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Abstract

Due to worldwide regulations on the application of the high production volume industrial chemical bisphenol A (BPA) in various consumer products, alternative bisphenols such as bisphenol F (BPF) and bisphenol S (BPS) are increasingly used. To assess human exposure to these chemicals, biomonitoring of urinary concentrations is frequently used. However, the short-term variability of alternative bisphenols has not been evaluated thoroughly yet, which is essential to achieve a correct estimation of exposure. In this study, we collected all spot urine samples from ten healthy adults for five consecutive days, and an additional 24 h pooled sample. We measured the concentrations of seven bisphenols (BPAF, BPF, BPA, BPB, BPZ, BPS and BPAP) in these samples using gas chromatography coupled to tandem mass spectrometry. BPA, BPF and BPS were frequently found in spot samples (>80%), while bisphenol AP (BPAP) was detected in 43% of spot samples. Calculations of intra-class correlation coefficients (ICCs) showed that reproducibility of these four bisphenols was relatively poor (<0.01-0.200) but improved when concentrations were corrected for urine dilution using creatinine levels (0.128-0.401). Of these four bisphenols, BPF showed the best reproducibility (ICC 0.200-0.439) and BPS the most variability (ICC <0.01-0.128). In general, the within-participant variability of bisphenol levels was the largest contributor to the total variance (47-100%). We compared repeated first morning voids to 24 h pooled urine and found no significantly different concentrations for BPA, BPF, BPS, or BPAP. Levels of BPA and BPF differed significantly depending on the sampling time throughout the day. The findings in this study suggest that collecting multiple samples per participant over a few days, in predefined time windows throughout the day, could result in a more reliable estimation of internal exposure to bisphenols.

Keywords: bisphenols, variability, urine, intra-class correlation coefficient, biomonitoring

Capsule:

Levels of BPA, BPF and BPS differed significantly depending on the sampling time throughout the day and between days.

1. Introduction

Bisphenol A (BPA) is a chemical that is widely used in the manufacturing of consumer products, e.g. plastics and coatings intended for packaging of food, receipts (Liao and Kannan, 2011; Geens et al., 2012a; Geens et al., 2012b; Vervliet et al., 2019), dental restoration materials (Vervliet et al., 2018; Xue et al., 2018), clothing (Xue et al., 2017; Li and Kannan, 2018) and electronics (Geens et al., 2011). Although BPA is polymerized in most applications, the monomer can leach from these products and thus, humans could be exposed, mainly through the dietary intake (Geens et al., 2010; European Food Safety Authority, 2015). The increasing evidence that BPA is harmful to humans because of its endocrine disrupting properties (Rochester, 2013; Gramec Skledar and Peterlin Masic, 2016) has led to bans on BPA in certain products worldwide (e.g. baby bottles, thermal paper) (European Union, 2011a; European Union, 2011b; Kawamura et al., 2014; European Union, 2016) and the increasing use of analogues, such as bisphenol F (BPF) and bisphenol S (BPS), in these applications instead (Liao et al., 2012c; Bjornsdotter et al., 2017; Vervliet et al., 2019). Several studies have found these BPA analogues in various matrices, e.g. indoor dust (Liao et al., 2012b), sediments (Liao et al., 2012d) and different foodstuffs (Grumetto et al., 2013; Liao and Kannan, 2013; Chen et al., 2016; Russo et al., 2019a; Russo et al., 2019b). Little is known about the potential toxicity of these BPA alternatives, but there are indications that they too could have endocrine-disrupting or other detrimental properties (Rochester and Bolden, 2015; Gramec Skledar and Peterlin Masic, 2016; Bjornsdotter et al., 2017; Pelch et al., 2019), illustrating the importance of monitoring the human exposure to these chemicals.

Biomonitoring of human biological matrices is a valuable tool to assess internal human exposure to chemicals coming from the environment, from product use and food consumption (Aylward et al., 2014). Recent biomonitoring studies have increasingly measured and detected various BPA analogues in human urine (Liao et al., 2012a; Hoffman et al., 2018; Lehmler et al., 2018; Frederiksen et al., 2020; Gys et al., 2020). BPA has a short half-life (<7 h) and is quickly and largely conjugated excreted in urine, thus making this the preferred matrix for measuring internal BPA exposure (Völkel et al., 2002; Thayer et al., 2015). Pharmacokinetics of BPA analogues are not so well characterized yet. So far, a small number of *in vivo* studies reported that BPF and BPS might be eliminated as glucuronide metabolites, in a similar way, but at a slower rate compared to BPA (Oh et al., 2018; Gayrard et al., 2019; Gingrich et al., 2019; Khmiri et al., 2020), but there is no consensus yet and more research is needed. Most biomonitoring studies generally use a single spot urine sample per participant, often a first-morning void, to characterize exposure levels. However, for non-persistent chemicals such as bisphenols, which have multiple or incompletely characterized sources and are subject to changes in physiological status, levels could vary greatly over time. Measuring bisphenols in single spot urine samples could thus result

in a poor estimation of exposure (Aylward et al., 2014; Koch et al., 2014; Casas et al., 2018; Vernet et al., 2019; Wang et al., 2019).

Over the past decade, the temporal variability of urinary BPA concentrations has been investigated in several studies, in different study populations (e.g. children, mother-child pairs, adults and multiple populations of pregnant women) and over varying timespans, ranging from 24 hours to 3 months. Depending on the set-up of the respective study, poor to fairly good reproducibility was reported (Teitelbaum et al., 2008; Lassen et al., 2013; Philippat et al., 2013; Heffernan et al., 2014; Koch et al., 2014; Casas et al., 2018; Morgan et al., 2018; Sakhi et al., 2018; Vernet et al., 2018; Wang et al., 2019). Studies evaluating shorter periods (e.g. 24 h, two days) often showed less variability (Heffernan et al., 2014; Sakhi et al., 2018). Far less research has been conducted on the variability of other bisphenols in the urine.

To the best of our knowledge, the variability of frequently measured and detected analogues, BPF and BPS, have been investigated in only one study per compound. Variability of urinary BPF, over five days and over three months, was evaluated in Chinese men and poor reproducibility was reported for spot samples, morning voids and 24-hour pools (Wang et al., 2019). In the available study on the variation of BPS and other phenolic compounds, variability was calculated within-day, within-week and between-week, based on pooling of spot samples and the reported reproducibility of BPS was poor (Vernet et al., 2018). Reproducibility of other bisphenols has not been reported yet, and also BPF and BPS need to be investigated more elaborately.

In this study, we aimed to assess the short-term variability in urinary concentrations of BPA and six bisphenol analogues (BPAF, BPF, BPB, BPZ, BPS, and BPAP) by collecting spot samples, including morning voids, of ten adult volunteers during five consecutive days, and a complete 24-hour pooled urine sample for an additional day. We calculated intra-class correlation coefficients (ICCs), comparing uncorrected to dilution-corrected urinary concentrations, using both specific gravity values and creatinine concentrations. Morning voids and 24-hour pooled urine were compared to investigate reproducibility in capturing internal exposure to bisphenols.

2. Materials and methods

2.1. Study population

To investigate the variability in urinary concentrations of non-persistent chemicals, ten participants were recruited in March 2018. The participants were healthy adults from our laboratory; students, researchers and technical assistants (8 men, 2 women, aged 20-50), who lived in Antwerp, Belgium. Participants showed a mean bodyweight of 72.2 ± 11.1 kg. None of the participants was occupationally

exposed to bisphenols. Participants were asked to provide a spot urine sample of every urination for five consecutive days. Sampling started with the first morning void (MV) on Monday and ended on Friday at 5 pm. MV was considered to be the first sample that was closely followed by a normal morning ritual of breakfast and personal hygiene. The following week on Wednesday, the participants collected the complete individual urinations for 24 h, which were later pooled into one sample. All samples were collected in clean polypropylene containers, labelled with a personal code and kept in a refrigerator until delivery to the laboratory, where the de-identified samples were stored at -20°C until analysis. In total, 319 samples were analyzed in this study: 309 spot samples (of which 50 were MV) and ten 24-hour pooled samples. The number of spot samples collected per participant during the five-day study period ranged from 23 to 38, with an average of 31 (approximately 6 samples per day). The Ethical Committee of the Antwerp University Hospital approved our study (EC Reference Number: 18/03/023, Belgian Registry Number: B300201835329). Written informed consent was acquired from each participant and all data were processed anonymously.

2.2. Measurement of bisphenols in urine

Samples were analyzed between November 2019 and February 2020. An overview of the reagents and standards that were used is available in Supporting Information (SI)-1. Concentrations of BPAF, BPF, BPA, BPB, BPZ, BPS and BPAP were quantified using a validated analytical protocol described elsewhere(Gys et al., 2020). Briefly, for preparation of a sample, 1 mL of urine was spiked with isotope-labelled reference standards (4 ng of $^{13}C_{12}$-BPA, 2 ng of $^{13}C_{12}$-BPF, $^{13}C_{12}$-BPS, and $^{13}C_{12}$-BPB). Next, 750 µL of sodium acetate buffer (1 M, pH 5) and 10 µL of ß-glucuronidase/arylsulfatase enzyme solution (30/60 U/mL, respectively) were added. Samples were incubated for 1 h at 37 °C and subsequently sonicated for 15 min. Next, they were extracted using Oasis WAX cartridges (3 mL, 60 mg, Waters, Milford, MA, USA) that were previously washed with 10 mL of methanol and conditioned with 2 mL of water. After the samples were loaded, the cartridges were washed with 2 mL of water with 5% methanol and dried on the vacuum manifold for 20 min. Elution of bisphenols was carried out using 2 mL of methanol, which was then evaporated to dryness under a gentle stream of nitrogen gas. Analytes were reconstituted in 100 µL of derivatization reagent (10% BSTFA in toluene) and samples were kept at 60 °C during 1 h to complete the formation of trimethylsilyl-derivates of the target compounds. Final extracts were transferred to glass vials with inserts for GC-MS/MS analysis.

Instrumental analysis was performed on an Agilent 7890B gas chromatograph coupled to an Agilent 7000D triple quadrupole mass spectrometer (Santa Clara, CA, USA). Chromatographic separation of the derivatized analytes was achieved using an Agilent DB-5MS capillary column (30 m x 250 µm, 0.25 µm; Santa Clara, CA, USA). Ionization was carried out in EI mode with an electron energy of 70 eV.

Target compounds and internal standards were measured using multiple reaction monitoring. Data acquisition and processing were carried out using the Agilent MassHunter software (version B.07.01, Santa Clara, USA). Limits of quantification (LOQs) were 0.02 ng/mL for BPAF, BPF and BPB, 0.04 ng/mL for BPZ, BPS and BPAP and 0.3 ng/mL for BPA. An overview of the target compounds, their internal standards, linear ranges and LOQs are provided in Table SI-1.

The specific gravity of urine samples was determined at room temperature using a handheld refractometer (Euromex RF.6612, Euromex Arnhem, Holland). An aliquot of every urine sample was sent to the Antwerp University Hospital (UZA) for the measurement of the creatinine concentration, using a Dimension Vista 1500 spectrophotometer (Siemens, Beersel, België).

2.4. Quality control and quality assurance

Urine samples were prepared and analyzed in batches consisting of 20 urine samples, two procedural blanks and two quality control (QC) samples. These QC samples were either obtained by participation in the aforementioned international inter-laboratory comparison exercises or by analysis of a spiked and matching non-spiked pooled urine sample, so that the detected concentration in the non-spiked sample could be subtracted. As BPA is a ubiquitous substance, it is inherently present in the lab environment. Therefore, two procedural blanks (ultrapure water) were included in every batch of 20 samples and these blank values were subtracted from concentrations found in samples. All glassware used in the procedure was heated to 400 °C for 2 h and all pipette tips were rinsed twice with methanol beforehand. SPE cartridges were pre-washed with 10 mL of methanol before conditioning and loading samples (Caballero-Casero et al., 2016). Field blanks (from polypropylene containers, used for storing urine samples) were analyzed and did not contain detectable levels of bisphenols. Results for QC samples and procedural blanks are presented in Table SI-2.

External quality control was assured through successful participation in inter-laboratory comparison exercises. This method was thoroughly evaluated in 1) the Human Biomonitoring for Europe External Quality Assurance Scheme (HBM4EU ICI/EQUAS) for BPA, BPF and BPS (four rounds in 2018, 2019 and 2020) and 2) the External Quality Assessment Scheme for Organic Substances in urine (OSEQAS) of the *Centre du toxicologie du Québec* for BPA, BPF, BPS and BPZ (four rounds in 2018, 2019 and 2020), and performance was satisfactory. Resulting Z-scores are presented in Table SI-3.

2.5 Statistical data analysis

Concentrations <LOQ were replaced by a value of LOQ x detection frequency (James et al., 2002). Concentrations were corrected for urinary dilution with individual specific gravity (SG) and creatinine (CRT) values. Specific gravity normalized concentrations were calculated as follows:

176 $conc_{SG}$ = conc*[(1.024-1)/SG-1)]

177 where $conc_{SG}$ is the normalized bisphenol concentration, conc is the uncorrected bisphenol
178 concentration, 1.024 is a standardized SG value and SG is the specific gravity level of the individual
179 sample (Duty et al., 2005; Pearson et al., 2009; Meeker et al., 2012). Compounds with a detection
180 frequency > 40% were considered in statistical analysis. To compare differences in mean bisphenol
181 concentrations between repeated morning void (5 days, n=50) and 24-hour pooled samples (n=10), a
182 linear mixed model was used, with posthoc Sidak correction for multiple comparisons (participant as
183 a random factor). Another linear mixed model was applied to evaluate the difference in
184 concentrations between daily time segments; morning (6-9h), noon (10-12h), afternoon (13-18h) and
185 evening samples (19-24h). Bisphenol concentrations were ln-transformed before introduction in
186 mixed models, to normalize their distributions.

187 Temporal variability of urinary bisphenol concentrations was investigated by linear mixed models with
188 random intercept per participant and sampling day as a fixed effect (Vernet et al., 2018). Separate
189 models were set up for all spot samples (n = 309) and morning void samples only (n = 50), using
190 uncorrected, specific gravity-corrected or creatinine-corrected concentrations. Three variance
191 components were calculated using these models: between-individual, within-individual between-day
192 and within-individual within-day variance (Preau et al., 2010). ICCs were calculated as the ratio of
193 between-individual variance to the total variance (sum of the between-individual variance and the
194 within-individual variance) (Vernet et al., 2018; Wang et al., 2019). ICC values can range from zero
195 (indicating poor reproducibility) to one (indicating less variability and high reproducibility) and were
196 categorized as poor (< 0.40), fair to good (0.40 < ICC < 0.75), and excellent (≥ 0.75) (Wang et al., 2016).
197 To compare the fitness of models between uncorrected values, specific gravity- and creatinine-
198 corrected values, Akaike Information Criterion (AIC) values were used; lower AIC values indicate better
199 fitness. Bivariate correlations among compounds were calculated by Spearman rank analysis (rho ρ).
200 Statistical significance was set at $p < 0.05$. Statistical analyses were carried out using SPSS software
201 (version 26, IBM Corp., Armonk, NY, US). Figures were prepared with the open-source software
202 package R (version 3.5.0).

203 **3. Results and discussion**

204 3.1. Urinary concentrations of bisphenols

205 The detection frequencies and distributions of measured urinary concentrations of bisphenols in spot
206 and 24-hour pooled samples are displayed in Table 1. Distributions were positively skewed and
207 statistical outliers for the urinary concentrations were retained as valid data points as their
208 concentrations were within the linear range of the calibration curve. Three bisphenols were frequently

detected in both sample types; BPS, BPA, and BPF (80-100%). Overall, BPS was the most frequently detected: in 82% of spot samples and all 24-h pooled urine samples. BPA was found in all pooled urine as well, and in 80% of spot samples, followed by BPF, in 80% of spot samples and 90% of pooled urine. BPAP was detected in 42% of spot samples, while it was found in 60% of 24-h pooled urine. Other measured bisphenols showed lower detection frequencies (≤ 30%). Bisphenols showing detection frequencies > 40% (i.e. BPA, BPAP, BPF and BPS) were included for analysis of temporal variability. Highest concentrations were excreted for BPA (median 0.92 ng/mL in spot and 1.36 ng/mL in 24-h pooled urine), followed by BPS and BPF. Highest measured maximal concentration was 20.62 ng/mL for BPA in a spot sample. Despite lower detection frequency and median concentration, a relatively high maximum concentration was detected for BPAP (10.55 ng/mL) in a spot urine sample, indicating the presence of a specific source. To our best knowledge, this is the first study to measure and report BPAP concentrations in multiple samples from the same person. Concentrations of evaluated bisphenols in spot samples were all weakly, but significantly correlated with each other (n = 309, Spearman's ρ= 0.19 to 0.46; p < 0.01, Figure SI-1). This result suggests that exposure to different bisphenols might have occurred simultaneously, either because of common sources or through certain behavior.

In comparison to a recent study evaluating the temporal variability of bisphenols in Chinese men using spot and 24 pooled samples, median levels for BPA were higher in our study population (0.92 ng/mL in our study *versus* 0.56 ng/mL in spot samples; and 1.36 ng/mL *versus* 0.70 ng/mL in 24-h pools), whereas the median BPF level was higher in their study population in spot samples (0.09 ng/mL), but lower in 24 h pooled urine (0.12 ng/mL) (Wang et al., 2019). Wang et al. reported a much lower detection frequency of BPS (13%) compared to our study (82%), although the LOQ was similar for both methods. This is likely due to differences in habits and lifestyle of the individuals in the various studies from China and Belgium and to the time of sampling (2012-2014 *versus* 2018). In other recent research that examined BPA reproducibility in the urine of 50 US adults, BPA median levels were higher in spot samples (1.75 ng/mL in morning voids and 2.04 ng/mL in bedtime voids) and 24 h pools (2.12 ng/mL) (Morgan et al., 2018). The only other study evaluating variability of BPS in French pregnant women found a higher median concentration (0.3 ng/mL) for BPS compared to ours (0.11 ng/mL) as well as for BPA (1.9 *versus* 0.92 ng/mL respectively) (Vernet et al., 2018). Comparison of concentrations with other studies, especially with large biomonitoring studies, has to be done with caution, as the number of participants in the current study is small and reported results depend on included compounds and their LOQs in the respective analytical methods. Moreover, as regulations on the application of BPA become stricter worldwide, its sources might become scarcer but more widespread for BPA

alternatives. As a result, the exposure profile to bisphenols might change over time and vary depending on regional differences in exposure.

Table 1 Distribution of uncorrected bisphenol concentrations (ng/mL), specific gravity values and creatinine concentrations (mg/dL) in spot urine samples (n = 309) and 24-h pooled urine (n = 10).

			ng/mL				
Compound		**% >LOQ**	**Minimum**	**25th**	**Median**	**75th**	**Maximum**
BPAF	spot	22	<LOQ	<LOQ	<LOQ	<LOQ	0.13
	24h	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BPF	spot	80	<LOQ	0.030	0.060	0.19	3.34
	24h	90	<LOQ	0.050	0.14	0.18	0.79
BPA	spot	80	<LOQ	0.42	0.92	1.94	20.62
	24h	100	0.68	0.74	1.36	2.25	4.60
BPB	spot	26	<LOQ	<LOQ	<LOQ	0.020	0.32
	24h	30	<LOQ	<LOQ	<LOQ	0.030	0.060
BPZ	spot	10	<LOQ	<LOQ	<LOQ	<LOQ	0.23
	24h	10	<LOQ	<LOQ	<LOQ	<LOQ	0.03
BPS	spot	82	<LOQ	0.060	0.11	0.21	7.64
	24h	100	0.10	0.13	0.19	0.44	0.79
BPAP	spot	43	<LOQ	<LOQ	<LOQ	0.080	10.55
	24h	60	<LOQ	<LOQ	0.080	0.13	0.89
Specific gravity	spot	100	1.02	1.01	1.01	1.02	1.04
	24 h	100	1.02	1.01	1.02	1.02	1.03
Creatinine (mg/dL)	spot	100	118.87	51.75	88.55	153.75	387

LOQ: limit of quantification

The five-day profiles of SG-corrected concentrations in spot urine samples of the most frequently detected bisphenols for each participant (P1-10) are displayed in Figure 1. These graphs show a typical trend for chemicals with a short half-life but which people are frequently exposed to; drops and rises in concentrations are frequent but rather flat (Aylward et al., 2014). All participants showed concentrations that were in the same range. However, concentrations within each participant varied

up to two orders of magnitude during the study period of five days. Our results indicate that, despite the emergence of alternative bisphenols, BPA is still the predominant compound in this study (samples dating from March 2018), as it shows the highest median levels and a high detection frequency. The visible peaks in the levels of the alternative bisphenols BPF, BPS and BPAP suggest the exposure to specific sources. On multiple occasions, concentrations of two or more bisphenols rose at the same time, indicating common exposure (e.g. for P2 at noon on Wednesday, for P3 on Tuesday morning, for P6 on Thursday afternoon, for P9 on Tuesday evening and for P10 on Wednesday morning). This observation is also highlighted by the significant correlations between different bisphenols, mentioned above (Figure SI-1). We did not collect a participants' diary to document the meals or the use of consumer products, so it was impossible to determine which exposure events took place. The statistical power of such analyses would be insufficient, because of the small number of participants and due to the many sources of bisphenols in our environment; both dietary and non-dietary (Geens et al., 2011; von Goetz et al., 2017).

In order to perform a risk assessment, the maximal estimated daily intakes (EDIs) were calculated for BPA, BPAP, BPF and BPS for every participant in this study population and compared to available health-based standards. EDIs were calculated according to the following equation:

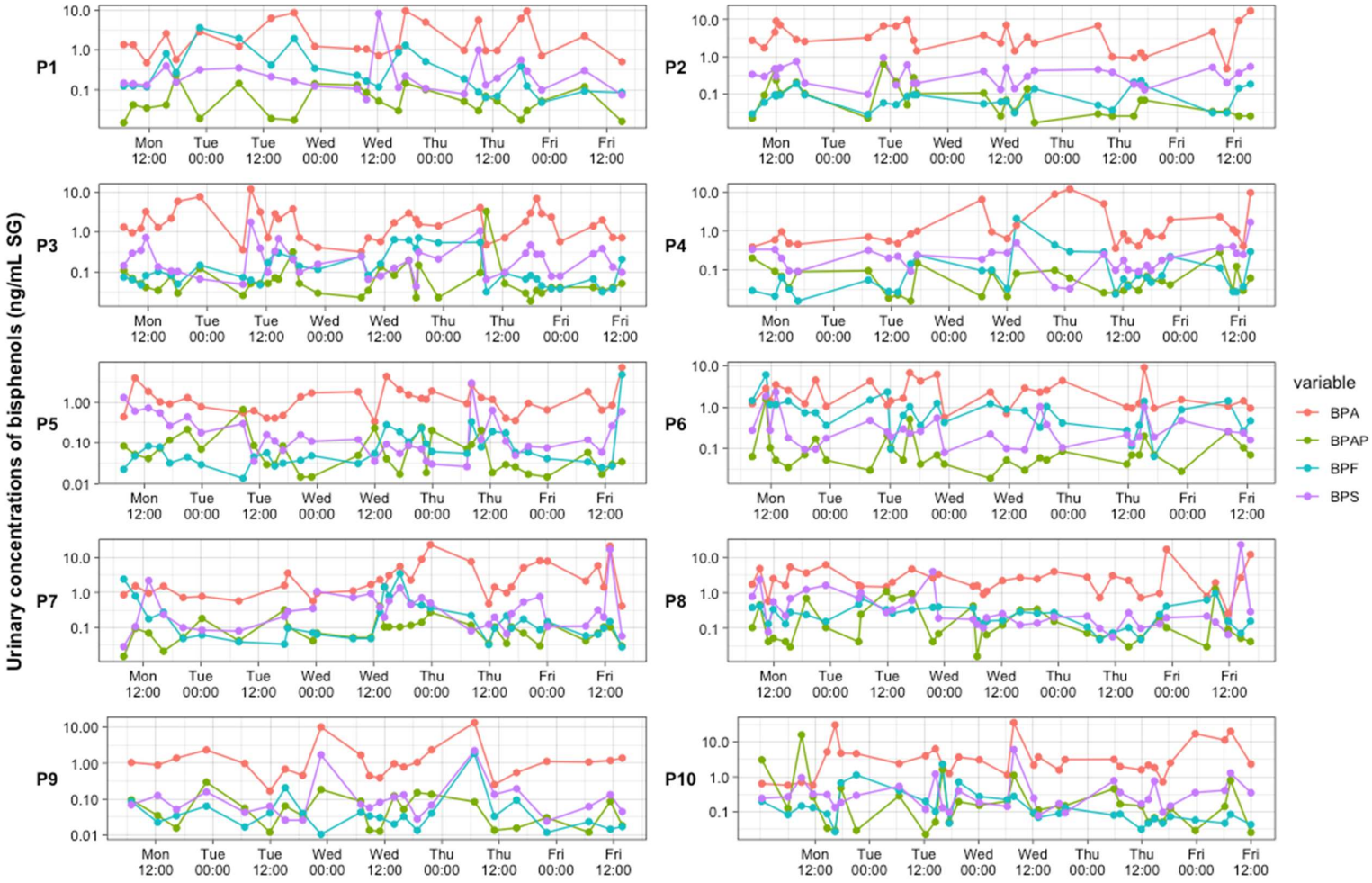
$$EDI = \frac{C_U \times V_U}{BW}$$

where the EDI is expressed in ng/kg bw/day, C_U is the maximal measured urinary concentration of the respective bisphenol (in ng/mL) in the participant, V_U is the daily urine excretion rate (mL/day) and bw is the body weight provided by the participant, expressed in kg (Lakind and Naiman, 2011; Geens et al., 2015; Chen et al., 2018; Zhang et al., 2020). The urine excretion rate was calculated as 22 mL/kg bw/day (Valentin, 2002; Lakind and Naiman, 2008). As these EDI values are calculated based on the measured internal exposure and bisphenols are short-lived chemicals (t ½ < 7 h) completely excreted in urine, they represent the intake from all exposure sources (Völkel et al., 2002; Dekant and Völkel, 2008; Thayer et al., 2015). The EDI values are available in Table SI-4. For BPA, a tolerable daily intake (TDI) of 4 µg/kg bw/day was established by the EFSA (European Food Safety Authority, 2015). Other institutions such as the U.S. Environmental Protection Agency (USEPA) and Health Canada provide higher TDI values for BPA: 50 and 25 µg/kg bw/day, respectively (Huang et al., 2017). For alternative bisphenols, no TDI values or reference doses are available yet, to our best knowledge.

As expected from the measured urinary levels and in accordance to other studies, the EDIs are highest for BPA compared to the other bisphenols (Zhang et al., 2020). However, even in this high-exposure scenario based on the maximal measured urinary concentration for every participant, the EDI is lower (factor 5 to 26) than the established TDI value of EFSA, indicating that there are no expected health

concerns for this study population. Remarkably, the participant showing the highest EDI for BPA, also had the highest value for BPAP. It is important to note that EDI values greatly depend on the period of sample collection, since urinary levels of BPA are decreasing during recent years (Frederiksen et al., 2020; Gys et al., 2020). The intake of alternative bisphenols is less investigated compared to BPA. Because of the small size of the study population and thus the lack of sufficient statistical power, these values should be interpreted with caution and should not be compared to cross-sectional studies which have assessed large study populations.

293 **Fig. 1:** Concentrations of the most frequently detected bisphenols, corrected for specific gravity in spot samples of 10 participants (P1-10) over 5 consecutive
294 days (log10 scale).

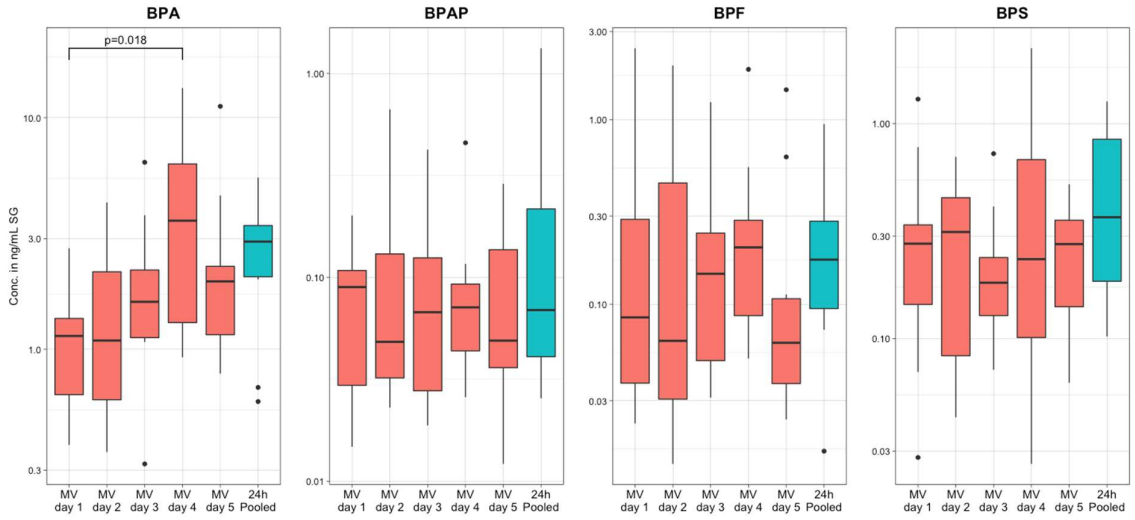


295

3.2. Sampling time

Generally, median concentrations measured in spot samples were slightly lower than those in 24 h pooled urine (Table 1), but both were in the same range. Using linear regression models, no statistically significant differences in mean concentrations were found between the repeated morning voids and 24-hour pooled urine samples (Figure 2). Only for BPA, a significant difference was found between the morning voids collected on day 1 (median 1.15 ng/mL, SG-corrected) and on day 4 (median 3.62 ng/mL, SG-corrected).

Fig. 2 Boxplots of bisphenol concentrations in repeated morning void samples (MV, n = 50) and 24-hour pooled urine samples (n = 10), corrected for specific gravity.



P-values were calculated using linear regression models, with sampling day as fixed effect and random intercept per participant.

BPA and BPF levels differed significantly depending on the sampling time throughout the day (divided into daily intervals, $p < 0.05$, Table 2). BPAP concentrations showed a similar pattern according to sample collection time, but less pronounced and not in a significant way. These three bisphenols showed higher concentrations in samples collected in the morning (6-9h), decreased around noon (10-12h) and went up again during the afternoon (12-18h) and the evening (19-24h). A similar trend was also reported for urinary BPA levels in a study population of 80 pregnant women (Fisher et al., 2015) and can be expected for chemicals mainly taken in through the diet (Vandenberg et al., 2007). BPS showed a slightly different profile: highest concentrations were measured for samples collected during the morning interval and levels decreased throughout the day, but not significantly. These slight differences in the concentration profiles throughout the day might be explained by the various specific sources of exposure to these respective bisphenols. BPA might be replaced by a certain

bisphenol analogue in one application, and by a different bisphenol in another application (Geens et al., 2011; Chen et al., 2016). Additionally, excretion rates of bisphenols also vary and have an influence on the fluctuation of their concentrations (Oh et al., 2018; Gayrard et al., 2019; Khmiri et al., 2020).

Table 2 Association of bisphenol concentrations (corrected for SG) with sampling time, divided into daily intervals (morning 6-9h, noon 10-12h, afternoon 13-18h, evening 19-24h).

	Sampling Time	n	Median (25th – 75th)	p-value
BPA	Morning	71	1.53 (0.95 – 3.16)	**0.001**
	Noon	81	1.15 (0.68 – 2.32)	
	Afternoon	91	1.82 (0.96 – 3.61)	
	Evening	66	2.18 (1.03 – 4.57)	
BPAP	Morning	71	0.070 (0.030 – 0.14)	**0.239**
	Noon	81	0.050 (0.030 – 0.10)	
	Afternoon	91	0.050 (0.030 – 0.10)	
	Evening	66	0.070 (0.030 – 0.15)	
BPF	Morning	71	0.090 (0.050 – 0.30)	**0.001**
	Noon	81	0.070 (0.040 – 0.16)	
	Afternoon	91	0.14 (0.060 – 0.34)	
	Evening	66	0.15 (0.060 – 0.42)	
BPS	Morning	71	0.27 (0.11 – 0.49)	**0.070**
	Noon	81	0.22 (0.12 – 0.36)	
	Afternoon	91	0.20 (0.11 – 0.31)	
	Evening	66	0.17 (0.080 – 0.35)	

P-values were calculated using linear mixed models with the participant as a random factor.

3.3. Variability of bisphenols over 5 days

To assess the temporal variability of bisphenol concentrations in urine over five consecutive days, intraclass correlation coefficients (ICCs) were calculated. To the best of our knowledge, this is the first study to calculate and report ICCs for BPAP overall and for BPS in the adult population. An overview of available studies reporting ICC values for bisphenols is presented in Table SI-6. Calculated ICC values for frequently detected bisphenols in all spot samples and MV samples only are presented in Table 3. Overall, relatively poor reproducibility was found for all evaluated bisphenols. The highest ICC values were observed for CRT-corrected BPF in MV samples (ICCs range: 0.200 – 0.439). In spot samples, CRT-corrected BPF showed an ICC value of 0.401 (range: 0.200 – 0.401), when CRT correction was applied.

BPS showed the poorest reproducibility, with ICCs ranging from <0.01 (spot and MV samples, both uncorrected and SG-corrected) to 0.128 (CRT-corrected, in spot samples). BPA and BPAP showed similar patterns for ICC values and variance proportions (for all samples and MV only). Also, the improvement of their ICCs by correcting for urine dilution was comparable. As the detection frequency of BPAP was relatively low, the overall calculated ICC value should be interpreted with caution, because it might be biased by the relatively higher number of imputed values <LOQ.

For this reason, we additionally calculated ICCs for BPAP using only the samples showing concentrations >LOQ (spot samples, n = 133). The outcome was an ICC of 0.059 for uncorrected, 0.174 for SG-corrected and 0.230 for CRT-corrected concentrations. Compared to the overall ICC values, considering only samples with levels >LOQ resulted in slightly higher reproducibility, when corrected for urine dilution. For MV samples, the ICC could not be calculated because the number of samples per participant was too low. Reproducibility did not always improve when only MV samples were considered, for BPS it even resulted in a poorer result (ICC all spot samples <0.01-0.128, ICC MV only <0.01-0.049). No ICCs were calculated for 24 h pooled urine, as this sample was only collected for one day. ICCs were reported in recent literature for BPA in 24 h pooled urine over the course of 6 (Lassen et al., 2013; Morgan et al., 2018) or 12 weeks (Lassen et al., 2013; Morgan et al., 2018), but did not show a substantially better reproducibility compared to other types of samples(Lassen et al., 2013; Morgan et al., 2018)(Lassen et al., 2013; Morgan et al., 2018)(Lassen et al., 2013; Morgan et al., 2018)(Lassen et al., 2013; Morgan et al., 2018).

Short-term reproducibility generally improved when urine dilution correction with either specific gravity or creatinine was applied, implying that differences in urine dilution could explain some of the observed variance. The influence of the dilution correction was studied by either using the SG- or CRT-corrected urinary bisphenol concentrations or by including the SG or CRT value as a covariate in the model, with the uncorrected bisphenol concentrations (data not shown). Both methods generated very similar results and the values included in Table 3 represent the former strategy, using the corrected concentrations. The same similarity between models was presented by (Wang et al., 2019). Creatinine levels and specific gravity were strongly and significantly correlated in spot samples (Spearman's ρ 0.93, $p < 0.01$, Figure SI-1). Despite creatinine levels being slightly less reproducible over five days than specific gravity values (Table SI-5), correction with creatinine concentration yielded a larger improvement in ICC values for the evaluated bisphenols. This discrepancy between the two correction methods was the most pronounced for BPS. This observation might be explained by the slightly stronger correlation between urinary bisphenols in spot samples and creatinine concentrations (Spearman's ρ 0.31 to 0.42, $p < 0.01$), compared to specific gravity (Spearman's ρ 0.25

to 0.41, $p < 0.01$, Figure SI-1). However, in the case of morning voids, correction for urine dilution did not always improve the reproducibility.

Table 3 ICC values and variance proportions of LN-transformed levels of BPA, BPAP, BPF and BPS, for all spot samples and MV only, collected during five consecutive days.

	BPA		BPAP		BPF		BPS	
Spot samples (n=309)								
Uncorrected	AIC	918	AIC	934	AIC	950	AIC	901
Between indiv. σ^2 (%)	0.043	(4%)	0.112	(9%)	0.319	(20%)	<0.01	
Within indiv., between day σ^2 (%)	1.068	(96%)	1.103	(91%)	1.007	(63%)	1.000	(96%)
Within indiv., within day σ^2 (%)	<0.01		<0.01		0.268	(17%)	0.042	(4%)
ICC [a]	*0.039*		*0.092*		*0.200*		*<0.01*	
SG-corrected	AIC	858	AIC	904	AIC	897	AIC	904
Between indiv. σ^2 (%)	0.130	(13%)	0.124	(11%)	0.581	(36%)	0.068	(8%)
Within indiv., between day σ^2 (%)	0.853	(87%)	0.994	(89%)	0.832	(51%)	0.952	(85%)
Within indiv., within day σ^2 (%)	0.000		0.000		0.216	(13%)	0.080	(7%)
ICC	*0.132*		*0.111*		*0.357*		*0.080*	
CRT-corrected	AIC	865	AIC	903	AIC	890	AIC	911
Between indiv. σ^2 (%)	0.236	(21%)	0.194	(16%)	0.695	(40%)	0.153	(13%)
Within indiv., between day σ^2 (%)	0.865	(79%)	0.992	(84%)	0.809	(47%)	0.986	(82%)
Within indiv., within day σ^2 (%)	<0.01		<0.01		0.230	(13%)	0.060	(5%)
ICC	*0.214*		*0.164*		*0.401*		*0.128*	
MV samples (n=50)								
Uncorrected	AIC	128	AIC	147	AIC	173	AIC	137
Between indiv. σ^2 (%)	0.109	(15%)	0.231	(20%)	0.675	(31%)	<0.01	
Within indiv. σ^2 (%)	0.625	(85%)	0.921	(80%)	1.531	(69%)	0.879	(100%)
ICC	*0.148*		*0.201*		*0.306*		*<0.01*	
SG-corrected	AIC	122	AIC	142	AIC	166	AIC	144
Between indiv. σ^2 (%)	0.057	(9%)	0.092	(9%)	0.856	(41%)	<0.01	(0%)
Within indiv. σ^2 (%)	0.582	(91%)	0.891	(91%)	1.219	(59%)	1.006	(100%)
ICC	*0.090*		*0.093*		*0.413*		*<0.01*	
CRT-corrected	AIC	126	AIC	140	AIC	163	AIC	143
Between indiv. σ^2 (%)	0.148	(20%)	0.063	(7%)	0.880	(44%)	0.049	(5%)
Within indiv. σ^2 (%)	0.578	(80%)	0.872	(93%)	1.127	(56%)	0.959	(95%)
ICC	*0.204*		*0.067*		*0.439*		*0.049*	

Mixed models used ln-transformed concentrations. ICC, intraclass correlation coefficient; σ^2, variance; AIC, Akaike Information Criterion values; AIC were used to assess fitness of the models; SG: specific gravity; CRT: creatinine; [(a)] ICC is the ratio of the between-individual variance to the total variance.

The contribution of between-individual and within-individual components to the variance are displayed in Table 3. For all bisphenols, the within-individual variance was the largest contributor to the total variance, also reflected in the low ICC values. Moreover, when considering all spot samples, the highest proportion was coming from the within-individual between-day variance. This finding is consistent for other chemicals mostly associated with food intake, as a person's food consumption typically changes from day to day (Preau et al., 2010; Aylward et al., 2014), and has been reported in other studies on BPA as well (Ye et al., 2011; Lassen et al., 2013; Fisher et al., 2015; Morgan et al., 2018). Previous research has reported that dietary ingestion of BPA is the major exposure route in non-occupationally exposed adults, although it is likely that not all non-food sources of exposure are elucidated yet (Geens et al., 2011; Geens et al., 2012a; European Food Safety Authority, 2015; von Goetz et al., 2017; Morgan et al., 2018). As BPA is such a widespread chemical with many different types of applications, fluctuation in exposure between days is expected (Preau et al., 2010; Geens et al., 2011; von Goetz et al., 2017). For other bisphenols, the major exposure route has not been determined yet, but as they are meant to replace BPA in certain regulated applications, it can be expected that sources are similar. Moreover, recent research has shown that several BPA alternatives are widespread and humans are extensively exposed to them (Chen et al., 2016; Lehmler et al., 2018), which is also reflected by the high detection frequencies found for BPF and BPS in our study participants. For BPF, Wang *et al*. reported that the within-individual component was the largest contributor to the total variance, to an even bigger extent (94-99%) compared to our results (Wang et al., 2019). It has to be taken into account that their sampling timeframe was much longer (12 weeks) compared to ours (5 days). To the best of our knowledge, variance components for BPAP and BPS have not been calculated and reported before.

Multiple studies have reported ICC values and variance components of BPA, mostly in pregnant women and in study populations including more participants but fewer samples per participant (Table SI-6). Most studies on the variability of urinary BPA corrected for urine dilution using creatinine concentration. Depending on the set-up of the study and the applied correction, reported ICCs ranged from 0.09 (adult population, over 6 weeks) to 0.70 (mother-child pairs, over 24 h) (Morgan et al., 2018; Sakhi et al., 2018). Studies evaluating shorter periods (e.g. 24 h, two days) often showed higher ICC values, e.g. 0.70 for 24 h timespan, 0.51 for two days, indicating that within-person variability increases as larger timespans are considered (Lassen et al., 2013; Heffernan et al., 2014; Sakhi et al., 2018). Several studies that also compared different dilution correction methods, reported results similar to our study, namely that SG corrected concentrations yielded lower ICCs compared to CRT corrected levels (Philippat et al., 2013; Koch et al., 2014; Stacy et al., 2016; Morgan et al., 2018; Vernet et al., 2018). However, contradictory results, showing that using CRT corrected concentrations lowers

409 the ICCs, have also been reported for BPA (Braun et al., 2011; Ye et al., 2011; Guidry et al., 2015;
410 Vernet et al., 2018).

411 In the only other study examining the reproducibility of urinary BPS levels, reported within-week ICC
412 values for BPS in daily pooled samples were in the same range as ours. Similarly to our observations,
413 this study described that correction for urine dilution with creatinine concentration yielded the best
414 reproducibility (ICC uncorrected 0.14 – ICC SG corrected 0.09 – ICC CRT corrected 0.20) (Vernet et al.,
415 2018). For BPF, the only available study on variability reported poorer reproducibility compared to our
416 results (ICC <0.01-0.06) (Wang et al., 2019). This might be explained by their larger timespan of
417 sampling, which is 12 weeks in Wang's study, compared to 5 days in our study. However, their
418 reported ICC values for BPA (CRT corrected, 0.27-0.28) were quite similar to ours.

419 The comparison with ICCs reported in other studies should be carried out with caution, as these values
420 can be influenced by several factors. There can be differences in study design, e.g. the sampling
421 window (ranging from one day to several months), the number and the type of samples collected
422 (spot, only morning voids, only (24 h) pooled samples). As some studies have evaluated urinary
423 bisphenol variability in specific populations (e.g. pregnant women, mother-child pairs) or different age
424 groups, the influence of physiological and behavioral properties should be taken into account
425 (Aylward et al., 2014). Additionally, differences in the applied analytical method can influence the
426 detection frequency and the urinary levels and distribution of the investigated bisphenols.
427 Measurements of chemicals in the laboratory environment also show an inherent variability. As these
428 compounds are usually present in urine in low concentrations (ng/mL or lower), differences in the
429 method LOQs can cause relatively large variations. Furthermore, various statistical models can be used
430 for calculating ICCs and a different method for correcting for urine dilution can be applied.

431 Overall, these findings suggest that biomonitoring through measurement of bisphenols in one single
432 spot urine sample might not accurately represent internal exposure over time, as reproducibility is
433 relatively poor (demonstrated by the low ICC values). No significant difference was found between
434 levels in 24 h pooled samples and morning voids, suggesting that it is not strictly necessary to collect
435 24 h pooled urine, which is also less practical. Two recent studies that examined BPA in 24 h pooled
436 urine samples also indicated that reproducibility is not improved compared to other types of samples
437 (Lassen et al., 2013; Morgan et al., 2018). However, the variability of other bisphenols in 24 h pooled
438 urine should be examined further, both short- and long-term. Median urinary levels of BPA and BPF
439 were influenced significantly by sampling time throughout the day and concentrations of BPAP and
440 BPS differed slightly depending on the time of sampling. Additionally, within-individual variance
441 appeared to be the major variance component, so it seems that collecting only one morning void or

multiple morning voids is not recommendable. When the biomonitoring study aims to reflect exposure over a longer period than just the few hours before sampling, a more reliable sampling strategy would be to collect multiple spot samples per participant for a week, in the same, predefined time windows for all participants, scattered over the respective days (e.g., each morning and evening).

3.4. Strengths and limitations

The major asset of this study is that 10 healthy adult participants provided a urine sample of every urination during five consecutive days. This allowed us to study day-to-day variation and calculate ICCs for bisphenols that have not been investigated before or only in pregnant women. Our study is complementary to other studies on urinary variability of bisphenols because of its timeframe of 5 days, compared to studies sampling only 1 day or multiple weeks/months and because of the new information on previously unevaluated bisphenols, e.g. BPAP, in the adult population. The improvement of reproducibility by correction for urine dilution was assessed by comparison of uncorrected, creatinine-corrected and specific gravity-corrected concentrations. Additionally, the successful performance of the analytical method was confirmed during several rounds of interlaboratory proficiency tests, which ensures the analytical validity of the measured concentrations. A limitation of our study was the lack of a diary of every participant, registering the timing of meals and knowledge about the use of consumer products. Our study participants included a small population consisting of healthy adults, implying that comparison and generalization should be carried out with caution, because of potential physiological and behavioral differences. The reproducibility of 24 h pooled samples could not be assessed because of the lack of a second sample. This should be investigated in future studies.

4. Conclusions

Internal exposure to bisphenols is extensive in healthy adults living in Belgium, as shown by the high detection frequencies of BPA, BPF and BPS. Urinary bisphenol levels are generally poorly reproducible, demonstrated by relatively low ICC values for all spot samples and repeated morning voids collected during 5 consecutive days. Of the four evaluated bisphenols, urinary BPS levels were the most subject to variation (ICC <0.01 to 0.128) and BPF showed the most reproducible concentrations (ICC 0.200 to 0.439). Reproducibility increased slightly when measured bisphenol concentrations were corrected for urine dilution, preferably with creatinine concentration. Within-individual variance was the major contributor to variance in urinary levels for BPA, BPAP, BPF, and BPS. A significant association was found between the time of sample collection and urinary levels of BPA and BPF: concentrations were high in the morning, lower throughout the afternoon and increased again towards the evening, as expected for chemicals mainly taken in through the diet. These findings suggest that measurement of

bisphenols in one single spot urine sample, including morning void urine, might not accurately represent exposure over longer periods. It would be more opportune to collect multiple spot samples per participant, at different, predefined time windows throughout the day. This is the first study evaluating the variability of BPAP and BPS in the adult population, so more research is needed to add to these findings.

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Conflicts of interest

The authors have no conflict of interest to declare.

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