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Association between dietary exposure to bisphenols and body mass index in Spanish schoolchildren

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Abstract

The increase in children obesity worldwide has been of particular concern in recent decades. Environmental factors have been proposed as contributors to obesity, and there is a growing concern over obesogens, environmental chemicals with potential obesity-related endocrine-disrupting properties. In this regard, bisphenol A (BPA) and its analogues are suspected to have obesogenic properties. Current document report on the activities of the fellow, undertaken during the fourth, 2020–2021 cycle of the EU-FORA programme at the University of Granada, Institute of Nutrition and Food Science, in Spain. The work programme offered by the hosting site was related to the extrapolation of bisphenols exposure following the determination of these compounds in food frequently consumed by children and in their biological samples. The fellow has participated in the recruitment of the study population in the health centres. In addition, she has participated in the collection of the children biological samples, anthropometric measurements and dietary surveys and in the optimisation of the laboratory methodology for the extraction of bisphenols in biological samples. All these activities also provided the fellow an opportunity to develop her data science related skills, which will benefit her professional development. In addition, the fellow gained an overview of various topics related to food safety risk assessment by attending the EU-FORA dedicated training modules.

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1. Introduction

Endocrine disrupter chemicals (EDCs) are a family of exogenous substances able to alter the hormonal equilibrium of the body, they can cause adverse effects in nervous, cardiovascular and reproductive system in both men and women (Kabir et al., 2015). In the last decade, some studies have demonstrated EDCs can also behave as obesogens altering the endocrine system and then leading to the onset of obesity (Vaamonde and Álvarez-Món, 2020). Obesity is worldwide increasing over the last decades in adults as well as in children. In 2016, more than 1.9 billion adults, 18 years and older, were overweight, of these over 650 million were obese. World Health Organization (WHO) estimates the prevalence of overweight and obesity among children and adolescents aged 5–19 has risen dramatically from just 4% in 1975 to just over 18% in 2016. Nowadays over 340 million children and adolescents aged 5–19 are overweight or obese (Vaamonde and Álvarez-Món, 2020). This is considered of particular concern as this population can develop diabetes mellitus type II and cardiovascular disease among others. Recently different scientific studies have started to consider additional factors playing a role in the onset of obesity including diet and lifestyle habits, genetics (Herbert, 2008; Heindel and Blumberg, 2019), living environment (Nappi et al., 2016) and exposure to obesogens. Obesogens can be found in food, dust, water, drugs and personal care products (PCPs), so they can enter the human body by oral and dermal contact or inhalation (Monneret, 2017).

One of the most studied EDCs is bisphenol A (BPA). Since 1930, BPA has been used in the manufacture of polycarbonate plastics and epoxy resins, and their use is widely extended as cans coating in processed and ultra-processed food, where BPA can migrate from food contact material to food, and enter the body by diet. The European Food Safety Authority (EFSA) established a limit of $4 \mu\text{g kg weight}^{-1} \text{ day}^{-1}$ (EFSA, 2015); however, recently, a new limit of $0.04 \text{ ng kg weight}^{-1} \text{ day}^{-1}$ has been established (EFSA, 2021). Because of legal limits, industries have started to use BPA analogues (bisphenols, BPs) with similar chemical structures and behaviour. However, specific limit values of exposure have not been established yet for these compounds. BPs more commonly used today are bisphenol S (BPS), bisphenol F (BPF), bisphenol E (BPE), bisphenol AF (BPAF), bisphenol P (BPP) and bisphenol B (BPB). However, some studies have demonstrated that BPA analogues show endocrine disrupting activities similar to BPA, and could act as obesogens at low concentrations (Andújar et al., 2019; Martínez et al., 2020; Reina-Pérez et al., 2021). We have recently demonstrated that some of those BPs including BPA are present in foodstuffs (Gálvez-Ontiveros et al., 2021; García-Córcoles et al., 2018).

Moreover, evidences of toxicity related to other BPA analogues used as substitutes of BPA are still missing due to their recent appearance; they include bisphenol C (BPC), bisphenol Z (BPZ), bisphenol AP (BPAP), bisphenol M (BPM) or bisphenol FL (BPFL). All chemical structures of target bisphenols are shown in Appendix A.

The interest in finding an association between BPA and analogues exposure with obesity is currently growing in the scientific community. In a recently published study, we have demonstrated an association between dietary exposure and both total bisphenols and BPA in overweight/obese adolescent girls (Robles-Aguilera et al., 2021). Therefore, the main objective of the fellow work program was to learn how to measure bisphenols exposure in a children population to establish a correlation between exposure and overweight/obesity.

1.1. Ethical considerations

This proposal was developed according to the Helsinki Declaration and human rights and biomedical research. This proposal respect UNESCO Universal Declaration about human genome and human rights. Moreover, the proposal was carried out according to the protocol established by the Organic Law 15/1999, 13 December, which includes data about Personal Data Protection Law 41/2002.

2. Description of work programme

2.1. Aims

As part of the EU-FORA fellowship, the focus of this study was for the fellow to be involved in all the activities required to obtain data, tools and possible biomarkers to correlate the estimation of dietary exposure to BPA and analogues with endocrine-disrupting activity (BPS, BPP, BPF, BPB, BPE and BPAF) in schoolchildren population and its impact in obesity. Her work was included the EFSA Partnering Grants 'OBEMIRISK-Knowledge platform for assessing the risk of Bisphenols on gut

microbiota and its role in obesogenic phenotype: looking for biomarkers' (Grant Agreement Number – GP/EFSA/ENCO/2018/03 – GA04). Changes were proposed due to delays in field work in the collection of samples due to the COVID-19 pandemic. The objectives were focus mainly on the estimation of BPA and analogues in foods frequently consumed by the population under study, to acquire knowledge in measuring the levels of these endocrine disruptors in different biological samples and to collaborate in the know-how platform database.

In support of this objectives, the fellow also learn how to determine individual SNPs from hormonal receptor genes. However, she does not participate in the genetic laboratory analyses due to COVID restriction of the use of the laboratory.

2.2. Activities/methods

As part of the fellowship, the priority of the hosting site was to provide the fellow with the basic theoretical background required to develop the questionnaires and perform the biological samples analyses. The fellow joined a working team with proved expertise in the subjects.

- Population recruitment: sample collection (swabs, urine, saliva, hair and nails), FFQ (Food frequency questionnaire) and anthropometric analyses in health centres.
- Experimental design (procedure of bisphenols extraction from saliva and urine, analytical methods validations)
- Software tools specific for design of experiments, databases and data interpretation (Statgraphics plus v.5.0; SPSS v.23; MassLynx v4.1).
- Analysis of bisphenols in food and biological samples.
- In addition, the fellow benefited from the EU-FORA dedicated training modules.

2.3. Food analysis

A total of 100 food products were purchased from the main Spanish supermarket chains. The selected foods represent 95% of the daily intake of energy, macronutrients and micronutrients of the children selected in this study (Gálvez-Ontiveros et al., 2021). Most of the foods were packaged in plastic containers, cans and tetra bricks. The foods were divided into three categories based on the NOVA classification (Monteiro et al., 2018). The categories recognised were minimally processed or unprocessed, processed and ultra-processed.

The bisphenols determined in food were a total of 7 (BPA, BPAF, BPB, BPE, BPF, BPP and BPS).

The fellow learned the extraction and quantification protocol for bisphenols in food along with the analysis and interpretation of the results (Gálvez-Ontiveros et al., 2021) and carried out the entire procedure for better understanding and consolidation.

Regarding the foods analysed (Appendix B. Food list), the presence of bisphenols was detected in 51% of samples. BPA was detected in the majority of food samples analysed (28%) followed by BPS (26%) and BPE (4%). The rest of the studied bisphenols were not detected. The concentrations ranged from 1 ng g⁻¹ (chocolate palm tree) to 409 ng g⁻¹ (canned tuna in oil). The Figure 1 and Table 1 below show the described results.

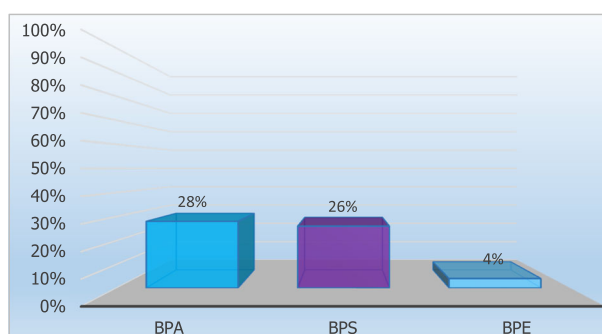


Figure 1: Percentage of detected bisphenols in analysed food samples

Table 1: Frequencies (%) and mean (ng g^{-1}) of bisphenols in analysed food samples (Gálvez-Ontiveros et al., 2021)

	Bisphenols in foods			
	BPS	BPE	BPA	ΣBPs
Unprocessed or minimally processed foods (n = 32)				
Frequency (%)	46.88	6.3	21.88	63
Mean (ng g^{-1})	17.27	< LOQ	6	18.35
Processed foods (n = 21)				
Frequency (%)	38.1	0	38.1	67
Mean (ng g^{-1})	39.49	0	86.3	35
Ultra-processed foods (n = 47)				
Frequency (%)	6.38	4.26	27.66	36
Mean (ng g^{-1})	47.48	< LOQ	35.3	38.34
All (n = 100)				
Frequency (%)	26	4	28	51
Mean (ng g^{-1})	28.99	< LOQ	43.28	30.4

ΣBPs: ΣBisphenols; LOQ: limit of quantification.

The obtained results agreed with other evidences obtained in previous scientific studies where it was observed that the highest concentrations of bisphenols have been found in canned foods (Gallart-Ayala et al., 2011; Alabi et al., 2014; Russo et al., 2019). This may be due to the food contact material covering the cans; it is usually epoxy resin obtained mainly from BPA (Abraham and Chakraborty, 2020) that can migrate from the container to the food. In addition, it was observed that the greater is the contact time of food with the container, the greater will be the migration towards food, with canned foods being the foods that spend the longest time stored in the pantries of our houses.

2.4. Recruitment of the study population

2.4.1. Study population

The fellow has participated in the recruitment of the studied population. The age of the children selected for this study was ranged between 6 and 12 years old. The recruitment of the population was carried out in different health centres where the fellow proceeded to collect the biological samples (hair, swab, nails, saliva and urine), to take anthropometric measurements (weight, height, circumference of waist and hip and bioimpedance) and to submit the survey containing questions related to dietary habits, physical activity and exposure to bisphenols (Appendix C). Moreover, the fellow learnt how to perform bisphenol exposure assessment using the data collected in the questionnaires.

Urine and saliva samples were stored at -80°C , while hair, nail and swab samples were stored at room temperature until laboratory analysis.

2.4.2. Anthropometry

The anthropometric measurements were collected taking into consideration the documents recommended by the WHO and the recommendations of the THAO program for the prevention of childhood obesity (Gomez et al., 2014). The measuring instruments applied included: Floor scale (model SECA 872), Tallimeter (model SECA 214 (20–207 cm)) measuring tape to measure the waist circumference (model SECA 201) and the Plicometer (model Harpenden Skinfold Caliper-0120) to measure skinfolds.

The fellow collaborated with the Dieticians-Nutritionists to learn how to make anthropometric measurements for the subsequent classification of the population.

2.4.3. Survey

A questionnaire has been designed and validated to assess the risk of schoolchildren exposure to BPA and its analogues through food consumption (Appendix C) (Robles-Aguilera et al., 2021). The fellow had the opportunity to improve the questionnaire and contribute to the final version.

2.5. Analysis of biological matrixes

Saliva, urine, nails and hair have been taken from children and new methodologies to determine target endocrine disruptors have been developed. Selected bisphenols to be determined in all samples were BPAF, BPF, BPE, BPA, BPC, BPB, BPZ, BPS, BPAP, BPM, BPP and BPFL (Appendix A).

2.5.1. Saliva

Procedures to analyse saliva were out-of-date, so first step was to develop a new original method to guarantee optimum extraction of all bisphenols from the samples. The fellow helped to design the experiments and to apply the extraction procedure. Final results about the optimisation and validation have been published recently (Moscoso-Ruiz et al., 2022). Briefly, 1 g of fresh saliva is subjected to a protein precipitation with ACN and acidic medium, followed by an ultrasound-assisted extraction with ethanol and re-extracted with acetone (30 min, 35% power). Finally, the dry residue was reconstituted with MeOH/H₂O (30/70) (v/v) and injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) equipment. The fellow also learned how to optimise parameters in the equipment and the theory behind spectrometry technique. Once the method was validated for the sensitivity and sensibility, it was possible to use it to analyse samples of children saliva.

A total of 74 samples were analysed following this protocol. Results in Figure 2 show that 38% of samples contained BPA, being the most frequently detected bisphenol. However, several BPA analogues have been detected as BPP (28%), BPAP, BPAF, BPM (11%), BPB (8%), BPE, BPC (5.4%) and BPZ (4%).

These results cannot be compared with other scientific studies, indeed as far as we know this is the first study analysing BPA and analogues content in children saliva. However, Gomes et al. (2020) reported evidences about BPA presence in saliva after dental treatment, as the dental composites and resins contain this compound.

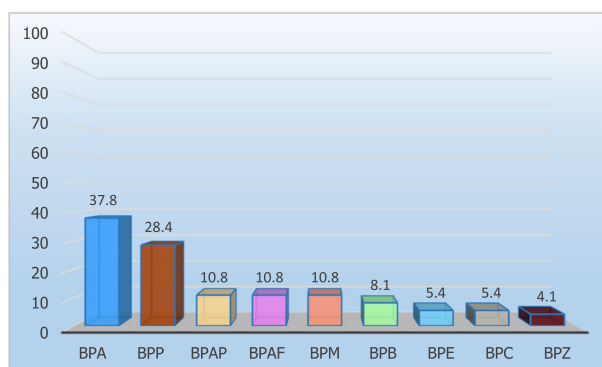


Figure 2: Results from children saliva

2.5.2. Urine

The procedure to analyse urine samples was taken from Vela-Soria et al. (2014) with some modifications. Analysis of urine was performed by duplicate, with and without enzyme (total and free BPs). Briefly, for free BPA, 4 mL of urine were taken and 4 mL of NaCl (aq) 10% (p/v) were added to provoke 'salting out' effect, plus 100 µL of HCl 6 N to adjust pH to 2. Extraction was made by injecting a solution of 400 µL of acetone and 600 µL of ethanol. The inferior portion of the mix was separated in a new tube and the extraction was repeated four times, mixing the inferior parts. After that, an evaporation of the mix was carried out, reconstituted in MeOH/H₂O (30/70) (v/v) and injected into the LC-MS/MS equipment. Total BPs was determined by adding to urine aliquot two different enzymes: 100 µL of β-glucuronidase from *Helix Pomatia* (solid) in acetate buffer and 25 µL of β-glucuronidase/sulfatase (liquid). The same procedure was repeated to determine free BPs. After the method validation, the procedure was carried out for all samples of urine collected. Results are illustrated in Figure 3. They show that 40% of urine samples contained detectable quantity of BPA, but also we found BPAF (5%) and BPF (1.3%) in different samples. These results are in concordance with other studies concerning BPs in children urine; some of them detected BPA in remarkable concentrations and

even established an association between BPA level in urine and body mass index (Tschersich et al., 2021; Mahfouz et al., 2021).

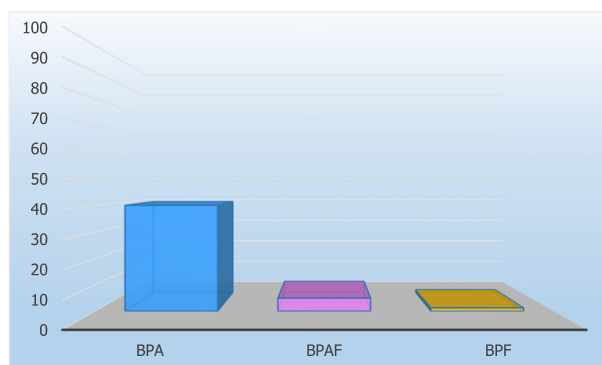


Figure 3: Results from children urines (free + conjugated)

3. Conclusions

3.1. Conclusions from the laboratory results

This project focused on the analysis and study of several matrices where BPA and its analogues can be accumulated and evaluated as potential non-invasive human samples. Furthermore, food analysis, in concordance with other studies, has demonstrated there are remarkable levels of BPs in foodstuffs. On the other hand, the presence of BPs in children biological samples confirmed that BPs bioaccumulate into the body, and further studies are needed to establish reliable biomarkers of exposure. The final objective of the project was to demonstrate whether exposure to bisphenols is associated to overweight and obesity in children. The statistical analysis of the results is still ongoing to evaluate the relationship between BPs dietary exposure, their presence in biological matrices and overweight and/or obesity in children.

3.2. Scientific activities during fellowship

During the fellowship, the fellow had the opportunity to participate to various scientific activities including participation in various conferences/seminars/webinars:

XXV Jornadas Internacionales Nutrición Práctica y el XIV Congreso Internacional de la SEDCA. She has contributed with the communication 'Consumo de los alimentos en niños en edad escolar para su uso en la estimación de la ingesta a disruptores endocrinos'. Held in Madrid, Spain, from April 20 to 22, 2021.

XXV Jornadas Internacionales Nutrición Práctica y el XIV Congreso Internacional de la SEDCA. She has contributed with the communication 'Concordancia entre la autopercepción del peso corporal y el índice de masa corporal en una muestra de adolescentes españoles'. Held in Madrid, Spain, from April 20 to 22, 2021.

3.3. Conclusions from the participation in the fellowship programme

The EU-FORA programme offered the fellow an opportunity to learn about BPs dietary exposure and its association with overweight/obesity in children. The fellow participated and learnt field work such as submitting questionnaires to children in health centres, collecting biological samples such as urine, saliva, hair and nails and performing chemical analyses to evaluate their presence in foods frequently consumed by the population under study and in the biological samples collected. The fellow learn about risk assessment of bisphenols reviewing literature related to the physico-chemical characteristics of the substances, their health effects and the regulatory framework they are framed, as well as practical hands-on exercises on risk assessment. This experience also provided the fellow an opportunity to develop her data science related skills, which will benefit her professional development as a data analyst.

In addition, the fellow gained an overview of various topics related to food safety risk assessment by attending the EU-FORA dedicated training modules.

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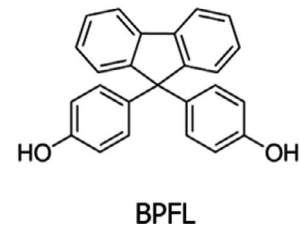
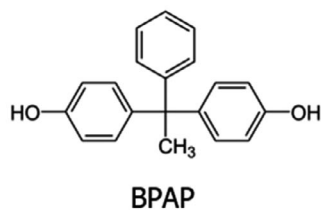
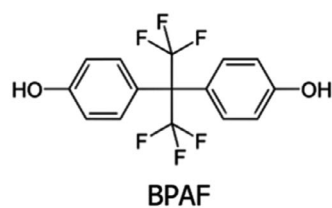
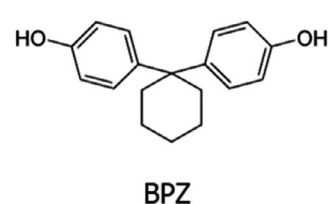
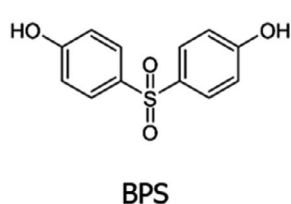
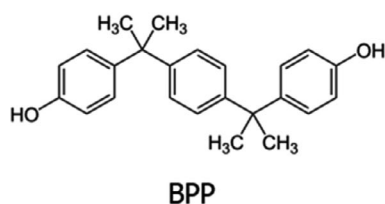
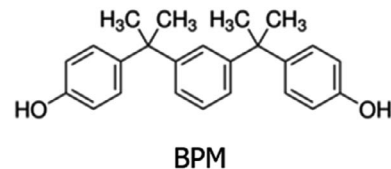
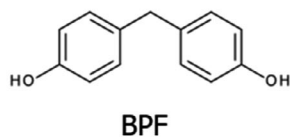
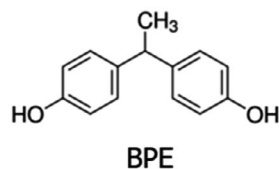
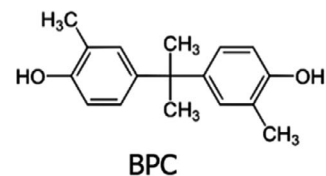
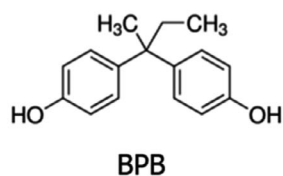
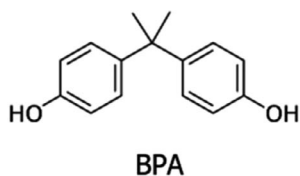
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Abbreviations

AESAN	Spanish Agency for Food Safety and Nutrition
BFPL	bisphenol FL
BPA	bisphenol A
BPAF	bisphenol AF
BPAP	bisphenol AP
BPB	bisphenol B
BPC	bisphenol C
BPE	bisphenol E
BPF	bisphenol F
BPM	bisphenol M
BPP	bisphenol P
BPS	bisphenol S
BPZ	bisphenol Z
Bw	body weight
ECHA	European Chemical Agency
HCl	hydrochloric acid
LC-MS	liquid chromatography–mass spectrometry
MeOH	methanol
MRM	multiple-reaction-monitoring
NaCl	sodium chloride
TDI	tolerable dietary intake

Appendix A – Structures of target bisphenols



Appendix B – Food List

FOOD LIST	
Chocolate doughnuts	Canned tuna in oil
Milk bread	Frozen hake
Croissants	Tuna dumplings (frozen precooked)
Chocolate palm (puff pastry with chocolate)	Battered hake sticks (frozen precooked)
Cacao-filled roll (Bollycao)	Bottle water
Chocolate rice pancakes	Pizza
Homemade cake	Ketchup
Muffins	Tomato sauce
Hamburger bun	Grape
Sandwich bread	Blueberries
Milk bread with chocolate (Weikis)	Pineapple
Cooked ham	Raspberries
Sausage (salchichon, chorizo, frankfurt sausages, turkey cold cut and mortadella)	Melon
Chicken burger	Apple
Chicken	Packaged apple
Serrano ham	Pear
Olive	Frozen red fruit mix
Anchovy stuffed olives	Frozen mango
Whole milk plastic bottle	Frozen chopped garlic
Tetra brick whole milk	Frozen chopped onion
Chocolate milkshake	Frozen chopped parsley
Semi-fermented milk	Frozen spinach
Yogurts (fruits and natural)	Tomato
Liquid yogurt	Packaged tomato
Cheese (slice, spread and melting)	striped carrot
Lentils	Packaged carrot
Pasta	packaged lettuce
Microwave rice cups	Packaged pumpkin
Candy jelly	packaged mushrooms
Corn snacks	Guacamole
Chips	Green pepper
Nachos	Packaged eggs
Canned sweet corn	

Appendix C – Exposure to Bisphenols Food Questionnaire

Link:

<https://docs.google.com/document/d/1Ryq2WwbnJRZgGP5qaHsCuJ1UQo62LqDR/edit?usp=sharing&ouid=112625456176696250428&rtpof=true&sd=true>