3. CONTENTS

3.1. Introduction

The current coronavirus pandemic precluded the collection of the target samples, as the hospital services were mostly entirely dedicated to treat Covid-19 patients. For this reason, the collection of urine samples from early menopause women could not be performed. As a contingency plan, previous urine and house dust samples already collected from volunteers living in the same geographical area (Aveiro district) were analyzed. Additionally, samples from volunteers undergoing an educational program on the impacts of environmental contaminants in human health were also collected and analyzed.

Hence, the aim of this report is to describe the levels of phenolic compounds, namely triclosan, triclocarban, parabens, benzophenones and bisphenols in urine and dust samples from volunteers living is Aveiro district and to describe exposure levels and to understand if educational programs are able to reduce exposure to these contaminants.

3.2. Procedure

3.2.1. Sampling

This study was approved by the Ethics Committee of Centro Hospitalar do Baixo Vouga and by the Portuguese National Data Protection Agency. Samples from volunteers inhabiting Aveiro district were collected between 2017 and 2019. Urine samples from (n=46) were collected directly into sterile containers and several aliquots of 1.5ml were prepared and immediately frozen. Two aliquots of each sample were transported to CMES in cool conditions and preserved at -20^{o}C until analysis. Whenever possible, matched dust samples were collected. Dust samples (n=41) were retrieved from the vacuum cleaner bag that was delivered to the volunteers and that was used for 60 days. The samples were then sieved trough a 63µm sieve and preserved at room temperature in dark conditions until chemical analysis.

A sub group of seven individuals were invited to participate in an intervention study. The participants were asked to provide a urine and house dust sample and to attend an educational program. This program focused on the impacts of exposure to environmental contaminants in human health and strategies to reduce exposure. After 30 days of the educational session, the participants were asked to provide another urine and dust sample. Details on the educational program are fully described elsewhere (Souto-Miranda et al. 2020).

3.2.2. Chemical analysis

At CMES, levels of triclosan, triclocarban, parabens (methyl, ethyl, propyl, butyl), benzophenones 2-OH-4-MeO-BP (BP-3); 2,4-diOH-BP (BP-1); 2,2'-diOH-4-MeO-BP (BP-8); 2,2',4,4'-tetraOH-BP

(BP-2); 4-OH-BP) and bisphenols (BPA, BPB, BPC, BPF, BPS, BPZ, BPAF, TBBPA) were quantified in urine samples. The target compounds were analyzed following the protocol described by Kunisue et al. (2010) after some modifications and optimization by the team members (see LaMer 2017/18 report). Briefly, 50 µL of Internal Standards (ISs) Mixture was added to each urine sample after being hydrolised with β-glucuronidase/aryl-sulfatase for 16h at 37^0C. Afterwards, cold methanol, ultrapure water and ammonia solution (5% NH_4OH) were added. The samples were then loaded into a pre-conditioned OASIS MAX cartridge (MTBE, Methanol and ultrapure water). Prior to elution with formic acid:MTBE:methanol = 0.2: 3: 7 (v/v/v), the cartridges were washed with 5% NH_4OH, 5% NH_4OH in methanol, Milli-Q-water: methanol = 0.2: 6: 4 (v/v/v) and afterwards completely dried for 15 min. The eluted target compounds were evaporated to dryness under nitrogen flux and re-dissolved with acetonitrile and 50 µL of mixture of naproxen-$^{13}C,d_3$ and ketoprofen-$^{13}C,d_3$ (20 ng/mL each) was added. The samples were preserved in amber LC glass vials at 4^0C and before injection into the LC-MS/MS, Milli-Q water was added to a final volume of 1mL.

For dust samples, ISs mixture was added to 10mg of dust sample. The target compounds were extracted twice by ultra-sonication with acetonitrile and methanol. After two centrifugations, the supernatants were combined and afterwards diluted with ultra-pure water and 5% NH_4OH. The sample was then loaded into a pre-conditioned OASIS MAX cartridge (MTBE, Methanol and ultrapure water). Prior to elution with formic acid : MTBE : methanol = 0.2: 3: 7 (v/v/v), the cartridge was washed with 5% NH_4OH and methanol and afterwards dried for 15 min. The eluted target compounds were evaporated to dryness under nitrogen flux and re-dissolved with acetonitrile and ultra-pure water. The samples were also preserved in amber LC glass vials at 4^0C until injection into the LC-MS/MS.

The internal standard (ISs) mixture contains 100 ng/mL of Triclosan-$^{13}C_6$, 20 ng/mL of Triclocarban-$13C_6$, 20 ng/mL of Methyl paraben-$^{13}C_6$, 20 ng/mL of Butyl paraben-$^{13}C_6$, 20 ng/mL of BP-3-d_5, 100 ng/mL of Bisphenol A-d_{16}, 100 ng/mL of Bisphenol F-$^{13}C_6$, 20 ng/mL of Bisphenol S-$^{13}C_{12}$, 20 ng/mL of Bisphenol AF-d_4 and 20 ng/mL of Tetrabromobisphenol A-$^{13}C_{12}$.

The activity of β-glucuronidase/aryl-sulfatase was 290 units per mL of urine. The solution was prepared twice a week by adding 4.7 ml of 1.0 mol/L ammonium acetate; 5.3 mL of 1.0 mol/L acetic acid and 50 µL of β-glucuronidase/aryl-sulfatase solution (116,000 units/mL). The crude mixture of β-glucuronidase/sulfatase from Helix pomatia (Type HP-2, aqueous solution, 116,000 units/mL glucuronidase and 1020 units/mL sulfatase) was purchased from Sigma-Aldrich (St. Louis, MO, USA); (G7017).

3.4. Results

Of the 19 compounds analyzed, only 3 bisphenols, namely BPB, BPC and BPZ were not detected in any type of sample, whereas triclocarban was not detected in urine samples. Generally, the highest detection frequencies were obtained in dust samples and the most frequent compound in both samples was Ethyl paraben (100% detection in both dust and urine). For dust samples, 14 of the 19 target compounds were detected in more than 50% of the samples, whereas for urine only five compounds were detected in 50% of the individuals. Furthermore, the profiles of the phenolic compounds were also different as it can be clearly noticed in figure 1.

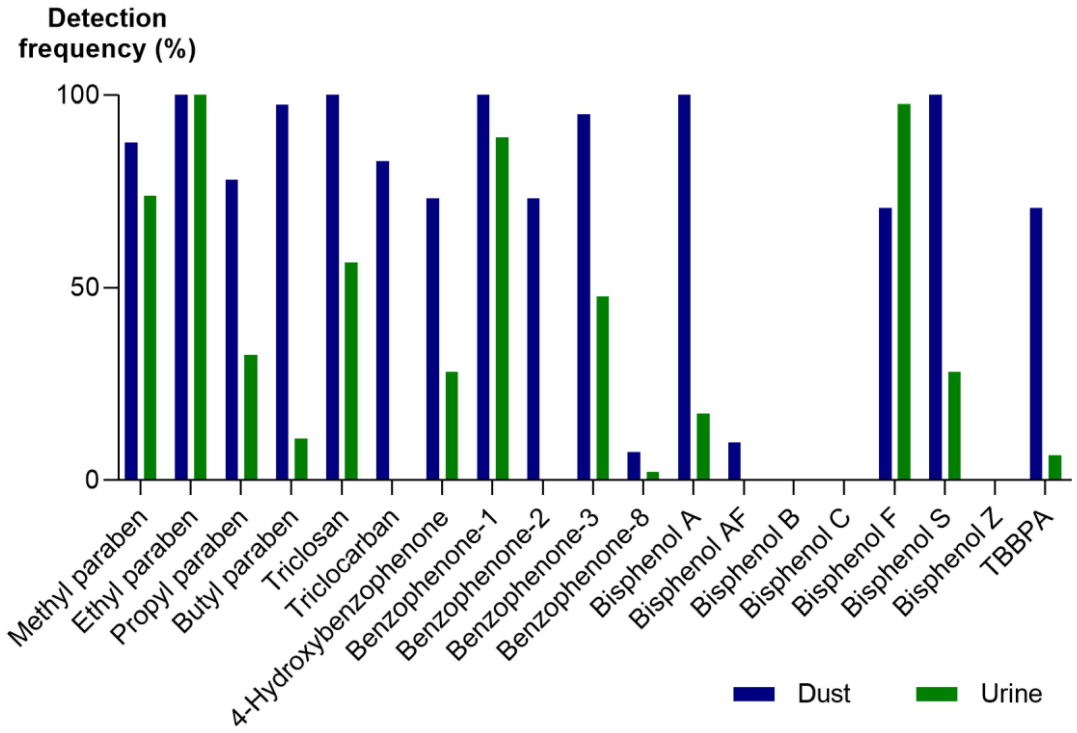

Figure 1. Detection frequency (%) of each EDC in all the dust (n=41) and urine (n=46) samples analyzed.

Overall, in urine samples there is a marked predominance of ethyl and methyl paraben, BPF and benzophenone-1, followed to a lesser extent by triclosan and benzophenone-3. In dust samples, as previously mentioned the frequency profiles varied in a lesser extent with most of the compounds being detected in the majority of the analyzed samples.

The profiles for each phenolic compounds detected in urine and dust samples are described in Figures 2 and 3. Overall, in urine samples the highest average concentration detected was for BPF with 8.7 ng/g, followed by methyl paraben with 4.0ng/g (figure 2). For dust samples, a different profile was obtained, with the highest average being found for BPA (2310 ng/g).

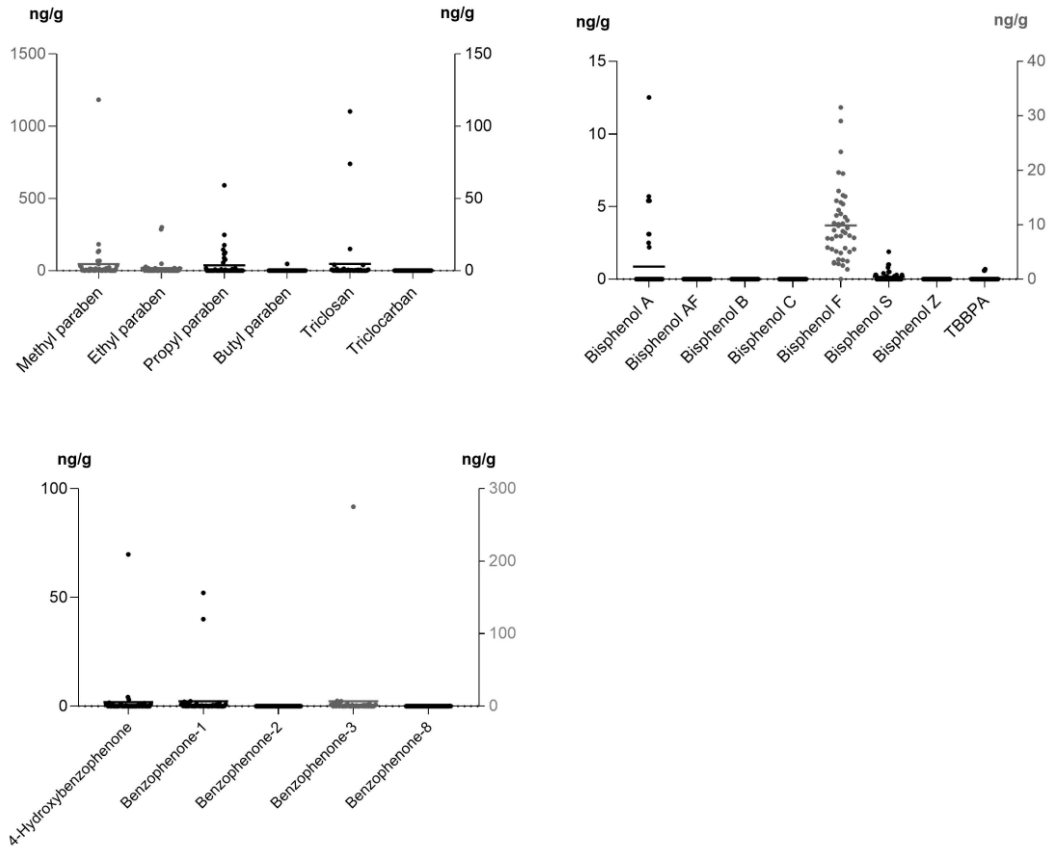

Figure 2. Scatter plot summarizing the values of parabens, antimicrobials, bisphenols and UV-filters in the analyzed urine samples.

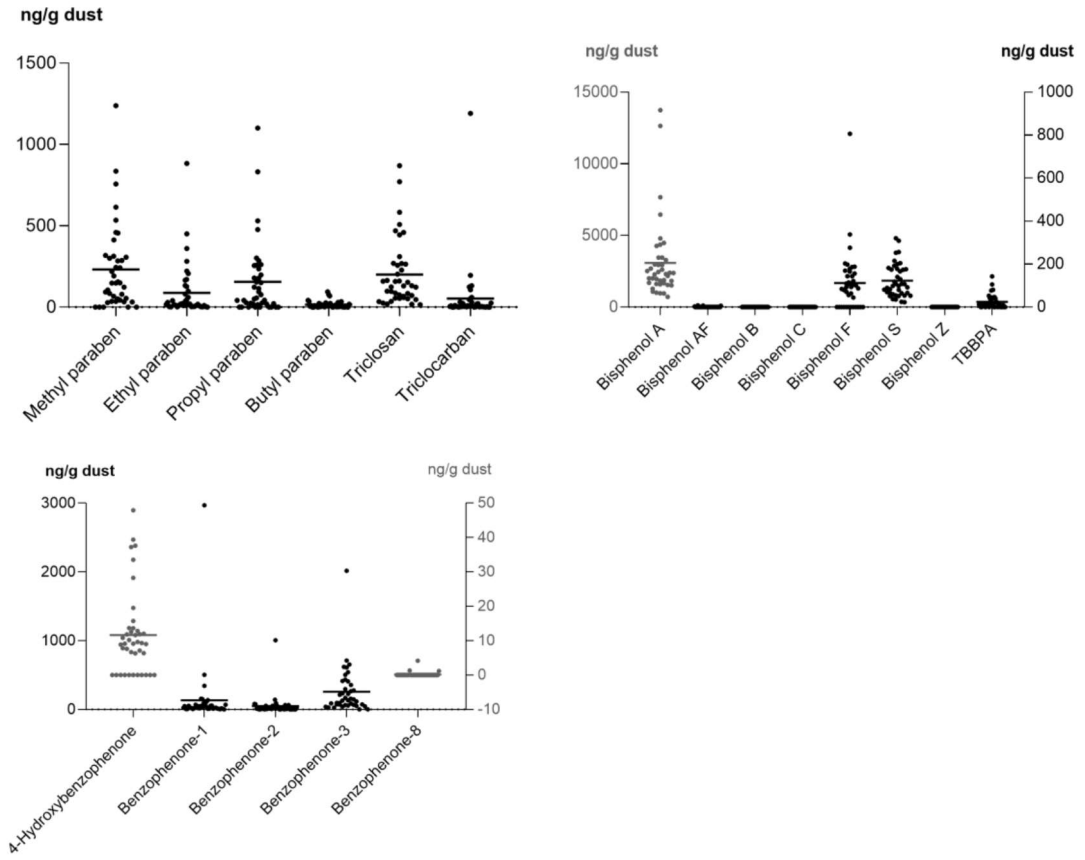

Figure 3. Scatter plot summarizing the values of parabens, antimicrobials, bisphenols and UV-filters in the analyzed dust samples.

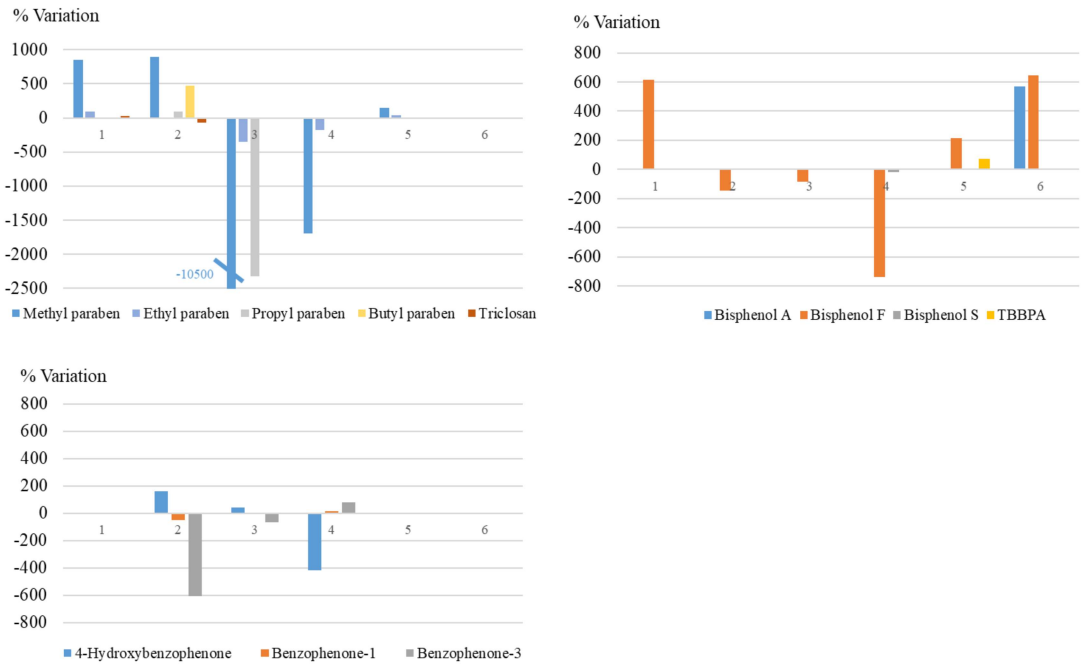

Figure 4. Variation (%) of the levels of parabens, antimicrobials, bisphenols and UV-filters in urine samples collected before and one month after the intervention.

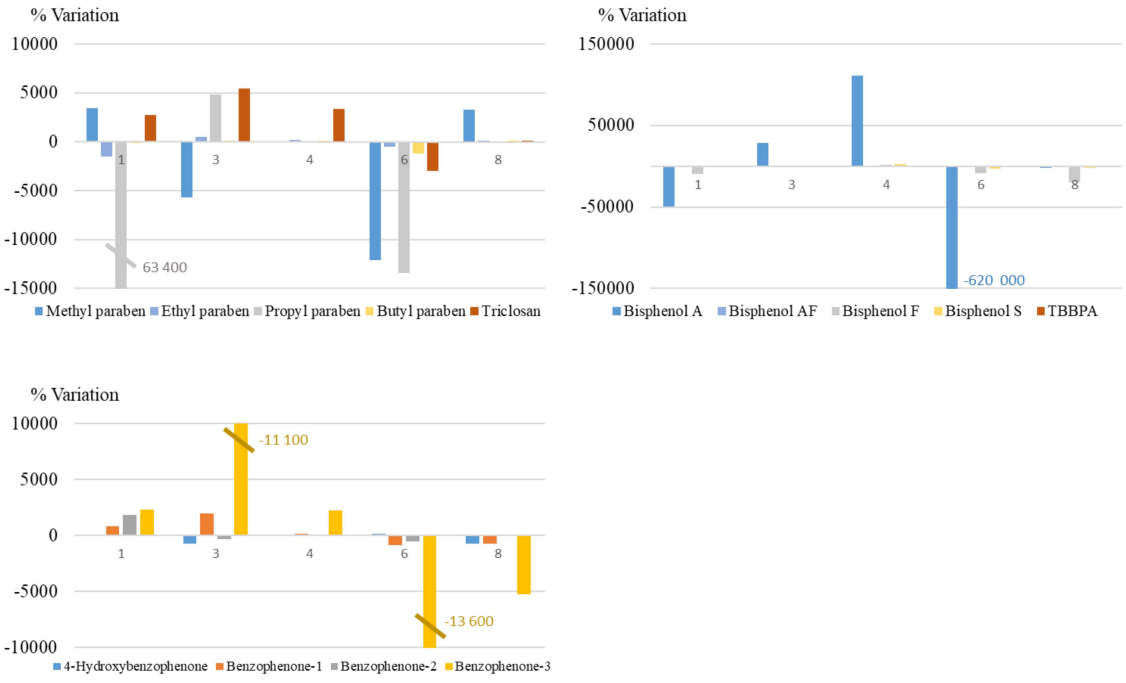

Figure 5. Variation (%) of the levels of parabens, antimicrobials, bisphenols and UV-filters in dust samples collected before and one month after the intervention.

Concerning the intervention study, the obtained results varied widely between participants and contaminants (figure 4). Generally, higher variations were noticeable in dust samples, with the levels of BPA in house dust from volunteer 6 registering the most spectacular decrease, yet for this same individual an increase in urine levels was observable. On the other hand, no clear increase or decrease tendency could be observed between contaminants. Such results stress the need for need for future interventions studies and with more volunteers.

3.5. Future perspectives

Clinical data from all the patients is now being computed and analyzed. The obtained results from the chemical analysis will be analyzed by means of multivariate statistics and correlated with clinical data and life-style questionnaires. We plan to write 2 research papers in 2021 with the obtained data.

3.6. Achievements

Oral Communications by Invitation

- Sousa ACA (2020) Multidisciplinary approach to understand the role of environmental contaminants in respiratory diseases – Overview of the RESPIRA project. JECS Special seminar. National Institute for Environmental Studies (NIES), Tsukuba, Japan, 25th January 2020.

Oral Communications

- Alda M, Sousa A, **Sousa ACA**, Souto-Miranda S, Valente C, Freitas C, Almeida C, Andrade B, Dias J, Dias S, Tavares A (2020) PRISMA - Pulmonary Rehabilitation: a response for patients with COPD in an InduStrialized environment and its implication on lung MicrobiotA. 7eme Colloque de Restitution OHM- Estarreja, 10 January 2020, Universit of Aveiro, Aveiro, Portugal, OHM-E/2018/Proj.2

Posters in Conferences/Workshops

- Madureira J, **Sousa ACA**, Silva AI, Tanoue R, Kunisue T, Tanabe S, Teixeira JP, Costa C (2020) Endocrine disrupting chemicals in indoor dust and the implications for human exposure: preliminary findings. 20th International Symposium on Environmental Pollution and its Impact on Life in the Mediterranean Region (MESAEP), 26-27 October 2020. P. 211
- Guimarães J, Barros R, Pastorinho MR, Lemos MC, **Sousa ACA** (2020) Evaluation of

mercury exposure in pregnant women from Aveiro District. XV annual CICS-UBI Symposium, 1-2 October 2020, Covilhã, Portugal, p.53

- Souto-Miranda S, Melo-Dias S, Valente C, Freite C, Sousa AM, **Sousa ACA**, Marques A (2020) Patients' perspectives on exposure to environmental contaminants: a missing piece in pulmonary rehabilitation? 30th European Respiratory Society Annual Congress 2020, 5-9 September 2020, Vienna, Austria

- Sousa ACA, Assunção R, Coelho SD, Kunisue T, Tanabe S, Pastorinho MR (2020) Dietary exposure to Cadmium and the associated burden – results from a duplicate diet study in Portugal (2012). Glob 1st WG meeting & 2nd MC meeting Burden of Disease in Europe Taking stock and moving forward. National Food Institute, Technical University of Denmark, 18-19 February 2020, Copenhagen, Denmark, p. 21

Peer-reviewed abstracts in international conferences

- Sousa ACA, Assunção R, Coelho SD, Kunisue T, Tanabe S, Pastorinho MR (2020) Dietary exposure to Cadmium and the associated burden – results from a duplicate diet study in Portugal (2012). Glob 1st WG meeting & 2nd MC meeting Burden of Disease in Europe Taking stock and moving forward. National Food Institute, Technical University of Denmark, 18-19 February 2020, Copenhagen, Denmark, p. 21, https://www.burden-eu.net/docs/m1wg-abstract-book.pdf

Book chapters

- Pastorinho MR, Sousa ACA (2020) Pets as sentinels of human exposure to neurotoxic metals. *In* Pastorinho MR, Sousa ACA (Eds) Pets as Sentinels, Forecasters and Promoters of Human Health. Springer International Publishing AG, Switzerland, ISBN: 978-3-030-30733-2, p.83-106; https://doi.org/10.1007/978-3-030-30734-9_5

Editorial activities

- Special issue: "Obesogens in the XXI century: emerging health challenges", Frontiers in Endocrinology, Guest Associate Editor (publication scheduled October 2021)
- Special Issue: "Environmental Exposures and Epidemiological Studies on Maternal and Child Health", International Journal of Environmental Research and Public Health, Guest Associate Editor (publication scheduled October 2021)

Media Coverage – TV

- Participation in the primetime program "Linha da Frente", a prime-time national television broadcast in Portugal, about the impacts of cosmetics in humans. 2020.01.16 https://www.rtp.pt/play/p6595/e450672/linha-da-frente

References:

Kunisue T, Wu Q, Tanabe S, Aldous KM, Kannan K (2010) Analysis of five benzophenone-type UV filters in human urine by liquid chromatography-tandem mass spectrometry. Analytical Methods 2 (6):707-713. doi:10.1039/B9AY00324J

Souto-Miranda S, Gonçalves A-C, Valente C, Freitas C, Sousa ACA, Marques A (2020) Environmental Awareness for Patients with COPD Undergoing Pulmonary Rehabilitation: Is It of Added Value? International Journal of Environmental Research and Public Health 17 (21):7968