldwide detected in environmental, biological and food samples, such as in indoor dust (Fan et al., 2021), sediment and sludge (Šauer et al., 2021), human urine (Lucarini et al., 2020), blood serum (Li et al., 2020), breast milk (Deceuninck et al., 2015; Niu et al., 2017), drinking water (H. Wang et al., 2020; J. Wang et al., 2020; H. Zhang et al., 2019), food and beverages (Cunha et al., 2020; Liao & Kannan, 2013, 2014; van Leeuwen et al., 2019; Y. Yang et al., 2014). The acknowledged ubiquitous occurrence and the BPA-related structures of these BPs required an evaluation of their toxicological profile and activity as EDCs. Furthermore, EDCs can transfer from the polluted environment to the raw materials used as food ingredients, or from contaminated soil, water, or atmospheric deposition from nearby industrial activity (Careghini et al., 2015; Michałowicz, 2014). This way, animals fed with contaminated feed can bioaccumulate EDCs in their adipose tissue, because of their good lipophilicity, and then can eliminate them through major excretion routes, including milk (Q. Wang et al., 2017, 2020; Zhao et al., 2019). Whereas the presence of BPA and other bisphenols in breast milk has been widely studied, there are scarce data about bisphenol contamination in bovine milk (Grumetto et al., 2013; Mercogliano et al., 2021; Santonicola et al., 2019, 2021), and no data at all about buffalo milk. Moreover, even though some researchers have studied bisphenol concentrations in feed (R. Wang, Dong, et al., 2021; R. Wang, Huang, et al., 2021; R. Wang, Tan, et al., 2021; Xiong et al., 2020), none have simultaneously analyzed bovine milk, sera, and drinking water from the same farms.

In recent years, many studies have been carried out, proving that other BPs, apart from BPA, exhibited estrogenic and androgenic activity. A lot of these compounds acted as pure agonists, antagonists, or mixed agonists/antagonists towards estrogen receptor α (Q. Chen et al., 2020) whereas some showed anti-androgen, anti-progestagenic and anti-thyroid activity (Cavaliere et al., 2020; Grimaldi et al., 2019; Šauer et al., 2021). An increasing number of studies have been carried out on BP toxicity and a variety of correlations between their occurrence and related detrimental effects on human health

have been found. For example, BPS, BPF and BPAF showed a role as breast cancer promoter, by interfering with several regulation pathways (Catenza et al., 2021); a strong relationship has been found between BPAF serum levels and thyroid cancer in human beings (Marotta et al., 2019). Also, BPS and BPAF have been associated with gestational diabetes mellitus (Duan et al., 2018; W. Zhang et al., 2019) and urinary BPS has been correlated to waist circumference and abdominal obesity (Y. Zhang et al., n.d., 2019). Furthermore, urinary BPF has been positively associated with asthma in adults, whereas BPS has been correlated only with asthma in men (Mendy et al., 2020). These adverse effects on human health and the increasing interest about BPs contamination levels, require more information about the exposure through the diet, that is the major intake route. The monitoring of the contamination levels of BPA and its analogues in food, beverages, and even in feedingstuff for food producing animals will improve the knowledge of the phenomenon and the risk assessment by the EFSA.

1.3. Heavy metals

Heavy metals are naturally occurring elements that have a high atomic weight and a density at least 5 times greater than that of water. Since the Earth's crust is rich in heavy metals, they can be often found in soil, rocks, sediment, and water, thus making them potential contaminants for the food and feed too (Fayiga & Saha, 2016; Nkwunonwo et al., 2020; Rai et al., 2019). In addition to that, anthropogenic activities can significantly increase the natural levels of the heavy metals into the environment (Santucci et al., 2018), thus posing a health risk to humans and all the living organisms. In fact, although their different chemical properties, they have multiple industrial, domestic, agricultural, medical, and technological applications, resulting in a wide distribution in the environment. Because of them being elements, they cannot be broken down and they will stay persistently into the environment, tending to accumulate especially in freshwater and sea sediments and then to be transported from one environment compartment to another. Some heavy metals are toxic for organisms even at low concentrations, but others are essential elements and their levels

inside the body are usually controlled by homeostasis in a way that fulfill the nutritional demand. On the other hand, for the toxic heavy metals several factors affect their toxicity, such as the dose, route of intake, chemical species, bioavailability, but we can consider arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg) to be the most toxic ones. In this work, the attention has been focused on Cd, Pb and Hg.

The cadmium is a toxic element and, when it enters human body, it is accumulated mainly in the gut (Tinkov, Gritsenko, et al., 2018) and in the kidneys (Satarug, 2018). The cadmium has toxic effects on different organs and can cause, among the other things, pulmonary edema, renal and hepatic dysfunction and it can damage the adrenals and hemopoietic system (Tinkov, Filippini, et al., 2018). It has also been proved to be a carcinogenic belonging to the group I of the International Agency for Research on Cancer classification (Humans, 1993; Tinkov, Filippini, et al., 2018). The accumulation of the cadmium in the food chain due to the uptake from contaminated soil and water, has already been reported by the World Health Organization (Organization, 2019) and it is also well known that the food is one of the major human intake source other than from smoking cigarettes (Zou et al., 2021).

The lead is another toxic element whose spread is mainly caused by industrial emissions, soils, car exhaust gases and contaminated foods (Alloway, 2012; Mielke et al., 2010; Rebezov et al., 2020). For example, when grown in the proximity of lead sources, some large leaf vegetables, such as spinach and cabbage, can contain very high levels of lead (Gama et al., 2006). The target organ for the toxicity of lead is the skeleton, and in particular the bone marrow; it can act as a neurotoxin and cause retards in intelligence and mental development and behavioural abnormalities (Needleman & Bellinger, 2001). In addition, lead can interfere with the metabolism of calcium and vitamin D and can cause anemia by affecting the formation of the hemoglobin (Memon et al., 2005).

Lastly, the mercury, is a very rare element in the Earth's crust and it has no known physiological role in humans. It exists in three different forms, each one with a different kind of toxicity: elemental mercury (valence state = 0), inorganic mercury (valence state = I or II) and organic mercury such as methylmercury and ethylmercury. The pharmacokinetics and the biological behavior vary basing on the chemical forms of the mercury. Also, the toxicity in humans is strongly dependent on different factors, such as the rate of exposure, the dose, and the chemical form itself. For example, if the mercury enters the body by inhalation, it is mainly accumulated in the brain, while in the salt form it is primarily damaging the kidneys and the gut (Berlin & Ullberg, 1963). On the other hand, the organic forms, such as the methylmercury, are evenly distributed in all the body without a particular target organ (Berlin & Ullberg, 1963). Also, it has been widely reported that mercury is responsible of neurodegenerative effects, including Alzheimer's disease and amyotrophic lateral sclerosis (Carocci et al., 2014). The human intake of mercury through the food chain, together with lead and cadmium, has been a public concern since a long time (Galal-Gorchev, 1993). Furthermore, because of its tendency to contaminate preferentially sea sediments and to bioaccumulate in the marine food chain, the mercury intake through fish and marine mammals has always been more investigated (Nogara et al., 2019; Renzoni et al., 1998).

In conclusion, it is evident that the levels of heavy metals, in particular the toxic ones, should be monitored in the food to preserve the consumers' health and to monitor the food intake exposure to humans, in particular to newborns and children because of their susceptibility (M. J. Lee et al., 2018; Zheng et al., 2023). Moreover, maximum limits for heavy metals in infant, follow-on and young-child formulae have been set by the European Commission since 2006 with the Commission Regulation (EC) No 1881/2006 (European Commission 2006), recently repealed by the more detailed Commission Regulation (EU) 2023/915 (European Commission 2023).

1.4. Microcystins

Cyanobacteria, improperly called blue-green algae, are photosynthetic organisms that are widely present in natural water sources both marine and freshwater (Codd et al., 2005; Huisman et al., 2018; Merel et al., 2013; Sivonen & Jones, 2021). In the last years, the climate change has played a big role into increasing these cyanobacteria blooms, both in terms of frequency and of intensity (Howard et al., 2017; Mantzouki et al., 2018; C. Zhang et al., 2019). Even if most of the cyanobacteria are considered innocuous and edible, some of them produce cyanotoxins, that is secondary metabolites harmful to humans (Paerl & Otten, 2013). The genera *Anabaena, Cylindrospermopsin, Lyngbya, Microcystis, Nodularia, Nostoc* and *Oscillatoria* (*Planktothrix*) are some of the more than 35 genera responsible of the production of cyanotoxins (Van Apeldoorn et al., 2007) and of microcystins (Khomutovska et al., 2020; Mantzouki et al., 2018; Massey & Yang, 2020). Microcystins (MCs) are heptapeptide compounds relatively stable in natural environments thank to their cyclic structure; in fact, they are very resistant to physical and chemical changes, such as extreme temperatures, variations of pH, exposition to the sunlight and enzymatic degradation (Harada et al., 1996; Rastogi et al., 2014; Tsuji et al., 1994). During the years, a great number of scientific studies reported the toxicity of these compounds on humans and animals, and they are known to be hepatotoxic compounds (J. Chen et al., 2009; Shi et al., 2021). The general structure of microcystins (**Figure 1**) is cyclo-(-D-Ala-L-X-DisoMeAsp-L-Z-Adda-D-isoGlu-Mdha), where X and Z are highly variable amino acids, D-MeAsp is D-erythro-b-methylaspartic acid, Mdha is N-methyldehydroalanine and the Adda [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid] is a distinctive amino acid of the cyanobacteria (Massey et al., 2018; Wei et al., 2020; F. Yang et al., 2020).

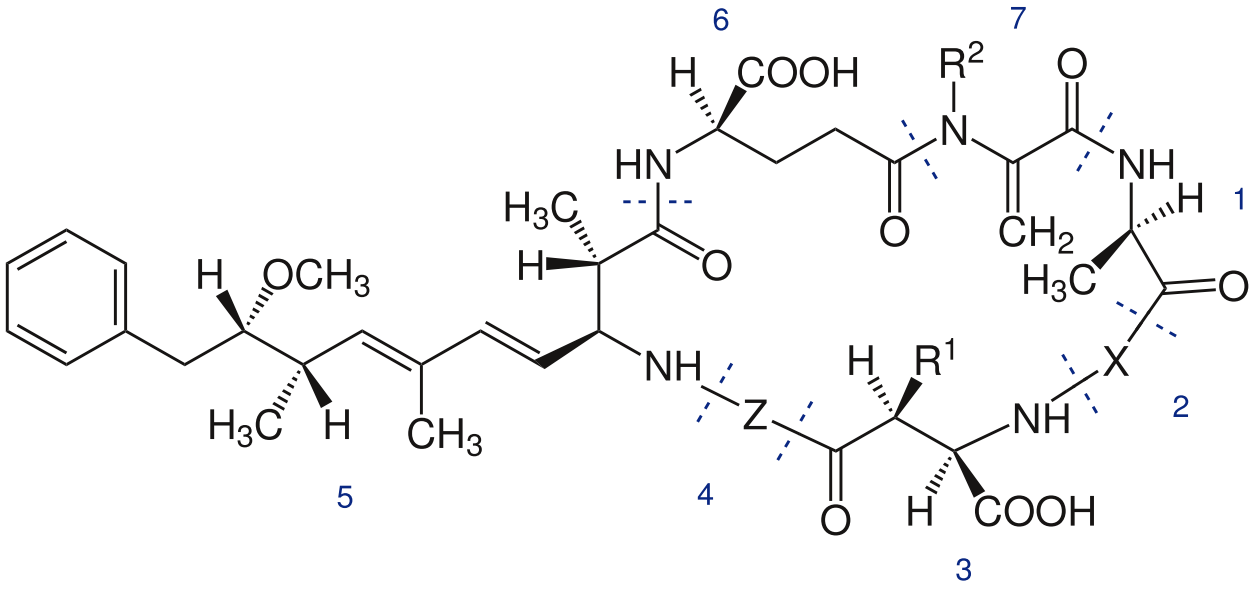


Figure 1. The cyclic heptapeptide structure of microcystins, with the variable amino acids X (2) and Z (4) and the characteristic ADDA amino acid (5).
Credit: Leyo, Public domain, via Wikimedia Commons

The amino acids present in the variable regions of the molecule are responsible for the name of the microcystin. For example, MC-LR, that is the most potent hepatotoxin among the known microcystins, has the amino acid leucine (L) in the X position and the amino acid arginine (R) in the Z position. Today more than 270 microcystin variants have been isolated during cyanobacteria algal blooms (Bouaïcha et al., 2019), including methylated and demethylated forms (Spoof & Catherine, 2016).

As said above, most of the cyanobacteria are considered edible and in the last years a wide number of food supplements containing cyanobacteria have been widely commercialized because of their putative beneficial effects for human health. In fact, it has become normal to buy in the supermarkets, pharmacies, herbalists, and online shops different forms of the food supplements containing blue-green algae (BGAS) in association with other nutrients such as vitamins, salts, minerals and amino acids. The most common claims found on these products are to improve the mood, to boost the energy levels, to have antioxidant and anti-cancer properties and to help in reducing body weight in obese persons (Hoseini et al., 2013; Khafaga & El-Sayed, 2018; Ku et al., 2013; Miczke et al., 2016; Persson

& Zakrisson, 2009; Zeinalian et al., 2017). The most used species in the production of BGAS are *Arthrospira maxima* and *platensis*, both commonly known as Spirulina, and *Aphanizomenon flos aquae*, commercialized as Klamath algae. These non-toxic cyanobacteria can coexist in the same environment with toxic strains thus leading to a contamination of the food supplement with the other organisms that are accidentally co-harvested (Grosshagauer et al., 2020). The presence of the hepatotoxic microcystins in food supplements containing cyanobacteria represents a risk for the consumers' health and the determination of these cyanotoxins should be implemented to protect the human health. At today, no maximum limits have been set by the European Commission for microcystins, except for the limit of 1.0 µg/L for MC-LR in water intended for human consumption, still to be measured only in the event of potential bloom in source water (European Commission 2020).

2. MATERIALS AND METHODS

2.1. Endocrine Disrupting Chemicals

In the frame of a research project funded by the Italian Ministry of Health (IZS ME 07-16 RC) I developed an analytical method to determine 17 bisphenols in bovine and buffalo milk and drinking water based on purification by molecularly imprinted polymers (MIPs) cartridges and determination by ultra high-performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (Di Marco Pisciottano, Albrizio, et al., 2022). The method was in-house validated according to the Regulation (EU) n. 2017/625 (European Commission, 2017) and the Regulation (EU) n. 2021/808 (European Commission, 2021). Then the method was applied to determine the contamination from bisphenols along the production cycle of buffalo milk collecting samples from 10 farms distributed in the Campania region and in 10 samples of retail bovine milk from Campanian markets (Di Marco Pisciottano, Guadagnuolo, et al., 2022).

In the frame of another research project funded by the Italian Ministry of Health (IZS ME 02-17 RC) I extended the validated method to the analysis of bisphenols in food supplements, verifying the analytical performance parameters in the new matrix by analyzing blank samples spiked at one concentration level and comparing the results with the validation ones. Afterwards, I applied the method to determine the bisphenols in 15 food supplements collected from the supermarkets of the Campania region to evaluate the contamination of bisphenols deriving from the different materials commonly used in the packaging of food supplements.

During a following research project (IZS ME 08-19 RC), always funded by the Italian Ministry of Health, the already validated method was extended to a new matrix, that is meat-based baby-food. In this case, the analytical performance on the new matrix were tested spiking blank samples at different concentration level and making sure that there was no statistically significant difference between the new matrices and the validation ones. Then the method was applied to the analysis of 40 samples of baby-food collected from Italian supermarkets.

2.1.1. Chemicals and Standard Reference Materials

The standard reference materials for the bisphenols (BPs) and internal standards (ISs) were supplied by Aldrich (Sigma-Aldrich, Milano, Italy), and were all analytical purity grade; the name, acronym, purity grade, CAS number, molecular weight and structure of each substance are shown in **Figure 2**. HPLC-grade methanol (MeOH), *n*-hexane (analytical purity grade) and glacial acetic acid (97% v/v, purity grade) were obtained from VWR International (Radnor, PA, USA). HPLC-grade acetonitrile (ACN) and ammonium acetate (analytical purity grade) were purchased from Carlo Erba (Milan, Italy). Ethylenediaminetetraacetic acid disodium salt (EDTA-Na2) 99% purity grade was from Bio-Rad Laboratories (Hercules, CA, USA). HPLC grade water was in-house produced using a Milli-Q laboratory system (Millipore, Bedford, MA, USA). The molecularly imprinted polymers AFFINIMIP® Bisphenols SPE cartridges in glass tubes were supplied by AFFINISEP (Normandy, France). The chromatographic column Kinetex Phenyl-Hexyl, 2.6 µm, 100 × 3.0 mm, the Security Guard Ultra Holder and cartridges with phenyl phase were supplied by Phenomenex (Torrance, CA, USA). To avoid any possible contamination deriving from plastic materials, only glassware, previously rinsed twice with both acetonitrile and methanol, was used during analysis.

2.1.2. Standard calibration curves and method validation levels

Individual standard stock solutions at 1.0 mg/mL were prepared dissolving the appropriate amount of each compound in 10.0 mL of methanol and stored in dark glass vials at -18°C until use. The purity grade, the chemical form and, in the case of the labelled internal standards, the relative percentage of isotope element (deuterium), were considered in the calculation of the amount to weight. A mix standard solution containing all the 17 bisphenols at 10.0 µg/mL and a mix standard solution at 10.0 µg/mL of BPA-d_{16}, and BPC2 were prepared in methanol every four months and stored in dark glass vials at -18°C until use.

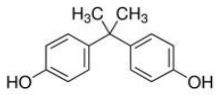
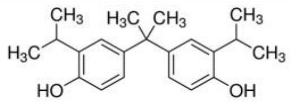
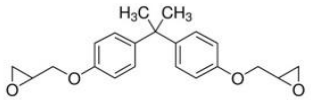
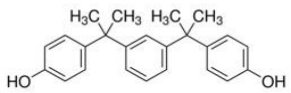
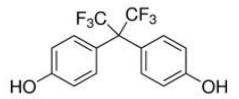
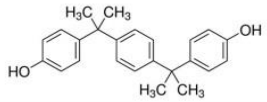
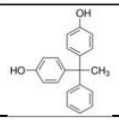
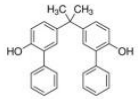
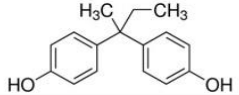
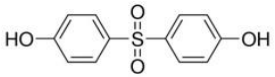
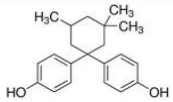
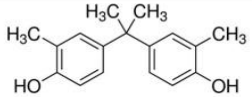
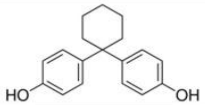
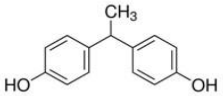
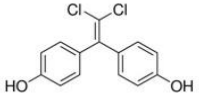
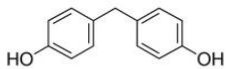
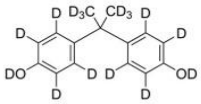
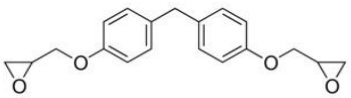
bisphenol A (BPA) 99.9% purity grade, CAS No. 80-05-7 MW: 228.29 g/mol		bisphenol G (BPG) 98.3% purity grade, CAS No. 127-54-8 MW: 312.45 g/mol	
bisphenol A diglycidyl ether (BADGE) 97.0% purity grade, CAS No. 1675-54-3 MW: 340.42 g/mol		bisphenol M (BPM) 99.9% purity grade, CAS No. 13595-25-0 MW: 346.46 g/mol	
bisphenol AF (BPAF) 100.0% purity grade, CAS No. 1478-61-1 MW: 336.23 g/mol		bisphenol P (BPP) 99.9% purity grade, CAS No. 2167-51-3 MW: 346.46 g/mol	
bisphenol AP (BPAP) 99.8% purity grade, CAS No. 1571-75-1 MW: 290.36 g/mol		bisphenol PH (BPPH) 99.6% purity grade, CAS No. 24038-68-4 MW: 380.48 g/mol	
bisphenol B (BPB) 98.4% purity grade, CAS No. 77-40-7 MW: 242.31 g/mol		bisphenol S (BPS) 99.0% purity grade, CAS No. 80-09-1 MW: 250.27 g/mol	
bisphenol BP (BPBP) 99.3% purity grade, CAS No. 1844-01-5 MW: 352.43 g/mol		bisphenol TMC (BPTMC) 99.1% purity grade, CAS No. 129188-99-4 MW: 310.43 g/mol	
bisphenol C (BPC) 99.6% purity grade, CAS No. 79-97-0 MW: 256.34 g/mol		bisphenol Z (BPZ) 99.4% purity grade, CAS No. 843-55-0 MW: 268.35 g/mol	
bisphenol E (BPE) 99.9% purity grade, CAS No. 2081-08-5 MW: 214.26 g/mol		bisphenol C2 (BPC2) 99.2% purity grade, CAS No. 14868-03-2 MW: 281.13 g/mol	
bisphenol F (BPF) 98.9% purity grade, CAS No. 620-92-8 MW: 200.23 g/mol		bisphenol A-d_{16} (BPA-d_{16}) 100.0% purity grade, CAS No. 96210-87-6 MW: 244.39 g/mol	
bisphenol F diglycidyl ether (BFDGE) 96.3% purity grade, CAS No. 2095-03-6 MW: 312.36 g/mol			

Figure 2. The name, acronym, purity grade, CAS number, molecular weight (MW) and structure of the 17 bisphenols (BPA, BADGE, BPAF, BPAP, BPB, BPBP, BPC, BPE, BPF, BFDGE, BPG, BPM, BPP, BPPH, BPS, BPTMC and BPZ) and 2 internal standards (BPC2, BPA-d16) used.
Credit: Di Marco Pisciottano, Albrizio, et al., 2022

The standard solutions for calibration curves were prepared at 0.1 – 0.25 - 0.5 – 1.0 – 2.5 – 5.0 – 10.0 – 50.0 ng/mL in methanol of all the tested BPs, all containing the internal standards at 10.0 ng/mL for all the matrices except for food supplements (100.0 ng/mL of internal standards). The water was the only matrix that did not require a thorough purification and the mean percentage recoveries were satisfactory for all the analytes, even without the use of internal standards. The method validation was carried out at 2.0 – 10.0 – 50.0 ng/mL spiking levels in drinking water, at 1.0 – 5.0 – 10.0 ng/mL of the BPs and at 2.0 ng/mL for internal standards for milk and milk-based baby-food and at 2.0 – 5.0 ng/g of the BPs, at 50.0 ng/g for the food supplements and at 5.0 ng/mL for meat-based baby-food.

All the intermediate standard solutions and calibration curve standards, specific for each matrix, were prepared daily from stock solutions.

2.1.3. Sample handling and cleanup

All the samples were collected, then stored in glass to avoid possible bisphenol contamination deriving from plastics. The drinking water was stored at T= 5°C ± 3°C for 3 days, whereas milk samples were kept at temperature ≤ -18°C until the analysis. The milk-based baby-food were stored at T < -18°C when liquid or at room temperature in a dry and dark place when in powder. The meat-based baby-food were stored at room temperature in a dark and dry place if sealed, and at T < -18°C after opening. The food supplements were stored at room temperature in a dry and dark place. Both the samples for the survey and samples spiked at the validation levels were cleaned up and analyzed following the procedure herein described.

The drinking water cleanup is rapid, consisting of a dilution of the sample with methanol, to allow the bisphenols to dissolve completely. The drinking water was warmed up to room temperature and mixed by inversion, then 1.0 mL of the sample was transferred in a glass tube and added with 1.0 mL of methanol. After mixing by vortex for 30 sec, the sample was transferred into an eppendorf tube and centrifuged at 13680 *g* at 4°C for 10 min. The supernatant was transferred in a glass vial for the UHPLC-MS/MS analysis (sample dilution factor = 2).

Milk samples were thawed at room temperature and thoroughly mixed by inversion to homogenize the lipidic and aqueous phases. Milk-based baby-food in powder were reconstituted weighing 1.00 ± 0.01 g of powder and dissolving it into 10.0 mL of water, then vortexed for 30 seconds to make the sample homogeneous. Afterwards, for all the milk-based samples 5.0 mL were transferred in a glass tube, added with 5.0 mL of acetonitrile. After mixing by vortex for 30 sec and centrifugation at 2246 *g* for 10 min, the supernatant was transferred in a clean glass tube, added with 10.0 mL of water, then mixed by vortex for 10 sec. Afterwards, the extract was purified by AFFINIMIP Bisphenols SPE cartridges, as described in the following subchapter.

The meat-based baby-food were homogenized manually by spatula, then, 2.00 ± 0.01 g of homogenized sample was weighted in a glass tube, added with 7.0 mL of Milli-Q water/acetonitrile solution 1/1 (v/v). The sample was mixed by vortex for 30 seconds, sonicated in an ultrasonic bath for 15 minutes, then centrifuged at 2246 *g* and 4°C for 15 min. The supernatant was transferred in a clean glass tube, added with 7.0 mL of Milli-Q water, and mixed by vortex for 30 sec. The solid residue was added again with 5.0 mL of acetonitrile, vortexed for 30 seconds, sonicated in an ultrasonic bath for 15 minutes and centrifuged at 2246 *g* and 4°C for 15 min. The supernatant was transferred in a clean glass tube, added with 15.0 mL of Milli-Q water, mixed by vortex for 30 sec and gathered with the previous extract. After mixing by vortex the total extract for 30 seconds, it was purified by AFFINIMIP Bisphenols SPE cartridges, as indicated in the following subchapter.

For the food supplement samples, 1.00 ± 0.01 g of homogenized sample was weighted in a glass tube, added with 100.0 µL of standard mix at 1.0 µg/mL of internal standards, 50.0 µL of 50 mM EDTA and 20 mM ammonium acetate solution, 1.0 mL of water and 9.0 mL of acetonitrile. The sample was mixed by vortex for 1 minute, centrifuged at 2246 *g* for 15 min, then the supernatant was transferred in a clean glass tube and added with 15.0 mL of water. After mixing by vortex for 30 seconds, the sample was purified by AFFINIMIP Bisphenols SPE cartridges, as indicated in the following subchapter.

2.1.4. Purification by AFFINIMIP Bisphenols SPE cartridges

The purification by the AFFINIMIP Bisphenols SPE cartridges is an additional step required in the cleanup of milk, milk-based and meat-based baby-food, because of the complexity of the matrix. These SPE cartridges are based on molecularly imprinted polymers selective for BPA that we have tested for its analogues, proving their efficacy also for compounds structurally related to BPA (Cirillo et al., 2019; Di Marco Pisciottano et al., 2020; Gallo et al., 2017). The use of the MIPs allowed us to eliminate all the interferences deriving from the matrix and even to concentrate the sample thus lowering the limits of quantification in this matrix. To evaluate possible interference from the cartridges and solvents used for the cleaning up step, a process and a reagent blank were also analyzed during each working session of validation. All the steps of the purification procedure were performed at atmospheric pressure, without applying vacuum, paying attention to never let the cartridges dry, if not indicated otherwise.

For milk, milk-based baby-food and food supplements the AFFINIMIP cartridge were conditioned with 3.0 mL of methanol containing 2% acetic acid, 3.0 mL of acetonitrile and 3.0 mL of Milli-Q water. The samples were loaded, and the cartridges washed with 10.0 mL of water and 6.0 mL of a water/acetonitrile 60/40 (v/v) solution. After the complete elution of the washing solvents, the cartridges were dried for 3 minutes applying a mild vacuum, to force out the solvent still adsorbed on the stationary phase. The BPs bound to the cartridge were eluted at atmospheric pressure by 3.0 mL of methanol followed by 3.0 mL of acetonitrile; after all the acetonitrile was eluted by gravity, the vacuum was applied for 1 minute. The two eluates were collected in a glass tube, evaporated to dryness under gentle nitrogen flow at room temperature, then the residue was dissolved in in 1.0 mL of water/methanol 50/50 (v/v) solution (sample dilution factor = 0.2). The sample was filtered on a 0.22 μm porosity PTFE filter, then transferred in a glass vial for the UHPLC-MS/MS analysis.

For meat-based baby-food, the AFFINIMIP cartridge were conditioned with 5.0 mL of methanol containing 2% acetic acid, 5.0 mL of acetonitrile and 5.0 mL of Milli-Q water. The samples were

loaded, and the cartridges washed with 10.0 mL of water and 6.0 mL of a water/acetonitrile 60/40 (v/v) solution. After the complete elution of the washing solvents, the cartridges were dried for 3 minutes applying a mild vacuum, to force out the solvent still adsorbed on the stationary phase. The BPs bound to the cartridge were eluted at atmospheric pressure by 3.0 mL of methanol followed by 3.0 mL of acetonitrile; after all the acetonitrile was eluted by gravity, the vacuum was applied for 1 minute. The two eluates were collected in a glass tube, evaporated to dryness under gentle nitrogen flow at room temperature, then the residue was dissolved in in 1.0 mL of water/methanol 50/50 (v/v) solution (sample dilution factor = 0.5) and transferred in a glass vial for the UHPLC-MS/MS analysis.

2.1.5. Method validation

The method was in-house validated according to the Regulation (EU) N. 2017/625 (European Commission, 2017) and to the Regulation (EU) 2021/808 (European Commission, 2021), evaluating the specificity, the trueness, the intermediate precision, the ruggedness for slight variations, the limits of quantification, the limits of detection and the linearity of the detector response for each compound, and the criteria for analyte unambiguous identification by mass spectrometry. Method specificity was assessed analyzing uncontaminated samples for each matrix and verifying that there were no interfering peaks both in the –MRM and in the +MRM chromatograms at the retention times of the analytes, considering a signal-to-noise ratio > 3/1. To evaluate method precision and trueness, at least 4 blank samples of each matrix were spiked at several concentration levels. The spiked samples were cleaned up and analyzed over different working sessions, performed during different days, to evaluate both the intermediate precision and the ruggedness of the test method. Moreover, to assess method ruggedness, further slight variations were introduced, such as different batches of solvents and reagents, different batches of standard stock solutions and different analysts. Method trueness, in terms of mean percentage recoveries, and intermediate precision, expressed as percentage relative standard deviations (RSD%), were calculated for each BP and in each matrix. The limit of quantification (LOQ) for each analyte was measured analyzing blank samples spiked at decreasing

concentrations, down to the lowest content showing the chromatographic peaks of both quantifier and quantifier ions, with a signal-to-noise ratio > 10/1 in respect to the baseline bias of a blank sample. The limit of detection (LOD) for each compound was calculated according to the equation: LOD = LOQ/3.3. The linearity of the detector response was assessed during each working session and for each compound, by verifying the determination coefficients (r2) to be greater than 0.95 for all the calibration curves. The analytical performance parameters resulting from the validation of the method are reported in **Table 1-3**. As said before, the method was first validated on milk and drinking water, therefore a full validation at three concentration levels was performed. When the meat-based baby-food and the food supplements were introduced, the method was not fully validated again, but just extended to the new matrices: the performance parameters obtained during the first validation were verified at one (food supplements) or two (meat-based baby-food) contamination levels.

Table 1. Method trueness, calculated as mean percentage recoveries at each spiking level for all the bisphenols, in milk and milk-based baby-food, in meat-based baby-food, in food supplements and in drinking water.

	Milk and milk-based baby-food			**Meat-based baby-food**		**Food supplements**	**Drinking water**		
Spiking levels	**1.0 ng/mL**	**5.0 ng/mL**	**10.0 ng/mL**	**2.0 ng/g**	**5.0 ng/g**	**50 ng/g**	**2.0 ng/mL**	**5.0 ng/mL**	**10.0 ng/mL**
BPA	110.9	102.9	102.3	73.9	84.9	82.6	94.2	97.4	108.7
BADGE	97.3	106.5	97.9	81.4	86.7	88.9	91.8	103.8	110.0
BPAF	101.8	107.2	103.2	95.6	94.9	81.0	94.9	111.9	105.7
BPAP	105.0	106.8	102.2	79.1	75.9	79.4	91.7	104.0	107.2
BPB	119.4	112.5	115.4	83.9	93.0	57.1	98.3	105.6	109.1
BPBP	80.9	107.2	78.4	57.3	53.9	53.8	100.0	106.7	107.3
BPC	108.9	112.9	112.2	71.8	85.4	62.2	101.1	99.9	109.1
BPE	105.6	85.4	95.6	96.8	98.3	98.4	89.0	105.3	107.5
BPF	85.5	77.0	78.6	63.9	80.8	71.5	94.8	108.0	108.8
BFDGE	92.9	98.8	92.8	64.4	99.2	108.7	104.0	105.0	103.1
BPG	98.9	89.2	98.3	71.6	81.3	58.0	105.5	106.7	105.5
BPM	93.2	100.0	99.9	69.2	90.4	81.3	97.5	94.2	104.8
BPP	81.2	85.3	84.9	51.2	54.5	92.9	105.5	96.4	97.8
BPPH	37.2	50.4	38.5	27.3	18.5	71.1	93.3	99.5	105.1
BPS	-	-	-	-	-	55.1	88.8	111.0	112.9
BPTMC	88.6	85.1	89.2	58.6	63.3	124.1	102.3	109.0	106.9
BPZ	89.9	105.0	93.7	66.6	68.9	83.5	102.5	98.1	102.7

Table 2. Method precision, as percentage relative standard deviation (RSD), at each spiking level for all the bisphenols, in milk and milk-based baby-food, in meat-based baby-food, in food supplements and in drinking water.

	Milk and milk-based baby-food			Meat-based baby-food		Food supplements	Drinking water		
Spiking levels	**1.0 ng/mL**	**5.0 ng/mL**	**10.0 ng/mL**	**2.0 ng/g**	**5.0 ng/g**	**50 ng/g**	**2.0 ng/mL**	**5.0 ng/mL**	**10.0 ng/mL**
BPA	3.8	9.1	7.4	4.2	11.4	1.7	12.4	7.1	14.2
BADGE	19.8	14.4	13.7	19.7	6.1	13.3	9.4	6.9	10.5
BPAF	11.5	8.3	12.3	13.7	10.2	5.2	12.9	9.3	8.4
BPAP	10.1	9.1	11.5	17.2	5.2	7.5	16.7	11.6	10.4
BPB	3.3	8.0	7.4	3.4	11.2	3.9	10.0	9.5	12.4
BPBP	10.1	9.1	6.6	5.0	6.7	11.8	17.0	9.1	11.2
BPC	14.7	11.4	6.6	22.7	7.0	10.1	10.0	16.2	11.6
BPE	9.2	9.3	7.2	5.5	2.5	12.1	10.4	10.7	12.0
BPF	9.5	5.9	6.2	21.2	9.0	9.2	15.7	10.5	11.9
BFDGE	15.7	9.8	12.9	16.0	16.2	8.4	14.7	10.0	9.3
BPG	15.2	11.6	18.5	4.4	14.9	12.1	8.2	10.4	11.2
BPM	18.0	19.6	15.9	13.1	6.9	16.4	13.1	14.6	11.3
BPP	11.7	10.4	9.7	6.5	23.2	18.1	13.7	10.0	18.6
BPPH	20.5	18.2	9.5	6.7	23.1	22.5	9.9	14.5	14.3
BPS	-	-	-	-	-	11.4	11.7	11.3	9.3
BPTMC	12.1	13.5	11.3	13.0	9.5	12.6	14.4	12.9	11.1
BPZ	12.3	12.1	12.7	20.2	13.1	9.2	11.1	12.0	12.1

Table 3. Limits of quantification (LOQ) and limits of detection (LOD) for all the bisphenols, in milk and milk-based baby-food, in meat-based baby-food, in food supplements and in drinking water.

	Milk and milk-based baby-food (ng/mL)		Meat-based baby-food (ng/g)		Food supplements (ng/g)		Drinking water (ng/mL)	
	LOQ	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**
BPA	0.5	0.15	1.0	0.3	1.0	0.3	0.1	0.03
BADGE	0.1	0.03	1.0	0.3	1.0	0.3	0.04	0.01
BPAF	0.5	0.15	1.0	0.3	1.0	0.3	0.01	0.003
BPAP	0.5	0.15	1.0	0.3	1.0	0.3	0.04	0.01
BPB	0.5	0.15	1.0	0.3	2.0	0.6	0.02	0.006
BPBP	0.5	0.15	2.0	0.6	2.0	0.6	0.02	0.006
BPC	1.0	0.3	1.0	0.3	5.0	1.5	1.0	0.3
BPE	0.5	0.15	1.0	0.3	2.0	0.6	0.2	0.06
BPF	0.5	0.15	2.0	0.6	5.0	1.5	0.25	0.08
BFDGE	0.5	0.15	2.0	0.6	1.0	0.3	0.1	0.03
BPG	0.5	0.15	1.0	0.3	5.0	1.5	0.07	0.02
BPM	0.1	0.03	2.0	0.6	1.0	0.3	0.01	0.003
BPP	0.5	0.15	2.0	0.6	2.0	0.6	0.1	0.03
BPPH	2.0	0.6	2.0	0.6	2.0	0.6	0.1	0.03
BPS	5.0 (CCβ)		5.0 (CCβ)		2.0	0.6	0.01	0.003
BPTMC	0.5	0.15	2.0	0.6	1.0	0.3	0.1	0.03
BPZ	0.5	0.15	2.0	0.6	2.0	0.6	0.1	0.03

Actually, no reference is available regarding the analytical performance of test methods to determine bisphenols in food; they can be considered both as environmental contaminants and industrial and process contaminants, therefore my considerations about the method performance are referred to the document EURL-MP-guidance doc_003 (version 1.1)- Guidance document on performance criteria (draft 17th September, 2021) from Wageningen (EURL, 2021). The mean recoveries of BPs, except for BPS and BPPH, are overall high or excellent in all the matrices, ranging from 51.5% to 124.1; RSD% values < 23 % are very good considering the matrix complexity. Thank to their excellent intensity in the mass spectrometry signals, BPS was detectable but not quantifiable, therefore for this EDC a detection capability (CCβ) of 5.0 ng/mL in milk and milk-based baby-food and of 5.0 ng/g in meat-based baby-food was established. Anyway, all the results were corrected for the percentage recovery of the spiked sample analyzed during each working session for quality control of the process; in this way the quantification always considers the trueness of the method for every single analyte.

2.1.6. UHPLC-MS/MS analysis

Analyses were performed using a UHPLC-MS/MS system, consisting of a UHPLC ExionLC™ AD series chromatograph (Sciex, Framingham, MA, USA), equipped with a binary pump, a column oven and an autosampler with temperature control, coupled to a QTRAP 6500^{+} mass spectrometer (Sciex). Both the UHPLC system and the mass spectrometer were managed by the software Analyst 1.7 (Sciex), acquiring and processing the experimental data, provided with the MultiQuant™ software (version 3.0.3) for post-run data elaboration. Tandem mass spectrometry analyses were carried out in multiple-reaction monitoring mode both in negative (-MRM) and in positive ionization (+MRM). The QTRAP technology is based on a triple quadrupole mass analyzer, where the third quadrupole can be configured as a Linear Ion Trap (LIT) to provide additional scan functions. In this way, confirmation or identification of the analytes can be performed simultaneously with data quantification, without losing sensitivity. The mass spectrometer was periodically calibrated in the

mass range 112.99 – 2833.87 Da with the reference standard mix solutions G2421A/G2421-60001, provided by the manufacturer.

During the preliminary study the fragmentation pattern of all BPs and internal standards were examined and all the experimental parameters were optimized to maximize the signals of precursor and product ions. The full scan mass spectrum of each compound was acquired by injecting the individual standard solution at 500.0 ng/mL in Milli-Q water/methanol 50/50 (v/v) at 40.0 µL/min and applying the LC flow conditions with a mobile phase composition of 50/50 (A/B). The following experimental parameters were optimized: curtain gas at 35 psi, ion spray voltage ±4500 V, source temperature at 400 °C, ion source gas-1 at 55 psi, ion source gas-2 at 60 psi, resolution Q1= 1.0 amu, resolution Q2 = 1.0 amu, collision-activated dissociation CAD gas = medium. All the BPs showed better ionization in negative mode, because of the acidity of the phenolic hydroxyl group that loses a proton (H^+) forming the corresponding anion. The oxygen atom of the hydroxyl group has a partial positive charge due to resonance, and the anion formed by the proton loss is stabilized by resonance too, thus improving the ionization in negative mode. On the contrary, in BADGE and BFDGE the oxygen of the hydroxyl group is bonded to an epoxide group through a methylene bridge. This way, the deprotonation site is no longer there, but both the ether oxygens can act as nucleophile, thus favoring the ionization in positive mode. As expected, BADGE and BFDGE showed a much better ionization in positive mode; in particular, the ammonium adduct $[M+NH_4]^+$ was the prevalent ion in the mass spectra of both compounds, therefore it was selected as precursor ion for the MRM transitions. The +MRM and -MRM experimental parameters, optimized for each BP and IS, are reported in **Table 4** for both quantifier and qualifier ions.

Table 4. The -MRM and +MRM experimental parameters of the internal standards (IS) and both quantifier (Q) and qualifier (q) transitions of the BPs, optimized for the UHPLC-MS/MS analysis.

Ion	Q1 → Q3, *m/z*	Declustering Potential (DP), V	Collision energy (CE), eV	Collision cell exit potential (CXP), V
BPA (Q)	227.2 → 212.2	-61	-24	-10
BPA (q)	227.2 → 133.2	-61	-33	-12
BPAF (Q)	335.0 → 265.2	-50	-29	-12
BPAF (q)	335.0 → 315.2	-50	-26	-16
BPAP (Q)	289.2 → 274.2	-59	-27	-12
BPAP (q)	289.2 → 273.3	-59	-41	-12
BPB (Q)	241.2 → 212.3	-54	-24	-10
BPB (q)	241.2 → 211.2	-54	-38	-10
BPBP (Q)	351.1 → 274.3	-87	-32	-13
BPBP (q)	351.1 → 273.3	-87	-34	-13
BPC (Q)	255.2 → 240.2	-70	-25	-12
BPC (q)	255.2 → 147.2	-70	-35	-12
BPE (Q)	213.0 → 198.3	-56	-23	-10
BPE (q)	213.0 → 197.1	-56	-37	-13
BPF (Q)	199.0 → 105.1	-58	-28	-12
BPF (q)	199.0 → 93.1	-58	-28	-12
BPG (Q)	311.3 → 295.3	-70	-46	-14
BPG (q)	311.3 → 175.3	-70	-39	-14
BPM (Q)	345.2 → 133.2	-109	-46	-11
BPM (q)	345.2 → 251.3	-109	-39	-12
BPP (Q)	345.0 → 330.3	-105	-36	-15
BPP (q)	345.0 → 315.2	-105	-49	-15
BPPH (Q)	379.3 → 209.2	-85	-46	-10
BPPH (q)	379.3 → 364.2	-85	-33	-10
BPS (Q)	249.0 → 108.1	-70	-34	-10
BPS (q)	249.0 → 92.1	-70	-43	-14
BPTMC (Q)	309.0 → 215.3	-46	-38	-15
BPTMC (q)	309.0 → 200.2	-46	-45	-9
BPZ (Q)	267.2 → 173.3	-78	-36	-13
BPZ (q)	267.2 → 145.2	-78	-47	-13
BPA-d_{16} (IS)	241.3 → 223.3	-60	-27	-11
BPC2 (IS)	278.7 → 71.2	-35	-18	-11
BADGE[1] (Q)	358.2 → 191.2	38	19	10
BADGE[1] (q)	358.2 → 135.2	38	40	12
BFDGE[1] (Q)	330.2 → 163.2	28	17	8
BFDGE[1] (q)	330.2 → 133.2	28	22	16

[1] Ammonium adduct $[M+NH_4]^+$

During method development, also several reversed phase HPLC columns were tested to find the one giving the best chromatographic separation of all the BPs tested. At first, a HPLC method, previously developed for the determination of BPA, BPB, BPF, 4-OP and 4-NP (Di Marco Pisciottano et al., 2020; Gallo et al., 2019), was tested, using a linear gradient elution with Milli-Q water/2mM ammonium acetate and methanol as mobile phases on a Kinetex® Pentafluorophenyl, 2.6 µm, 100 × 3.0 mm, column (Phenomenex, Torrance, CA, USA). Although some variations introduced to improve resolution for all the BPs (percentage of the organic phase, gradient slope, flow rate), the isobaric BPM and BPP were not separated at all. Then, the following columns were also tested introducing the same variations as above: a) Synergi™ Polar-RP, 2.5 µm, 50 × 3.0 mm; b) Kinetex® XB-C18, 2.6 µm, 100 × 3.0 mm; c) Gemini® Phenyl-Hexyl, 3.0 µm, 100 × 4.6 mm, all from Phenomenex (Torrance, CA, USA). Among them, the only stationary phase that showed a complete chromatographic separation of all the BPs, even the isobaric compounds, was the Gemini® Phenyl-Hexyl. The Phenyl-Hexyl stationary phase consists of phenyl groups bound to the particle surface through a hexyl bridge; the presence of both an aromatic and an aliphatic moiety allows to have a dual selectivity, thus using a reversed phase column with an increased retention of polar and aromatic compounds such as bisphenols. After choosing the best stationary phase, a Kinetex® Phenyl-Hexyl, 2.6 µm, 100 × 3.0 mm, column based on core-shell silica particles technology, was tested. The core-shell technology is based on the use of silica particles with a non-porous core, surrounded by a porous layer, thus increasing the theoretical plates of about 50% in respect to a totally porous particle column. Therefore, using the Kinetex® column allows us to have a higher number of theoretical plates in respect to the Gemini® column, thus improving analyte resolution. The optimal flow rate was set at 0.600 mL/min, with 5.0 µL injection volume and column temperature at 40°C. Both mobile phases contained 2 mM ammonium acetate, adding acetic acid (0.02% v/v) to buffer the pH at about 5.0, being the column stable between 1.5 and 8.5 units of pH. The final two-step linear gradient was optimized for the earl-eluting compounds.

The -MRM and +MRM chromatograms of a standard mix solution at 10.0 ng/mL in methanol and a process blank are showed in **Figure 2**; as can be seen, the chromatographic peaks are very sharp (about 0.1 min width at the baseline) and all the BPs are well separated, thus improving the method sensitivity. The process blank chromatograms showed no interference deriving from materials and solvents used.

Chromatographic separation was performed on a Kinetex® Phenyl-Hexyl, 2.6 μm, 100 × 3.0 mm stainless steel column (Phenomenex, Torrance, CA, USA) equipped with a pre-column Security Guard Ultra Holder and a phenyl stationary phase cartridge (Phenomenex). The column oven was set at 40°C and the chromatography was performed by linear gradient elution with Milli-Q water (A) and methanol (B) as mobile phases, both containing 2 mM ammonium acetate and 0.02% acetic acid, at 0.600 mL/min flow rate. The chromatographic run started from 50% B for 0.5 minute, then it was increased to 70% in 1 min, to 95% in the next 3.5 min and held at 95% for 5 min. Finally, the mobile phase B was decreased to 50% in 0.5 min, then equilibrated at 50% for further 4.5 min. The injection volume was 5.0 μL of the sample.

For each chemical, the most two abundant product ions (Q1→Q3 transitions) were selected as quantifier (Q) and qualifier ion (q), respectively, for effective quantification and unambiguous identification. The presence of each analyte was confirmed on the basis of the relative retention times (tr) of both quantifier and qualifier ion peaks, with a tolerance of retention time tr ± 1% in respect to the reference standard peaks and to the tr of the internal standard in each sample.

For water and meat-based baby-food samples, the BPs were quantified by calculating and interpolating the linear regression external calibration curve of the chromatographic peak area vs the standard solution concentrations. On the contrary, the quantification of each analyte in milk, milk-based baby-food and food supplements was carried out by calculation of the linear regression internal calibration curves, plotting the BP-to-IS chromatographic peak area ratio *vs* the standard solution concentrations and interpolating the same ratio from the sample.

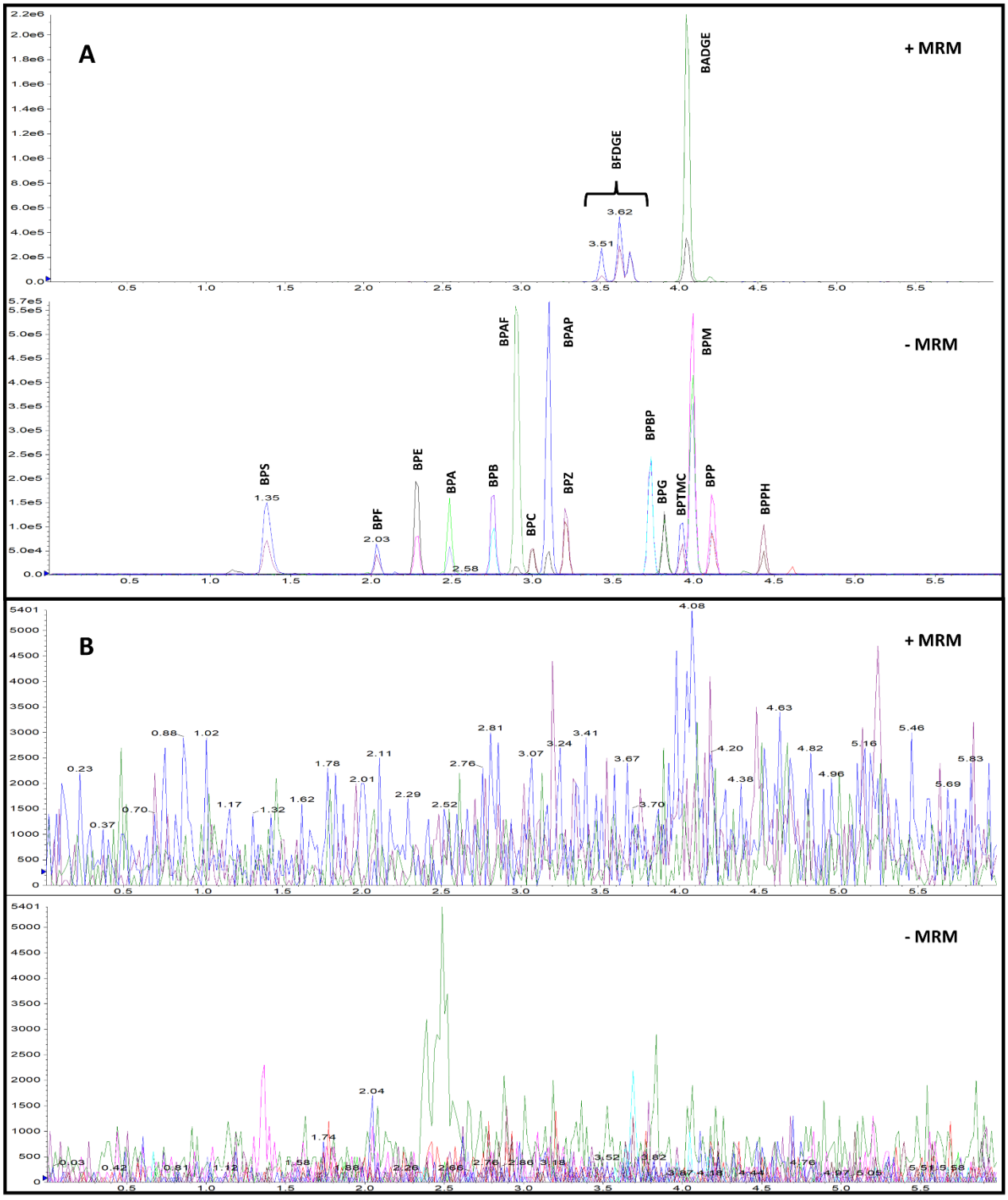


Figure 3. The –MRM and +MRM chromatograms of a standard mix solution at 10.0 ng/mL in methanol of the 17 bisphenols (A) and a process blank (B), showing no interference deriving from materials and solvents used.
Credit: Di Marco Pisciottano, Albrizio, et al., 2022

2.1.7. Food survey: Sample Collection and Storage

The milk and drinking water samples tested were collected from 10 buffalo farms in the Campania region, over the period 2019–2020; for each farm, 4–7 samples were taken during different periods and buffalo lactation phases. A total of 62 drinking water (for buffaloes in both dry and lactation phases) and 46 raw milks were collected from the selected farms and analyzed. All samples were collected in glass containers to avoid any contact with plastics, except for those already present in the milk production cycle steps; in this way, undesired contamination by EDCs during sampling was avoided. Furthermore, 10 samples of retail bovine milk, packaged both in PET and in Tetra Pak containers, were collected from supermarkets of Campania region and analyzed to evaluate consumers' possible exposure to EDCs due to environmental pollution or to migration from the packaging.

Regarding the baby-food samples, 22 milk-based baby-food and 16 meat-based baby-food were collected from the Italian supermarkets, pharmacies and all the stores selling those products. Two fruit-based baby-food were also collected and tested even if no spiked samples were performed on the different matrix to check that the analytical performance was still satisfactory. The fruit- and meat-based baby-food were all packed in glass jars with metal lids, whereas the milk-based baby-food were in different plastic packages.

The 15 food supplements were collected from supermarkets, pharmacies, and herbalists of the Campania region to evaluate the contamination of bisphenols deriving from the different materials commonly used in the packaging of food supplements. In fact, they were all packed in different materials, such as aluminum, polystyrene, plastic, glass, paper and high-density polyethylene.

2.1. Heavy metals (lead, cadmium, and mercury)

In this section is reported an overall description of the analytical methods used to analyze lead, cadmium, and mercury in baby food. The methods are validated, accredited, and used for official

control routine analysis by the Department of Chemistry of the Istituto Zooprofilattico Sperimentale del Mezzogiorno (Portici, Naples, Italy), therefore they are under company's Quality Management System.

2.1.1. Sample handling and microwave-assisted digestion

The milk-based baby-food were stored at T < -18°C when liquid or at room temperature in a dry and dark place when in powder. The meat-based baby-food were stored at room temperature in a dark and dry place if sealed, and at T < -18°C after opening. Both the meat- and the milk-based baby-food were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) after microwave-assisted digestion of the sample using an ETHOS Microwave Digestion System (Milestone, Denmark) to determine the concentration level of lead and cadmium. Furthermore, the meat-based baby-food were also analyzed using a Direct Mercury Analyzed (DMA-80, Milestone, Denmark) to also determine the concentration of mercury. The microwave-assisted digestion is a technique commonly used to destroy the organic matter present into sample until only the heavy metals are left into the extract. It is usually performed in strong acidic conditions, putting both the sample and a strong acid, such as nitric, hydrochloric or hydrofluoric acid, in a polypropylene vessel. Then, the vessel is closed, and the sample undergoes an increase of pressure and temperature obtained by using pulsed microwave irradiation. Usually, the temperature is not just linearly increased, but goes through different ramps while the temperature inside the vessel is constantly monitored with an infrared external sensor or an optic fiber probe. The combination of high temperature, high pressure and the low pH causes a faster thermal decomposition of the sample leading to a liquid extract in which all the heavy metals are now dissolved. There is no need to say that the vessel should contain at least one solvent that is able to absorb microwave radiation, and that is usually water. The total digestion time with the microwave system is about one hour, a dramatically decreased time if compared to dry ash digestion systems that took almost 24 hours to perform the whole process.

2.1.2. Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is an analytical technique used to determine elements in several different matrices, such as food, feed, pharmaceuticals, clinical research, after the sample being digested and the elements being dissolved in the liquid extract. The ICP-MS, as the name says, utilizes an inductively coupled argon (Ar) plasma to ionize the sample; in this case, it is not a molecule that is being ionized and detected, but the sample is rather atomized creating both atomic and polyatomic ions that are then measured using a mass spectrometer. Since this technique is also able to detect isotopes of the same element and it is also very useful in the isotopic labeling. Usually, the ICP-MS system has two parts at different pressure, that is atmospheric pressure into the source (ICP) and vacuum into the mass spectrometer. The ICP-MS can measure theoretically every natural element and also the non-natural "radiogenic" isotopes, but it cannot measure hydrogen (H), helium (He), Ar, nitrogen (N), oxygen (O), fluorine (F) and neon (Ne). For the first two (H and He), it is because they are below the lowest detection limit of the instrument; For Ar, N and O it is because they are present at very high levels due to the argon plasma and the air that is inside the atmospheric pressure source; lastly for F and Ne it is because the argon plasma is not enough to ionize them. Nevertheless, the ICP-MS is a widely used system because it provides low detection limits (0.1 part per trillion) for a significant number of elements but, on the other hand, it can also measure very high concentrations, in the magnitude of parts per million. No other analytical technique has such a wide detection range in terms of concentration and in panel of elements that can be detected.

2.1.3. Direct Mercury Analysis (DMA)

The Direct Mercury Analysis is the most advanced technique for the determination of the mercury in all different kind of matrices. The mercury is a high volatile element and that can lead to analyte loss during the digestion process if it is not correctly performed. Moreover, the mercury is well known to easily adhere to the containers or to the parts of the instrument it comes into contact with, thus causing

cross-contamination between the samples or contamination of the system itself. That requires thorough washings of the system or, in the case of the ICP-MS analysis, the use of gold as strong oxidizer to prevent the mercury to contaminate everything. The Direct Mercury Analysis, on the other hand, allows to measure mercury directly from the sample, without the need for sample preparation or dissolution of the element in a solution. This is possible thank to the decomposition furnace that is located into the instrument and that allows the mercury to be released as vapor from the sample itself without acid digestion, saving the time of the analysis and of the cleaning. During DMA, the sample goes through thermal decomposition, catalytic conversion, amalgamation and finally it is detected by atomic absorption detection. The sample is first dried and then thermally decomposed and an oxygen flow carries the products of the decomposition through a heated plate that acts as a catalyst, trapping oxides, sulfur, halogens and nitrogen. All the mercury species are reduced to elemental mercury, then a reaction gas transports the atoms to a gold amalgamator that traps them while all the unwanted products are carried away by a continuous gas flow. Afterwards, this amalgamator is heated to release the trapped mercury that is detected by means of an atomic absorption spectrophotometer. Moreover, the system is able to recognize when a too high level of mercury is present in a sample, and then it will automatically perform some blank cycles to clean the system. This technique has the advantage of being fast and time saving, accurate to quantify mercury, matrix independent, clean and easy to use.

2.1.4. Food survey: sample collection and storage

For the determination of the lead, cadmium, and mercury, 16 meat-based baby-food and 2 fruit-based baby-food were collected from Italian markets and pharmacies and stored at room temperature in a dry and dark place until sealed; after opening they were stored at temperature ≤ -18°C. For the determination of lead and cadmium, 22 milk-based baby-food, including powder milk and liquid milk, were also collected from the Italian supermarkets and pharmacies; the powder samples were stored

at room temperature in a dry and dark place, whereas the liquid ones were stored at temperature of (5 ± 3) °C until opening, then they were stored at temperature ≤ -18°C.

2.2. Microcystins

In the frame of a research project funded by the Italian Ministry of Health (IZS ME 02-17 RC) I developed and validated a method for the determination of 9 microcystins in food supplements containing cyanobacteria, verifying the analytical performance parameters in the matrix by analysing spiked blank samples. Afterwards, I applied the method to determine the microcystins in 7 algae-based food supplements collected from the pharmacies and herbalists of the Campania Region to evaluate the contamination due to these cyanotoxins.

2.2.1. Chemicals and Standard Reference Materials

The standard reference materials for the microcystin MC-LR, MC-RR, MC-YR, MC-WR, MC-LA, MC-LF, MC-LW, MC-LY and MC-demLR were supplied by Enzo Life Sciences (Farmingdale, New York), and were all with a purity grade > 95%. HPLC-grade methanol (MeOH), formic acid (analytical purity grade) and glacial acetic acid (97% v/v, purity grade) were obtained from VWR International (Radnor, PA, USA). HPLC-grade acetonitrile (ACN) and sodium hydrogen carbonate (NaHCO3) analytical purity grade were purchased from Carlo Erba (Milan, Italy). HPLC grade water was in-house produced using a MilliQ laboratory system (Millipore, Bedford, MA, USA). The ISOLUTE® C18 SPE columns were supplied by Biotage (Uppsala, Sweden). The chromatographic column Kinetex Phenyl-Hexyl, 2.6 µm, 100 × 3.0 mm, the Security Guard Ultra Holder and cartridges with phenyl phase were supplied by Phenomenex (Torrance, CA, USA).

2.2.2. Standard calibration curves and method validation level

Individual standard stock solutions were prepared by dissolving the powder reference materials in the appropriate volume of methanol. In particular, the microcystins with a weight of 25 µg were dissolved in 5.0 mL of methanol to have individual stock solutions at a concentration of 5.0 µg/mL. For the

reference materials with a content of 50 and 100 µg, the powder was dissolved in 10.0 mL of methanol to have individual stock solutions at the concentration of, respectively, 5.0 and 10.0 µg/mL. Then, a mix standard solution at 500 ng/mL of all the microcystins is prepared by diluting the appropriate amount of each individual stock solution in 5.0 mL of methanol. All the stock solutions and the mix standard solution were stored in dark glass vials at -18°C until use.

The standard solutions for calibration curves were prepared at 1.0 – 2.5 – 10.0 – 50.0 ng/mL of all the microcystins both in methanol and in a blank sample extract, to evaluate the possible signal suppression/enhancement due to the presence of the matrix in mass spectrometry. The method validation was carried out spiking blank samples of algae-based food supplements at a concentration level of 100.0 ng/mL. All the intermediate standard solutions and calibration curve standards were prepared daily from the mix standard solution.

2.2.3. Sample handling and cleanup

All the samples were collected, then stored in a dry and dark place until the analysis. Both the samples for the survey and the spiked samples were cleaned up and analyzed following the procedure herein described.

The powder food supplement samples were homogenized manually by using a spatula whereas the tablet ones were pulverized by mortar and pestel, then 0.50 ± 0.01 g of were weighted in a 15 mL polypropylene centrifuge tube and added with 2.5 mL of methanol/Milli-Q water 1/1 (v/v) mixture 0.01% of acetic acid. The sample was mixed by vortex for 30 seconds, sonicated in an ultrasonic bath at room temperature for 10 minutes, then centrifuged at 1146 *g* for 15 minutes. The supernatant is withdrawn and transferred in a clean 15 mL polypropylene centrifuge tube and the sample is added again with 2.5 mL of methanol/Milli-Q water 1/1 (v/v) mixture 0.01% of acetic acid. After mixing by vortex for 30 seconds, the sample is sonicated in an ultrasonic bath at room temperature for 10 minutes and centrifuged at 1146 *g* for 15 minutes. The supernatant is collected and gathered with the first extract, then mixed by vortex for 10 seconds. From the total extract, 2.0 mL are transferred into an

eppendorf tube and centrifuged at 13680 *g* and 4°C for 15 minutes. The supernatant is diluted with 18.0 mL of a 20 mM $NaHCO_3$ buffer with a pH value between 6.3 and 6.5 and then purified by ISOLUTE® C18 SPE columns, previously conditioned with 5.0 mL of methanol and 5.0 mL of Milli-Q water. After sample loading, the SPE columns are rinsed with 5.0 mL of a 20 mM $NaHCO_3$ buffer/methanol 95/5 (v/v) solution and the vacuum is applied to completely remove the rinsing solvent from sorbent. The elution of the microcystins is carried out with 5.0 mL of methanol 0.1% acetic acid and then 1.0 mL of the eluate is transferred into a glass vial for the UHPLC-MS/MS analysis (dilution factor = 25).

2.2.4. Method validation

The method was in-house validated evaluating the specificity, the trueness, the precision, the ruggedness for slight variations, the limits of quantification, the limits of detection and the linearity of the detector response for each compound, and the criteria for analyte unambiguous identification by mass spectrometry. Method specificity was assessed analyzing uncontaminated food supplements (not containing cyanobacteria) and verifying that there were no interfering peaks both in the –MRM and in the +MRM chromatograms at the retention times of the analytes, considering a signal-to-noise ratio > 3/1. To evaluate method precision and trueness 6 blank food supplements were spiked at 100.0 ng/g, cleaned up and analyzed over three analytical sessions during different days, thus assessing both method precision and ruggedness. To further evaluate the method ruggedness, slight variations were introduced during each working session, such as different batches of solvents and reagents and different analysts. The method trueness was calculated in terms of mean percentage recoveries, while the intermediate precision was expressed as percentage relative standard deviations (RSD%). The limit of quantification (LOQ) for each analyte was measured on a signal-to-noise ratio > 10/1 for each microcystin and limit of detections were calculated from LOQs according to the equation: LOD = LOQ/3.3. During each working session, the linearity of the detector response, in terms of determination coefficients (r2), was verified to be greater than 0.95 for all the calibration curves. The

analytical performance parameters resulting from the validation of the method are reported in **Table 5**.

Table 5. The analytical performance parameters of trueness, expressed as mean percentage recovery, intermediate precision, in terms of relative standard deviation (RSD%) and limits of quantification of the 9 microcystins in algae-based food supplements.

Microcystin	Mean recovery %	RSD %	LOQ (ng/g)
MC-LR	81.0	4.4	1.0
MC-RR	67.7	9.1	1.0
MC-YR	67.6	12.7	1.0
MC-WR	68.9	9.3	1.0
MC-LA	75.0	12.1	1.0
MC-LF	70.3	9.9	1.0
MC-LW	66.7	7.9	1.0
MC-LY	68.7	12.1	1.0
MC-demLR	80.7	19.2	1.0

The mean recoveries of the microcystins in the food supplements are overall satisfactory, ranging from 66.7% to 81.0%, as well as the RSD% values all below 19.2%. Also in this case, all the results found in the samples were corrected for the percentage recovery of the spiked sample analyzed during the working session for quality control of the process, thus considering every possible loss of the analytes.

2.2.5. UHPLC-MS/MS analysis

Analyses were performed using a UHPLC-MS/MS system, consisting of a UHPLC ExionLC™ AD series chromatograph (Sciex, Framingham, MA, USA), equipped with a binary pump, a column oven and an autosampler with temperature control, coupled to a QTRAP 6500^{+} mass spectrometer (Sciex).

Both the UHPLC system and the mass spectrometer were managed by the software Analyst 1.7 (Sciex), acquiring, and processing the experimental data, provided with the MultiQuant™ software (version 3.0.3) for post-run data elaboration. Tandem mass spectrometry analyses were carried out in multiple-reaction monitoring mode both in negative (-MRM) and in positive ionization (+MRM). The mass spectrometer was periodically calibrated in the mass range 112.99 – 2833.87 Da with the reference standard mix solutions G2421A/G2421-60001, provided by the manufacturer.

During the preliminary study, the fragmentation pattern of all the microcystins were examined and all the experimental parameters were optimized to maximize the signals of precursor and product ions. The full scan mass spectrum of each compound was acquired by injecting the individual standard solution at 500.0 ng/mL in Milli-Q water/methanol 50/50 (v/v) at 40.0 µL/min and applying the LC flow conditions with a mobile phase composition of 50/50 (A/B). The following experimental parameters were optimized: curtain gas at 35 psi, ion spray voltage ±4500 V, source temperature at 600 °C, ion source gas-1 at 50 psi, ion source gas-2 at 60 psi, resolution Q1= 1.0 amu, resolution Q2 = 1.0 amu, collision-activated dissociation CAD gas = medium. Out of 9 microcystins, four of them showed a better ionization in negative mode and the other five showed a better precursor ion signal in the positive mode. It is interesting to notice that all the microcystins containing an arginine amino acid in the variable part of the cyclic peptide showed a better ionization in positive mode. That could be explained by the higher isoelectric point of the arginine amino acid (10.76) in respect of all the other amino acids present in the analyzed microcystins (all below 6). In fact, at the pH of the $NaHCO_3$ buffer the arginine would be preferentially in the protonated form (positive) while all the other amino acids would be in the deprotonated form (negative). The +MRM and -MRM experimental parameters, optimized for each microcystin, are reported in **Table 6** for both quantifier and qualifier ions.

Table 6. The -MRM and +MRM experimental parameters of the internal standards (IS) and both quantifier (Q) and qualifier (q) transitions of the microcystins, optimized for the UHPLC-MS/MS analysis.

Ion	Q1 → Q3, *m/z*	Declustering Potential (DP), V	Collision energy (CE), eV	Collision cell exit potential (CXP), V
MC-LR (Q)	995.6 → 102.8	70	165	6
MC-LR (q)	995.6 → 135.2	70	139	11
MC-RR (Q)	519.9 → 135.1	70	35	9.3
MC-RR (q)	519.9 → 103.1	70	96	9.5
MC-WR (Q)	1068.6 → 103.0	70	165	9
MC-WR (q)	1068.6 → 134.9	70	150	11
MC-YR (Q)	1045.5 → 103.1	70	160	6
MC-YR (q)	1045.5 → 135.4	70	139	10
MC-demLR (Q)	981.7 → 102.8	70	165	6
MC-demLR (q)	981.7 → 135.2	70	139	11
MC-LA (Q)	908.4 → 890.4	-40	-50	-42
MC-LA (q)	908.4 → 283.3	-40	-64	-13
MC-LF (Q)	984.4 → 966.4	-24	-54	-40
MC-LF (q)	984.4 → 283.3	-24	-67	-16
MC-LW (Q)	1023.4 → 1005.4	-35	-55	-45
MC-LW (q)	1023.4 → 283.2	-35	-70	-19
MC-LY (Q)	1000.4 → 982.4	-30	-54	-45
MC-LY (q)	1000.4 → 283.0	-30	-69	-13

During method development, the following reversed phase HPLC columns were tested to find the one giving the best chromatographic: 1) Ascentis® Express RP-Amide 2.7 µm, 50 × 4.6 mm, column (Supelco, Bellefonte, Pennsylvania, USA); 2) Synergi MAX-RP 2.5 µm, 50 × 3.0 mm, column (Phenomenex, Torrance, CA, USA); 3) Synergi Polar-RP 2.5 µm, 50 × 3.0 mm, column (Phenomenex, Torrance, CA, USA); 4) Synergi Fusion-RP 4.0 µm, 50 × 2.0 mm, column (Phenomenex, Torrance, CA, USA); 5) Kinetex® Phenyl-Hexyl, 2.6 µm, 100 × 3.0 mm, column (Phenomenex, Torrance, CA, USA). Also, different gradients using Milli-Q water as mobile phase A and acetonitrile/water 94/6 (v/v) solution as mobile phase B, both acidified (0.1% acetic acid and 0.1% formic acid) and not acidified were tested to find the best conditions for improving chromatographic separation and peak shape. Finally, the Kinetex® Phenyl-Hexyl, 2.6 µm, 100 × 3.0 mm, column was selected and Milli-

Q water (A) and acetonitrile/water 94/6 (v/v) solution (B) both containing 0.1% formic acid were chosen as mobile phases.

The -MRM and +MRM chromatograms of a standard mix solution at 10.0 ng/mL in methanol are showed in **Figure 4**; as can be seen, the chromatographic peaks are all well separated and with good peak shape, thus improving the method sensitivity.

Chromatographic separation was performed on a Kinetex® Phenyl-Hexyl, 2.6 μm, 100 × 3.0 mm stainless steel column (Phenomenex, Torrance, CA, USA) equipped with a pre-column Security Guard Ultra Holder and a phenyl stationary phase cartridge (Phenomenex). The column oven was set at 40°C and the chromatography was performed by linear gradient elution with Milli-Q water (A) and acetonitrile/water 94/6 (v/v) solution (B) as mobile phases, both containing 0.1% formic acid, at 0.500 mL/min flow rate. The chromatographic run started from 10% B for 0.5 minute, then it was increased to 55% in 5.5 min, to 95% in the next 3 min and held at 95% for 2 min. Finally, the mobile phase B was decreased to 10% in 1 min, then equilibrated at 10% for further 3 min. The injection volume was 2.0 μL of the sample.

For each chemical, the most two abundant product ions (Q1→Q3 transitions) were selected as quantifier (Q) and qualifier ion (q), respectively, for effective quantification and unambiguous identification. The presence of each analyte was confirmed on the basis of the relative retention times (tr) of both quantifier and qualifier ion peaks, with a tolerance of retention time tr ± 1% in respect to the reference standard peaks.

The microcystins were quantified by calculating and interpolating the linear regression external calibration curve of the chromatographic peak area vs the standard solution concentrations.

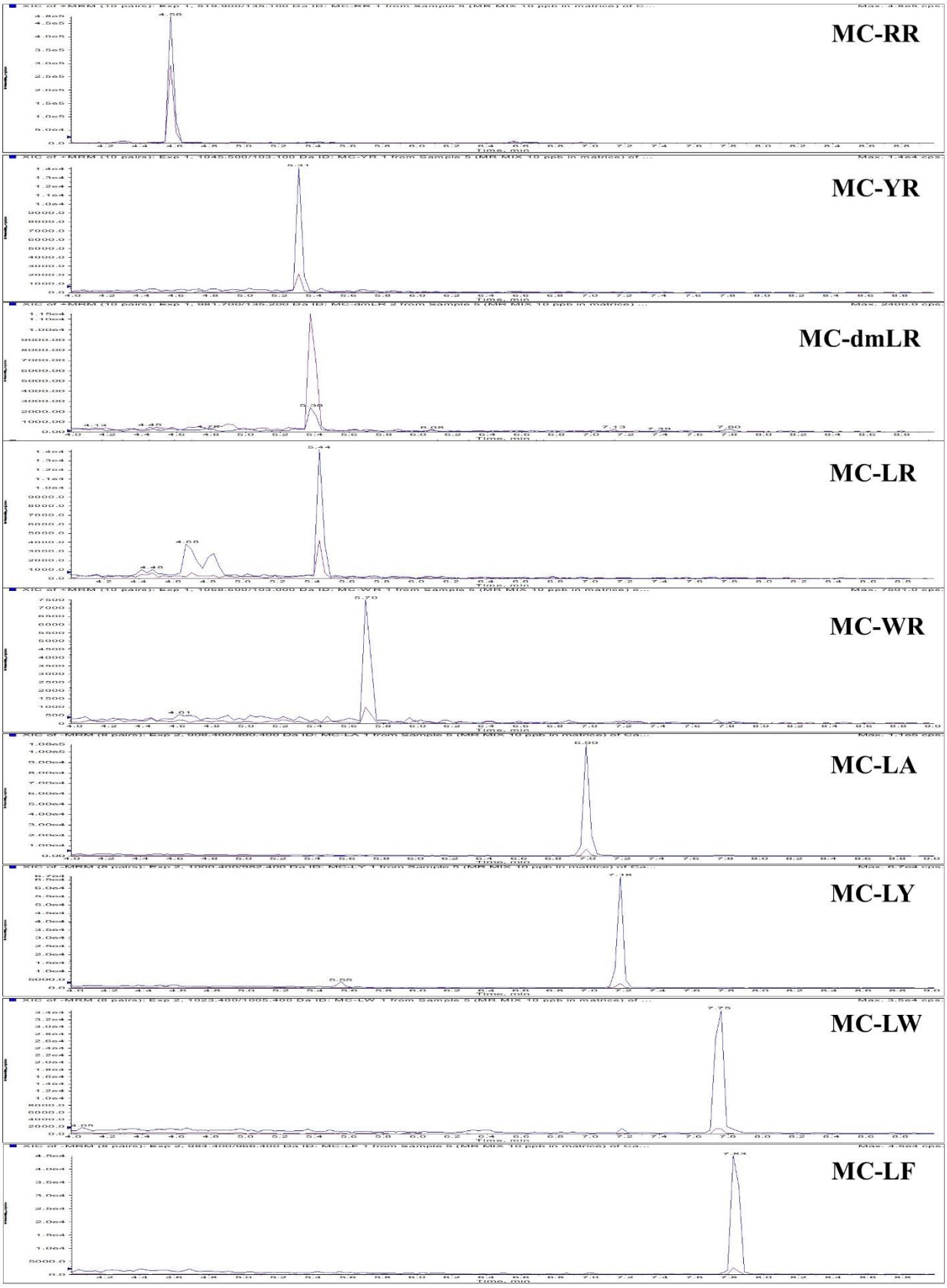


Figure 4. The –MRM and +MRM chromatograms of a standard mix solution at 10.0 ng/mL in methanol of all the 9 microcystins.

2.2.6. Food survey: Sample Collection and Storage

The 7 algae-based food supplements were collected from pharmacies and herbalists of the Campania Region to evaluate the content of the 9 microcystins. The details about the analyzed samples are reported in the **Table 7**.

Table 7. List of the 7 algae-based food supplement analyzed for the microcystin detection.

Algae-based food supplement	***Cyanobacterium***
Klamath	*Aphanizomenon flos-aquae*
Spirulina #1	*Arthrospira platensis*
Spirulina #2	*Arthrospira platensis*
Spirulina #3	*Arthrospira platensis*
Fucus motivador	*Fucus vescicolosus*
Fucus	*Fucus vescicolosus*
Spirulina BIO	*Arthrospira platensis*

2.2.7. The case of the Lake Avernus

Furthermore, besides the planned activities of the PhD project, in the month of March a massive *Planktothrix rubescens* bloom was observed in the Lake Avernus, a volcanic lake located in Campania Region (Southern Italy) (**Figure 5**). *Planktothrix rubescens* is a cyanobacterium known to produce several cyanotoxins, such as microcystins, anabaenopeptins, oscillamides, and modified microcystins (Du et al., 2019; Messineo et al., 2006; Monteiro et al., 2021). Bloom episodes of this cyanobacterium have already been observed in Italy through the years (Ferranti et al., 2009, 2011; Funari et al., 2020; Rita et al., 2014) and the analysis of water, mussel and cyanobacterium samples have always given evidence of the presence of the above-listed cyanotoxins. The cyanobacterial mass from the Lake Avernus migrated, through a channel, to the near Gulf of Pozzuoli, causing the contamination of two marine sites dedicated to mussel farming, thus posing a potential risk for consumers' health. Mussel and water samples, from both the sea and the lake, were weekly collected and analyzed by liquid chromatography coupled to tandem mass spectrometry for identification and quantification of the 9 microcystins, after extension of the method to the new matrices. The analytical performance

parameters were assessed on the new matrix by analyzing blank mussel and water samples spiked, respectively, at 20.0 ng/g and 5.0 ng/mL with all the 9 microcystins. Moreover, the samples were also analyzed by high resolution mass spectrometry in untargeted mode to determine other cyanotoxins basing on the accurate mass of the precursor ions and their MS/MS spectra. The untargeted analysis was performed using the same chromatographic separation of the LC-MS/MS method but coupled with a high-resolution mass spectrometer operating in Full Scan/data dependent MS^2 mode. Using this instrumental technique, all the precursor ions belonging to a set m/z range are collected in the orbital trap and fragmented in the collision cell; all the product ions are, then, registered in the form of a MS/MS spectrum. This way, the identification of each cyanotoxin is performed basing on the accurate mass of both the precursor ion and one of its product ions, with a maximum error of 5 parts per million.

Figure 5. The *Planktothrix rubescens* bloom observed in the Lake Avernus in March 2022.
From: https://casertaweb.com/notizie/lago-daverno-la-fenomenologia-dellacqua-rossa/

3. RESULTS AND DISCUSSION

3.1. Endocrine Disruptors

3.1.1. Milk and water samples from buffalo farms

The UHPLC–MS/MS analysis of the samples collected from the 10 selected buffalo farms in the Campania Region showed that the most abundant EDC in the milk was the BPA. In fact, 58.7% of the analyzed samples were contaminated by BPA, at concentrations ranging from 0.5 ng/mL to 5.6 ng/mL. The second most detected bisphenol was bisphenol F (BPF), identified in 17.4% of the samples, in the concentration range 0.5-8.7 ng/mL. No EDC contamination was observed in the drinking water samples. **Table 8** shows the contamination ranges, along with the mean and median values of the EDCs determined by UHPLC–MS/MS, expressed as ng/mL for raw milk, and the frequency of detection, expressed as the percentage of positive samples. As can be seen, only BPA, BPF and BPAF were identified in at least one of the samples tested; moreover, the drinking water samples were not contaminated by any bisphenol.

Table 8. The concentration ranges, the percentage of positive samples, and the mean and median values of the EDCs determined in the buffalo milk samples by UHPLC–MS/MS. No EDCs were detected in any of the 62 drinking water samples. (nd = not detected).

		Buffalo milk, ng/mL (*n* = 46)
BPA	*Range*	0.5 – 5.6 (58.7%)
	Mean	1.4 ng/mL
	Median	0.9 ng/mL
	LOQ	0.5 ng/mL
	LOD	0.2 ng/mL
BPF	*Range*	0.5 – 8.7 (17.4%)
	Mean	3.1 ng/g
	Median	1.6 ng/g
	LOQ	0.5 ng/mL
	LOD	0.2 ng/mL
BPAF	*Range*	3.0 (2.2%)
	Mean	3.0 ng/mL
	Median	3.0 ng/mL
	LOQ	0.5 ng/mL
	LOD	0.2 ng/mL

The results here reported seem to be coherent with the other scientific literature data about bisphenol contamination in milk. Santonicola et al. (Santonicola et al., 2019) analyzed 72 milk samples collected from cow farms via different milking techniques and found BPA in the concentration range 0.035-2.776 μg/L. Mercogliano et al. (Mercogliano et al., 2021) also investigated BPA levels in 92 cow milk samples collected from a dairy company at different steps of the production chain. BPA was identified in 35% of the samples, at concentration between 0.1 and 2.833 μg/L, showing results consistent with the levels found by Santonicola et al. (Santonicola et al., 2019), but slightly lower than the results herein reported for milk samples. The group of Santonicola et al. (Santonicola et al., 2021) also studied BPF contamination in 84 cow milk samples collected from a dairy company at various levels of the production chain. Their results showed that more than 50% of the cow milk samples were contaminated by BPF at concentrations ranging from 0.1 to 2.686 μg/L; in this case, the concentration levels were also slightly lower than those determined during this work.

3.1.2. Retail milk samples from supermarkets.

To evaluate consumers' exposure to EDCs from retail bovine milk as well, 10 samples, packaged both in Tetra Pak and in PET, were collected from supermarkets of Campania Region. The results of the UHPLC–MS/MS analysis (**Table 9**) confirmed that BPA is the most detected contaminant among the studied bisphenols; moreover, BPF was the only other bisphenol quantified, thus confirming the contamination profile already observed in the analysis of buffalo milk samples. The contamination profile in retail milk was quite similar to that observed by Grumetto et al. (Grumetto et al., 2013), but the bisphenol concentrations they quantified were much higher than those herein reported. In fact, Grumetto et al. determined BPA, BPF, and BPB in 68 retail milk samples at concentrations up to 521, 67, and 26.2 ng/mL, respectively; BADGE and BFDGE were not determined at all. The small number of samples did not allow us to perform a statistically significant evaluation, but apparently there was no correlation between the packaging materials and the BPA levels.

Table 9. The bisphenols determined in 10 samples of retail bovine milk collected from markets in the Campania region and analyzed by UHPLC–MS/MS. The other bisphenols were not detected at all (*nd* = not detected; > *LOD* = detected but not quantified).

Sample	Packaging	BPA (ng/mL)	BPF (ng/mL)
Milk #1	Tetra Pak	1.1	10.6
Milk #2	Tetra Pak	0.6	*nd*
Milk #3	Tetra Pak	> *LOD*	*nd*
Milk #4	Tetra Pak	1.3	2.1
Milk #5	Tetra Pak	*nd*	1.5
Milk #6	Tetra Pak	1.3	*nd*
Milk #7	Tetra Pak	> *LOD*	> *LOD*
Milk #8	PET	0.6	0.6
Milk #9	PET	2.8	> *LOD*
Milk #10	PET	0.5	*nd*

3.1.3. Milk- and meat-based baby-food

During this work, 22 milk-based baby-food and 16 meat-based baby-food were analyzed to determine the occurrence of 17 bisphenols in these products intended for newborns. In addition, also the results from two fruit-based baby-food are reported, even if the analytical performance of the method was not tested on this kind of matrix. The fruit- and meat-based baby-food were all packed in glass jars with metal lids, whereas the milk-based baby-food were packed in various plastic packaging materials. As for the buffalo milk samples and the retail milk samples from the markets, also in the case of the milk-based baby-food it is very clear that BPA is the most abundant contaminant since it has been quantified in 12 out of 22 samples at concentrations above the limit of quantification (0.5 ng/mL); no other bisphenols were detected in the analyzed baby-food. Furthermore, it has been detected at concentrations below the limit of quantification but above the limit of detection (0.2 ng/mL) in 3 samples (13.6% of the total samples) and a chromatographic peak below the limit of detection has also been observed in 4 samples (18.2% of the total samples). Only in 3 out of 22 samples the BPA has not been detected at all. Regarding the contamination levels (**Table 10**), they range from 0.53 ng/mL to 11.6 ng/mL, with an average value of 5.30 ng/mL, slightly higher than the concentration found in the buffalo and retail milk samples.

Table 10. The BPA determined in the 22 samples of milk-based baby-food collected from markets in the Campania region and analyzed by UHPLC–MS/MS. The other bisphenols were not detected at all (*nd* = not detected; > *LOD* = detected but not quantified; < *LOD* = detected at level below the LOD).

ID sample	Matrix	Target age (months)	BPA, (ng/mL)
Milk-based baby-food #1	Liquid milk	6-12	2.24
Milk-based baby-food #2	Powder milk	6-	7.64
Milk-based baby-food #3	Liquid milk	6-	4.21
Milk-based baby-food #4	Powder milk	6-	> *LOD*
Milk-based baby-food #5	Powder milk	0-6	> *LOD*
Milk-based baby-food #6	Powder milk	6-	< *LOD*
Milk-based baby-food #7	Powder milk	6-	4.71
Milk-based baby-food #8	Powder milk	0-	< *LOD*
Milk-based baby-food #9	Powder milk	0-	0.53
Milk-based baby-food #10	Powder milk	12-36	3.73
Milk-based baby-food #11	Powder milk	6-	< *LOD*
Milk-based baby-food #12	Powder milk	0-6	< *LOD*
Milk-based baby-food #13	Powder milk	6-12	> *LOD*
Milk-based baby-food #14	Powder milk	0-	11.6
Milk-based baby-food #15	Powder milk	0-6	6.5
Milk-based baby-food #16	Powder milk	0-	3.29
Milk-based baby-food #17	Powder milk	6-12	*nd*
Milk-based baby-food #18	Powder milk	0-	6.97
Milk-based baby-food #19	Powder milk	7-12	*nd*
Milk-based baby-food #20	Powder milk	0-6	*nd*
Milk-based baby-food #21	Powder milk	0-	2.2
Milk-based baby-food #22	Powder milk	0-6	10.0

Even in the case of the meat-based baby-food (**Table 11**), the only bisphenol determined in the samples was the BPA, but both a lower percentage of positive samples and a lower contamination level were observed. In fact, the BPA was quantified only in 5 out of 16 samples (31.2% of the analyzed samples), with an average value of 1.18 ng/g and a contamination range going from 0.94 ng/g to 1.83 ng/g, way lower than what was found in the milk-based baby-food. Moreover, in the case of the meat-based baby-food, the number of samples in which BPA was not observed at all is of 7 out of 16, that is the 43.8% of the total amount, while BPA was observed below the limit of quantification (1.0 ng/g) in 18.8% of the samples and below the limit of detection (0.3 ng/g) in the 6.2% of the samples.

Table 11. The BPA determined in the 16 samples of meat-based baby-food and 2 samples of fruit-based baby-food collected from markets in the Campania region and analyzed by UHPLC–MS/MS. The other bisphenols were not detected at all (*nd* = not detected; > *LOD* = detected but not quantified; < *LOD* = detected at level below the LOD).

ID sample	Type of meat	Target age (months)	BPA, (ng/g)
Meat-based baby-food #1	Cow	4-	1.15
Meat-based baby-food #2	Gilthead bream	6-	< *LOD*
Meat-based baby-food #3	Turkey	4-	> *LOD*
Meat-based baby-food #4	Lamb	4-	> *LOD*
Meat-based baby-food #5	Turkey	4-	*nd*
Meat-based baby-food #6	Cow	4-	1.83
Meat-based baby-food #7	Chicken	4-	*nd*
Meat-based baby-food #8	Ham	4-	1.00
Meat-based baby-food #9	Chicken and cow	4-	0.99
Meat-based baby-food #10	Ham	4-	*nd*
Meat-based baby-food #11	Rabbit	4-	*nd*
Meat-based baby-food #12	Plaice	4-	> *LOD*
Meat-based baby-food #13	Chicken and cow	6-	0.94
Meat-based baby-food #14	Trout	6-	*nd*
Meat-based baby-food #15	Cow	4-	*nd*
Meat-based baby-food #16	Chicken	4-	*nd*
Fruit-based baby-food #1	Apple and apricot	4-	*nd*
Fruit-based baby-food #2	Pear	4-	< *LOD*

The results from the bisphenol determination in baby-food are coherent with the different packaging materials used for the meat-based and the milk-based baby-food, that is, respectively, glass and plastic. Nevertheless, we cannot state that the BPA occurrence observed in the analyzed baby-food is deriving from the packaging and not from the raw materials used in food production.

For the two samples of fruit-based baby-food, one contained the BPA below the limit of detection and in the other one the BPA was not detected at all. The results herein reported for the BPA contamination are quite coherent with those of the work of Cao et al. (Cao et al., 2009), who found BPA in 99 out of 122 glass jar baby-food, with the highest level at 7.2 ng/g of BPA in two different products. Also, they observed the fruit-based baby-food to be overall less contaminated than the mixed-dish ones.

3.1.4. Food supplements

The bisphenol occurrence was evaluated also in 15 food supplements collected from supermarkets, pharmacies, and herbalists of the Campania region packed in different materials such as aluminum, polystyrene, plastic, glass, paper and high-density polyethylene.

In the **Table 12** are reported only the concentration levels of the quantified bisphenols and, for each sample, is indicated the packaging material that was in contact with the food supplement. The limits of quantification for all the bisphenols are between 1.0 ng/g and 5.0 ng/g and, in particular, for the bisphenols determined in the samples they are 1.0 ng/g for BPA and BPTMC, 2.0 ng/g for BPB and 5.0 ng/g for BPC. As it can be seen, 7 food supplements contained some bisphenols (46.7% of the analyzed samples), most of them by BPA (4 out of 7 samples) that is always the most abundant contaminant. The BPA has been quantified in a concentration range going from 3.2 ng/g to 92.1 ng/g, while the BPB has been determined at 189.5 ng/g in one sample, that is an impressively high concentration level. Similarly to the case of the baby-food, the contamination seems to derive mainly from the plastic materials used for the packaging, even if the BPA and BPTMC occurrence in two food supplements packed in glass containers suggested that the EDCs could also come from the environment.

Table 12. The EDCs determined in the 15 samples of food supplements collected from markets in the Campania region and analyzed by UHPLC–MS/MS. The other bisphenols were not detected at all (*nd* = not detected).

Sample ID	Composition	Packaging	BPA (ng/g)	BPB (ng/g)	BPC (ng/g)	BPTMC (ng/g)
Food supplement #1	*Fucus vescicolosus*	Glass	37.0	*nd*	*nd*	*nd*
Food supplement #2	Soy lecithin	Paper, alluminum, plastic	3.2	*nd*	*nd*	*nd*
Food supplement #3	Multivitamin powder	Single-dose aluminum bag	*nd*	*nd*	*nd*	*nd*
Food supplement #4	Herbal preparation	Plastic, aluminum	*nd*	189.5	*nd*	*nd*
Food supplement #5	Proteic bar	Aluminium	*nd*	*nd*	*nd*	*nd*
Food supplement #6	Proteic bar	Aluminium	*nd*	*nd*	*nd*	*nd*
Food supplement #7	*Arthrospira platensis*	High-density polyethylene	5.0	*nd*	*nd*	*nd*
Food supplement #8	Baobab powder	Polystyrene	*nd*	*nd*	*nd*	*nd*
Food supplement #9	*Arthrospira platensis*	Polystyrene	*nd*	*nd*	*nd*	*nd*
Food supplement #10	Hempseeds	Polystyrene	92.1	*nd*	*nd*	*nd*
Food supplement #11	*Moringa oleifera*	Polystyrene	*nd*	*nd*	29.7	*nd*
Food supplement #12	*Fucus vescicolosus*	Plastic, aluminum	*nd*	*nd*	*nd*	*nd*
Food supplement #13	*Aphanizomenon flos-aquae*	Plastic, aluminum	*nd*	*nd*	*nd*	*nd*
Food supplement #14	*Arthrospira platensis*	Plastic	4.7	*nd*	*nd*	1.5
Food supplement #15	*Arthrospira platensis*	Glass	*nd*	*nd*	*nd*	*nd*

3.1. Heavy metals

3.1.1. Milk- and meat-based baby-food.

The determination of cadmium, lead and mercury was performed on the 16 meat- and 2 fruit-based baby food, whereas only cadmium and lead were determined in the 22 milk-based baby-food. In the **Table 13** and **Table 14** are reported the results of the monitoring activity performed on the baby-food, with the limit of quantification of the method for each heavy metal. Overall, the contamination of baby-food by these metals is negligible. Lead and cadmium were determined in a few samples, below the maximum limits set by the European Commission for these products (European Commission, 2023). As it can be seen, apart for one sample of meat-based baby-food, containing 0.007 mg/kg of cadmium, no sample was contaminated by cadmium, that being coherent with what is reported in the scientific literature (de Paiva et al., 2019). Lead is present in 2 out of 22 milk-based baby-food (9.1% of the analyzed samples), presumably because of the milk used as ingredient. Mercury was not determined in the tested samples.

It is quite evident that the cadmium, lead and mercury contamination in baby-food does not represent a real risk for the health of such a susceptible population group, that is the newborns and the children, That is also expected since the European Commission set maximum limits on those kind of products (European Commission, 2023) and that the official control laboratories are constantly performing analysis to check all the different kind of food for their heavy metal content.

Table 13. The cadmium and lead levels determined in the 22 samples of milk-based baby-food collected from markets in the Campania region and analyzed by UHPLC–MS/MS. The LOQ is 0.005 mg/kg for both cadmium and lead.

ID sample	Cadmium (mg/kg)	Lead (mg/kg)
Milk-based baby-food #1	< *LOQ*	< *LOQ*
Milk-based baby-food #2	< *LOQ*	0.014
Milk-based baby-food #3	< *LOQ*	< *LOQ*
Milk-based baby-food #4	< *LOQ*	< *LOQ*
Milk-based baby-food #5	< *LOQ*	< *LOQ*
Milk-based baby-food #6	< *LOQ*	< *LOQ*
Milk-based baby-food #7	< *LOQ*	< *LOQ*
Milk-based baby-food #8	< *LOQ*	< *LOQ*
Milk-based baby-food #9	< *LOQ*	< *LOQ*
Milk-based baby-food #10	< *LOQ*	< *LOQ*
Milk-based baby-food #11	< *LOQ*	0.044
Milk-based baby-food #12	< *LOQ*	< *LOQ*
Milk-based baby-food #13	< *LOQ*	< *LOQ*
Milk-based baby-food #14	< *LOQ*	< *LOQ*
Milk-based baby-food #15	< *LOQ*	< *LOQ*
Milk-based baby-food #16	< *LOQ*	< *LOQ*
Milk-based baby-food #17	< *LOQ*	< *LOQ*
Milk-based baby-food #18	< *LOQ*	< *LOQ*
Milk-based baby-food #19	< *LOQ*	< *LOQ*
Milk-based baby-food #20	< *LOQ*	< *LOQ*
Milk-based baby-food #21	< *LOQ*	< *LOQ*
Milk-based baby-food #22	< *LOQ*	< *LOQ*

Table 14. The cadmium, lead and mercury levels determined in the 16 samples of meat-based baby-food and 2 samples of fruit-based baby-food collected from markets in the Campania region and analyzed by UHPLC–MS/MS. The LOQ is 0.005 mg/kg for cadmium, 0.004 mg/kg for lead and 0.010 mg/kg for mercury.

ID sample	Cadmium (mg/kg)	Lead (mg/kg)	Mercury (mg/kg)
Meat-based baby-food #1	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #2	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #3	< *LOQ*	*0.012*	< *LOQ*
Meat-based baby-food #4	*0.007*	< *LOQ*	< *LOQ*
Meat-based baby-food #5	< *LOQ*	*0.015*	< *LOQ*
Meat-based baby-food #6	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #7	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #8	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #9	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #10	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #11	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #12	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #13	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #14	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #15	< *LOQ*	< *LOQ*	< *LOQ*
Meat-based baby-food #16	< *LOQ*	< *LOQ*	< *LOQ*
Fruit-based baby-food #1	< *LOQ*	*0.015*	< *LOQ*
Fruit-based baby-food #2	< *LOQ*	< *LOQ*	< *LOQ*

3.2. Microcystins

3.2.1. Cyanobacteria and algae-based food supplements

The method for the determination of the 9 microcystins has been applied to 7 algae-based food supplements collected from the supermarkets, pharmacies, and herbalists in the Campania region. The 7 samples were constituted by 4 food supplements containing Spirulina (*Arthrospira platensis*), 2 containing Fucus (*Fucus vescicolosus*) and 1 food supplement containing klamath algae (*Aphanizomenon flos-aquae*). The analyzed samples were not contaminated by the 9 microcystins tested except for the Klamath algae one in which MC-LA, MC-LR and MC-RR have been observed in the concentration of 111.56 ng/g, 26.62 ng/g and 1.14 ng/g, respectively.

Only food supplements containing algae were collected from the various shops and only a small quantity and variety of samples were found, therefore this survey cannot be considered as

representative of the market situation. Anyway, despite the small number of analyzed samples, the obtained results are coherent with those from the scientific literature. In fact, they showed that the most present microcystins are MC-LR and MC-LA and their occurrence has been observed mainly in the food supplements containing the Klamath algae. On the other hand, the spirulina-based food supplements have always been found to not be contaminated by microcystins, proving to be quite safe for the consumers' health (Brizio et al., 2013; Gallo et al., 2012; Roy-Lachapelle et al., 2017; Vichi et al., 2012).

3.2.2. The case of the Lake Avernus

The mussel and water samples, weekly collected from both the sea and the lake, were analyzed by liquid chromatography coupled to tandem mass spectrometry for identification and quantification of 9 microcystins and no contamination was found from these cyanotoxins. Moreover, the samples were also analyzed by high resolution mass spectrometry in untargeted mode to determine other cyanotoxins basing on the accurate mass of the precursor ions and their MS/MS spectra. Even without having the reference materials and without the need to develop a target method, other cyanotoxins, such as anabaenopeptins and oscillamide Y, were detected in all the samples except for the lake water. The identification was done on the matching between the exact and the accurate mass, with a mass error tolerance of 5 ppm, and on the presence of the most abundant ions reported in the scientific literature for those compounds. Indeed, the cyanotoxin occurrence observed in the samples was coherent with the contamination data and the toxicological profiles reported in the scientific literature for past *Planktothrix rubescens* bloom happened in the Lake Avernus (Ferranti et al., 2011). Of course, the lack of the reference standard materials of the suspect cyanotoxins didn't allow to quantify the cyanotoxins, but the untargeted analysis has still been helpful in identifying the compounds and protecting the consumers' health by stopping temporarily the commercialization of the contaminated mussels.

4. RISK EVALUATION FOR CONSUMERS' HEALTH

4.1 Risk assessment for bisphenol A in foodstuff

Following the request from the European Commission to re-evaluate the risks to public health from the presence of BPA in foodstuffs, in 2023 EFSA established a tolerable daily intake (TDI) for BPA (Lambré et al., 2023) taking into consideration the evidence from animal data and support from human observational studies. The CEP panel set a TDI of 0.2 ng BPA/kg bw per day, this way lowering 20000 times the previous t-TDI (Bolognesi et al., 2015). Afterward, the BPA dietary exposure estimated from the 2015 was compared with the new TDI resulting in a health concern for all age groups, including the more susceptible ones. Also, I participated at the "Stakeholder meeting on the draft scientific opinion on re-evaluation of bisphenol A (BPA)", held in 2022 after the public consultation as one of the two Italian representatives.

After the EFSA re-evaluation of the health risk associated to the BPA intake from diet, it has become even more important to understand which are the contamination levels that can be dangerous to human health, basing on the consumption data of the specific food. With the contamination data found during my PhD project, I decided to calculate the risk for human health using the Rapid Assessment of Contaminant Exposure (RACE) tool from EFSA (https://www.efsa.europa.eu/en/events/event/webinar-rapid-assessment-contaminant-exposure-race-tool).

The human exposure to BPA from consumption of the contaminated food has been evaluated on the different matrices, that is buffalo milk, bovine milk, meat-based infant formula, milk-based infant formula, and food supplements. The lowest contamination levels found during this survey, often corresponding to the method LOQs, were used to assess the risk for human health. When there was already an evident health risk associated to the lowest level, no further assessment was done using the highest contamination levels, since that would have obviously resulted in an even higher risk. The summary of the obtained results is reported in **Tables 15-17**; the risk assessment was evaluated only

for BPA, among the investigated compounds, both because of the available TDI and because it was the contaminant with a significantly high occurrence in the tested foodstuff.

Table 15. Exposure data for BPA in bovine and buffalo milk calculated using the lowest contamination level found, that is 0.5 ng/mL for both the matrices.

BPA in whole bovine milk

chronic consumer only output

Population group	Output Mean	Output 95	Output Unit
Infants	**9.078,335**	10.036,483	%
Toddlers	**7.439,412**	15.981,466	%
Other children	**3.503,996**	8.946,078	%
Adolescents	**1.313,725**	3.607,924	%
Adults	**941,267**	1.988,204	%
Elderly	**681,876**	1.769,231	%
Very elderly	**1.070,928**	1.436,389	%
Pregnant women	**299,208**		%
Lactating women	**708,414**		%

chronic country output

Survey's country	Output Mean	Output 95	Output Unit
Austria	**1.396,072**	3.738,885	%
Belgium	**3.678,177**	8.946,078	%
Bulgaria	**1.931,770**	4.166,667	%
Germany	**3.195,639**	9.491,026	%
Denmark	**2.950,752**	10.036,483	%
Estonia	**3.413,598**	4.829,423	%
Spain	**4.840,483**	10.681,818	%
Finland	**3.139,576**	3.991,431	%
France	**197,285**	376,202	%
United Kingdom	**7.439,412**	15.981,466	%
Greece	**708,414**		%
Croatia	**386,934**	897,436	%
Hungary	**1.070,928**		%
Ireland	**501,096**	1.598,602	%
Italy	**9.078,335**	5.064,000	%
Latvia	**2.274,751**		%
Netherlands	**3.216,631**	8.640,709	%
Portugal	**3.702,322**	13.748,750	%
Romania	**103,821**		%
Sweden	**1.832,350**	5.725,694	%

BPA in water buffalo milk

chronic consumer only output

Population group	Output Mean	Output 95	Output Unit
Elderly	27,747		%

chronic country output

Survey's country	Output Mean	Output 95	Output Unit
United Kingdom	27,747		%

BPA in European buffalo milk

chronic consumer only output

Population group	Output Mean	Output 95	Output Unit
Adults	60,976		%
Elderly	3,086		%

chronic country output

Survey's country	Output Mean	Output 95	Output Unit
Hungary	60,976		%

Table 16. Exposure data for BPA in milk-based and meat-based infant formulas, calculated using the lowest contamination level found, that is, respectively, 0.5 ng/mL and 0.94 ng/g.

BPA in milk-based infant formula

chronic consumer only output

Population group	Output Mean	Output 95	Output Unit
Infants	3.201,861	6.769,952	%
Toddlers	1.020,833		%
Other children	376,884		%

chronic country output

Survey's country	Output Mean	Output 95	Output Unit
Bulgaria	3.201,861	6.769,952	%
Germany	867,609		%
Estonia	1.509,716		%
Finland	480,984		%
France	2.616,597		%
United Kingdom	1.143,490		%
Italy	1.891,949		%
Portugal	1.942,124		%

BPA in meat-based infant formula

chronic consumer only output

Population group	Output Mean	Output 95	Output Unit
Infants	5.144,137	6.603,306	%
Toddlers	3.002,778	5.974,576	%
Other children	3.342,222		%
Very elderly	557,037		%

chronic country output

Survey's country	Output Mean	Output 95	Output Unit
Germany	657,822		%
Estonia	1.240,884		%
United Kingdom	2.581,380	6.603,306	%
Italy	5.144,137		%
Portugal	3.002,778		%

Table 17. Exposure data for BPA in food supplements, calculated using the lowest contamination level (3.2 ng/g) and the first level giving an exposure assessment higher than the established TDI (37.0 ng/g).

BPA in food supplements (level 3.2 ng/g)

chronic consumer only output

Population group	Output Mean	Output 95	Output Unit
Infants	270,741		%
Toddlers	124,709		%
Other children	285,205		%
Adolescents	58,629		%
Adults	81,741		%
Elderly	32,598		%
Very elderly	33,264		%

chronic country output

Survey's country	Output Mean	Output 95	Output Unit
Germany	285,205		%
Finland	135,756		%
United Kingdom	36,717		%
Italy	52,288		%
Latvia	270,741		%
Netherlands	8,737		%

BPA in food supplements (level 37.0 ng/g)

chronic consumer only output

Population group	Output Mean	Output 95	Output Unit
Infants	3.130,440		%
Toddlers	1.441,943		%
Other children	3.297,683		%
Adolescents	677,902		%
Adults	945,129		%
Elderly	376,909		%
Very elderly	384,611		%

chronic country output

Survey's country	Output Mean	Output 95	Output Unit
Germany	3.297,683		%
Finland	1.569,682		%
United Kingdom	424,545		%
Italy	604,575		%
Latvia	3.130,440		%
Netherlands	101,024		%

The mean output and 95 percentile output values are calculated as percentage of the estimated exposure in respect of the established TDI and the consumption data for the different population groups and the different countries.

As can be seen, the lowest contamination level found in bovine milk (0.5 ng/mL) is already way higher than the TDI, for all the population groups and for all the countries, included Italy. For the buffalo milk, both the available choices, that is water buffalo and European buffalo, did not have consumption data for Italy, therefore a real estimation on the human health risk could not be performed. Even if RACE did not report the consumption data, we know that buffalo milk is widely consumed in Italy in the form of milk-derived foodstuff in which the production process leads to a concentration of all the contaminants present in the milk. For that reason, we can presume that, even if the frequency of consumption of buffalo milk derived foodstuff is surely lower than the bovine milk and its by-products, there could be a risk for human health associated at least to the highest BPA contamination levels found in buffalo milk.

For the milk-based and meat-based infant formulas, that is a clear risk for the human health in all the age groups and in all the countries, including Italy. On the other hand, for food supplements the risk assessment is different basing on the contamination levels used for the RACE calculation; that was expected since the food supplements do not have as high consumption frequency as other foodstuff. In fact, for the food supplements, the first contamination level giving an estimated exposure higher than the established TDI was 37.0 ng/g, as it is reported in **Table 17**.

5. THE WAGENINGEN FOOD SAFETY RESEARCH EXPERIENCE

During the mandatory six-month period abroad, I worked as visiting PhD at the Wageningen University and Research (WUR) and, specifically, at the Wageningen Food Safety Research (WFSR), that is the part of the University involved in the official controls and the research activity about food safety in the Netherlands. The WFSR is also where two of the European Union Reference Laboratories (EURL), the mycotoxins and plant toxins one and the growth promotors one, are located. During the six-month spent into WFSR I was actively part of the team of Natural Toxins and I worked on the second part of a project about contaminant metabolites produced by plants. The starting idea was that the presence of contaminants, such as pesticides and pharmaceuticals, in soil and irrigation water may pose a risk for the safe production of food and feed. Since it is involved in official control activities and into the monitoring of both known and emerging toxins in feed and food, WFSR expertise is very high in the analysis of a variety of contaminants and a number of their animal/human metabolites. However, both at WFSR and in the scientific literature, there is an overall lack of knowledge about what is the uptake and metabolization of contaminants performed by plants which could be a way to bring contaminants back into the food chain. Since plant metabolites are not readily available as standards, the pilot study I worked on aimed at setting up a method to generate and identify them. The ultimate aim is indeed to build a model system that enables laboratories to characterize some metabolites by their elemental composition, chromatographic retention time and MS/MS fragmentation pattern so they can be added to an LC-hrMS database.

To this aim, WFSR explored a novel, quick and cheap method to produce these metabolites by exposing unicellular plants (algae) to a selection of model compounds in 2022. Algae are known to be able to metabolize contaminants and can easily be cultured. In the first experiment performed, the algae were exposed to a selection of insecticides (acetamiprid and imidacloprid) and pharmaceuticals (clindamycin and sertraline), some of which have already shown to be metabolized by plants. All four compounds contained Cl to be able to look at the isotopic pattern of Cl and easily identify the possible

metabolites. After incubation of the algae cultures with the contaminants, together with some control groups of course, the algal extracts were analyzed by LC-hrMS using an Orbitrap system according to the HBM4EU protocol (Huber et al., 2022). Data analysis was also similar to the HBM4EU protocol, which was very successful in pesticide metabolite analysis in human urines when Cl is present. The results of this first experiment were promising, metabolites that have not been described in the literature were detected in the extracts, but without being able to acquire the MS/MS spectra that are the golden standard for new compound annotation. After those results, the algal cultures were carried out again, by using clindamycin and imidacloprid from the first experiment and the adding doxycycline, flumequine and tilmicosin as pharmaceuticals with antibiotic activity. All the three new compounds do not have Cl atoms in their molecular formula, that being more challenging but still useful to check the applicability of the model to molecules that do not have a characteristic isotopic patter. Control groups of algal cultures without the adding of the contaminants were also performed during the second experiment to check that the annotated compounds are present only in the treated groups.

As visiting PhD at WFSR, my aim during the six-month period was to improve the system already used during the first experiment and, in particular, to optimize the extraction procedure in order to have higher concentrations of the putative metabolites and to acquire the MS/MS spectra.

Therefore, the first step has been to optimize the extraction procedure and to do that I tested three different extraction solvents, that is water, methanol and acetonitrile, and their different combinations both with and without formic acid. To test the trueness of the extraction procedure, I spiked a blank Chlorella supplement sample with 100.0 ng/g of all the 5 parent compounds and used LC-lrMS to quantify the compounds and their mean percentage recoveries. From this first step, I obtained information about the best extraction solvent that I then used for the next step, that was the actual extraction of the algal extract. The algae cultures were carried out in triplicates during the second experiment, therefore I chose to do some explorative analysis, by extracting and analyzing only one

of the triplicates for each compound and of the control groups, so that I could check that the method was properly working prior to proceed with the analysis of all the samples. I analyzed the samples by LC-hrMS on an Orbitrap IQ-X system (Thermo Fisher Scientific) acquiring the samples in Full scan/data dependent MS^2 mode and using also a scan mode that is prerogative of this Orbitrap model, that is the "deep scan" option. This acquisition mode is a way to go deeper into the sample, analysing all the compounds down to those at the lowest concentration levels. That is thank to the AcquireX Intelligent Data Acquisition technology (Thermo Fisher Scientific) that allows to perform several injections of the same sample, every time moving the previously detected compounds to the exclusion list. This way, the system will not be "blind" for those compounds in the next sample injection, and it will be "forced" to go deeper and look for the compounds with a lower concentration. By running the sample multiple times, it will be possible to see all of what is inside your sample, having a complete overview of its composition. After the hrMS acquisition, the raw data were processed with the Compound Discoverer software (Thermo Fisher Scientific) setting a workflow intended to generate the predicted metabolites of each parent compound and selectively look for them into the chromatogram. Nevertheless, the results obtained from Compound Discoverer were scrupulously checked to annotate the metabolites using the following criteria:

1) The mass error of the putative metabolite in respect to the predicted one did not have to be higher than 5 ppm.
2) The calculated isotopic pattern of the predicted metabolite had to match with the observed one.
3) The putative metabolite did not have to be present in the control group.
4) A MS/MS spectrum had to be acquired for the putative metabolite, to be able to match the fragmentation pattern of the metabolite with the one from the parent compound.

Putative metabolites were annotated for the five parent compounds, then all the other samples were extracted and analyzed with the same workflow, to check the presence of the annotated metabolites and to observe how their concentration change during the incubation period.

The results are still preliminary and need to be confirmed, therefore are not herein showed.

6. CONCLUSIONS

During my PhD project I developed and validated analytical methods to determine EDCs, heavy metals and microcystins in food and food supplements. After validation or extension of the validation, the methods were applied to the analysis of different food and food supplements to evaluate the occurrence of the contaminants of interest and, afterward, to perform a risk assessment for human health related to the consumption of these contaminated foods. The analytical performance parameters were satisfactory and fit for purpose for all the methods and in all the tested matrices, accounting for method reliability and ruggedness. The surveys were performed on bovine and buffalo milk for EDCs, on milk-based and meat-based baby-food for both EDCs and heavy metals, and on food supplements for both EDCs and microcystins. Additionally, the method for the determination of the microcystins was also applied in a food safety emergency case due to a cyanobacteria bloom in the Lave Avernus in March 2022, to determine microcystins in contaminated mussels, marine and lake water. Overall, the occurrence data obtained were significantly relevant for EDCs, especially for BPA that was the most abundant contaminant found in the analyzed samples. Because of that and because of the lowering of the TDI performed by EFSA in 2023, the risk assessment using the RACE tool was calculated for BPA in all the analyzed food basing on the contamination levels. As expected, the estimated exposure to BPA was higher than the TDI for all the foodstuff and in all the population groups even using the lowest contamination level. Only for food supplements, a higher contamination level was needed to exceed the set TDI. Furthermore, during the abroad period spent at Wageningen Food Safety Research (WFSR), I worked on an innovative research project aiming to use algal cultures as a model for obtaining and identifying metabolites of different contaminants. To this purpose, hrMS in untargeted mode and metabolite prediction tools were successfully used to annotate putative metabolites of three pharmaceuticals and two pesticides, proving the model to be effective and promising for building a plant metabolite database.

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