Determinants of exposure levels of bisphenols in Flemish adolescents

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Abstract

The broadly used industrial chemical bisphenol A (BPA), applied in numerous consumer products, has been under scrutiny in the past 20 years due to its widespread detection in humans and the environment and potential detrimental effects on human health. Following implemented restrictions and phase-out initiatives, BPA is replaced by alternative bisphenols, which have not received the same amount of research attention. As a part of the fourth cycle of the Flemish Environment and Health Study (FLEHS IV, 2016-2020), we monitored the internal exposure to six bisphenols in urine samples of 423 adolescents (14-15 years old) from Flanders, Belgium. All measured bisphenols were detected in the study population, with BPA and its alternatives bisphenol F (BPF) and bisphenol S (BPS) showing detection frequencies > 50%. The reference values show that exposure to these compounds is extensive. However, the urinary BPA level decreased significantly in Flemish adolescents compared to a previous cycle of the FLEHS (2008-2009). This suggests that the replacement of BPA with its analogues is ongoing. Concentrations of bisphenols measured in the Flemish adolescents were generally in the same order of magnitude compared to recent studies worldwide. Multiple regression models were used to identify determinants of exposure based on information on demographic and lifestyle characteristics of participants, acquired through questionnaires. Some significant determinants could be identified: sex, season, smoking behavior, educational level of the parents, recent consumption of certain foods and use of certain products were found to be significantly associated with levels of bisphenols. Preliminary risk assessment showed that none of the estimated daily intakes (EDIs) of BPA exceeded the tolerable daily intake, even in a high exposure scenario. For alternative bisphenols, no health-based guidance values are available, but in line with the measured urinary levels, their EDIs were lower than that of BPA. This study is, to the best of our knowledge, the first to determine internal exposure levels of other bisphenols than BPA in a European adolescent population.

Keywords: bisphenols, biomonitoring, adolescents, determinants of exposure, estimated daily intake

Highlights

- BPA and 5 alternatives were measured in a representative Flemish population
- BPA, BPF and BPS were detected in almost every participant (>80%)
- Urinary BPA levels decreased significantly from 2008 to 2018
- Socio-economic status, product use and food were associated with bisphenol levels
- No participants exceeded the available health-based guidance values for BPA

1. Introduction

Bisphenol A (BPA) is a high production volume industrial chemical, applied in various consumer products, e.g. polycarbonate plastic, epoxy resins used to coat food and beverage cans, thermal paper receipts (Geens et al., 2011; Liao and Kannan, 2011; Geens et al., 2012a; Geens et al., 2012b; Vervliet et al., 2019), dental restoration materials (Vervliet et al., 2018), clothing (Xue et al., 2017; Li and Kannan, 2018) and electronics (Geens et al., 2011). Despite being polymerized in most applications, some amount of free BPA monomer could be present or formed due to degradation. The present free BPA could then leach from these products and humans could thus be exposed, mainly through the dietary intake (Geens et al., 2012a; European Food Safety Authority, 2015). Because of increasing evidence that BPA is harmful to humans due to its endocrine disrupting properties (reproductive, developmental, metabolic toxicity), this extensively used chemical has been phased out of certain applications (e.g. baby bottles, thermal paper) in the past decade worldwide, including in Belgium (Japanese National Institute of Technology and Evaluation, 2003; European Union, 2011; Moniteur Belge, 2012; Kawamura et al., 2014; European Union, 2016). As a consequence, BPA is gradually being replaced by bisphenol analogues, such as bisphenol F (BPF) and bisphenol S (BPS) (Liao et al., 2012c; Bjornsdotter et al., 2017; Vervliet et al., 2019). However, recently some evidence has shown that these BPA-alternatives could have an endocrine disrupting potential similar to that of BPA (Rochester and Bolden, 2015; Gramec Skledar and Peterlin Masic, 2016). Several recent studies have found measurable urinary levels of various alternative bisphenols in different study populations (Liao et al., 2012a; Hoffman et al., 2018; Lehmler et al., 2018; Sakhi et al., 2018). However, data on European human exposure levels to these chemicals are limited, particularly in young people, who might be exposed in different ways and to a different extent than adults (Lehmler et al., 2018; Rocha et al., 2018; Frederiksen et al., 2020). It is suspected that endocrine disrupting chemicals could be more harmful during developmental phases such as puberty, so it is necessary to study exposure in such populations (Vandenberg et al., 2009; Vandenberg et al., 2010; Frye et al., 2012). Urine is the preferred matrix for measuring total internal BPA exposure, as BPA has a short half-life (<7 h) and is excreted quickly. Because it is excreted in the urine in its conjugated form, mainly as its non-toxic glucuronide metabolite, measurements typically include a deconjugation step (Völkel et al., 2002; Teeguarden et al., 2011; Christensen et al., 2012; Thayer et al., 2015). Toxicokinetics of BPA analogues are not yet well characterized, but the available studies on this topic suggest that the total urinary levels are also considered robust biomarkers for internal exposure (Koch et al., 2012; Song et al., 2017; Lehmler et al., 2018; Oh et al., 2018).

Since 2002, the Flemish government has established a human biomonitoring network as part of a program on environmental health surveillance. The Flemish human biomonitoring program aims to

investigate the complex relationship between environmental contamination and human health by monitoring selected biomarkers of exposure and certain health effects (Schoeters et al., 2012). In previous cycles of the Flemish Environment and Health Study (FLEHS), adolescents were already included. However, only during the second survey (FLEHS II) in 2008-2009, BPA concentrations were monitored (Geens et al., 2014). In the current 4th cycle of FLEHS (2016-2020), a new biomonitoring survey was set up, to repeat measurements and report updated reference values for some chemicals and to report Flemish reference values for other, emerging chemicals for the first time (Steunpunt Milieu en Gezondheid, 2020).

The objectives of this study were: 1) to report Flemish reference values of frequently detected emerging bisphenols, 2) to compare the obtained results with international literature and with previously reported levels within FLEHS 3) to evaluate demographic and dietary characteristics as potential determinants of exposure, 4) to compare the observed bisphenol levels with available health-based guidance values from literature for a preliminary risk assessment. This study is, to the best of our knowledge, the first to determine internal exposure levels of other bisphenols than BPA in Flanders and in a European adolescent population.

2. Materials and methods

2.1. Study population

The samples in this study were collected from a group of 423 adolescents who took part in FLEHS IV. The program generates representative reference values for a selected set of chemicals, identifies determinants of exposure, and examines the relation between the exposure measurements and potential effects on human health. Adolescents (14 – 15 years old) were recruited through 20 schools from all five Flemish provinces. The number of schools per province was proportional to the population size of the province and schools in the same province had to be separated at least 20 km from each other. Inclusion criteria were as follows: participants and their parents had to provide written informed consent, participants had to reside in Flanders for at least 5 years and they had to be able to fill in an extensive questionnaire in Dutch.

All participants provided a spot urine sample on a school day between September 2017 and June 2018 and their body weight (bw) and body height (bh) were measured by trained nurses with calibrated equipment. The urine samples were collected in clean polyethylene (PE) containers and immediately processed during the fieldwork. Samples were divided into aliquots in glass vials and kept at -20 °C until analysis. The adolescents and their parents completed an extensive, self-administered questionnaire at home on health status, food consumption, use of cosmetics, tobacco and alcohol, housing conditions and socio-economic status (e.g. educational level of the parents, household

income). Participants filled in an additional short questionnaire including questions on recent exposure (i.e. within the last three days) to smoke, medication and food and on urine collection (e.g. time since last void). The study protocol was approved by the ethical committee of the Antwerp University Hospital (Belgian Registry Number: B300201732753). Collection, storage, transfer, and use of data were carried out in accordance to the European General Data Protection Regulation (GDPR). All data were pseudonymized.

In a previous round of the Flemish human biomonitoring program, assessment of BPA exposure was included. In the second cycle of the FLEHS (2008-2009), BPA concentrations were measured in urine of 196 adolescents (Geens et al., 2014). Similar to FLEHS IV, participants were recruited from all 5 Flemish provinces in order to examine a representative sample of the population. All adolescents were 14-15 years old at the time of sampling. As measurements were carried out at only two points in time, a real temporal trend could not be modelled, but a comparison between the two was made.

2.2. Measurement of bisphenols in urine

Bisphenols in urine were measured between July and September 2019. An overview of the used reagents and standards is available in the Supplementary Information (SI)-1. The sample preparation and GC-MS/MS analysis were performed according to the previously validated procedure described elsewhere (Gys et al., 2020a). Briefly, for sample preparation, 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. Then, 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 loading the samples, the cartridges were washed with 2 mL of water with 5% methanol and dried for 20 min on the vacuum manifold. 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 trimethylsilyl-derivatization 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). Target compounds and internal standards were measured using multiple reaction monitoring (Gys et al., 2020a). Limits of quantification (LOQs) were 0.02 ng/mL for BPAF, BPF

and BPB, 0.03 ng/mL for BPZ, 0.04 ng/mL for BPS 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.

Specific gravity (SG) of urine samples was determined by refractometry at Algemeen Medisch Laboratorium (AML, Antwerp, Belgium).

FLEHS II and FLEHS IV analyses, both carried out at the Toxicological Centre of the University of Antwerp, employed different analytical methods (Geens et al., 2009; Geens et al., 2014; Gys et al., 2020a). To allow comparison between the two cycles, three blinded duplicate samples from FLEHS II were measured again during analyses for FLEHS IV. As such, their comparability was evaluated. Linear regression was carried out using the results of the two measurements of these three samples. The regression coefficient R^2 was 0.909 and the slope of the curve was 1.119, indicating good accordance between the two measurements.

2.3. Quality control and quality assurance

Urine samples were prepared and analyzed in batches consisting of twenty urine samples, two procedural blanks and two quality control (QC) samples. These QC samples were either obtained by participation in international inter-laboratory comparison exercises (see below) 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 of the 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. The resulting Z-scores are shown in Table SI-3.

2.4. Statistical data analysis

Reference values for analytes with a detection frequency of at least 60% were calculated as geometric means (GM) with 95% confidence intervals. Concentrations below the LOQ were imputed with a random value (between 0 and the LOQ), drawn from the estimation of the lognormal distribution of all values by fitting a truncated lognormal distribution using only values above the LOQ. For analytes detected in less than 60% of the samples, no imputations were applied, and no reference values were calculated. Statistical outliers of urinary bisphenol concentrations were retained as valid data points. For further statistical analysis, concentrations were corrected for urinary dilution with individual specific gravity (SG) values using the following formula (Duty et al., 2005; Pearson et al., 2009; Meeker et al., 2012): $conc_{SG}$ = [conc * (1.024-1)/(SG-1)], where $conc_{SG}$ is the normalized bisphenol concentration, conc is the uncorrected bisphenol concentration, 1.024 is a standardized SG value and SG is the specific gravity level of the individual sample. Corrected concentrations were then transformed by the natural logarithm due to the skewness of the exposure data.

For comparison of BPA concentrations between FLEHS II and FLEHS IV, a multiple linear regression model was fitted to test the significance of cohort, corrected for gender, age, smoking and SG. Imputation of values below LOQ was carried out for the FLEHS II values in the same way as for the current FLEHS IV.

Associations between questionnaire data (i.e. personal, dietary, socio-economic characteristics) and bisphenol levels were first examined by performing univariate analysis (ANOVA) on the SG-corrected, natural logarithm-transformed concentrations. Variables showing a p-value < 0.2 were subsequently included in a stepwise multiple linear regression model. The SG value was additionally included in the model as a separate, independent variable, to make sure the statistical significance of the relation with other variables in the model was independent of the SG (Barr et al., 2005). During the backward step-by-step building of the multiple regression models the cut-off for the p-value was set at < 0.05 and non-significant variables were consecutively excluded until a set of significant variables was retained. Collinearity among independent variables was evaluated beforehand by calculation of the variance inflation factor (VIF), for which the cut-off was set at 0.8. The R-square of the model reflects the percentage of variation in bisphenol levels that could be explained by the remaining independent variables in the final model. Spearman ρ rank correlation was applied to evaluate correlations between analytes. Statistical analyses were carried out using SPSS Statistics software (version 26.0, IBM Corp, Armonk, USA).

3. Results and discussion

3.1. Study population characteristics

The distribution of characteristics of the adolescents who provided a urine sample (n = 423), such as body mass index (BMI, calculated as bw/bh^2 (kg/m^2)), gender, smoking habits and educational level of the parents and the participant is available in Table 1. In this study, slightly more females (53.4%) participated compared to males (46.6%), but equal distribution between the sexes is approached. The adolescents had a mean age of 14.8 ± 0.5 years. Mean BMI of the study population was 21.0 ± 3.7 kg/m^2 and 72.5% of participants had a normal weight (BMI between 18.5 and 25 kg/m^2). The proportion of adolescents being overweight or obese (BMI > 25 kg/m^2) has increased, compared to previous FLEHS cycles (Geens et al., 2014; Steunpunt Milieu en Gezondheid, 2020). The distribution over school types of the participants accorded well with that of Flanders in general. The educational level of the parents was high in comparison with the general Flemish population, which was a typical finding in previous FLEHS surveys as well, due to better response rates in this group (Morrens et al., 2012; Geens et al., 2014; Steunpunt Milieu en Gezondheid, 2020). Only 4.3% were active smokers, which is a decrease compared to previous cycles of FLEHS and in line with the general Flemish numbers (Rosiers, 2019). Because recruitment was carried out in collaboration with the schools, no samples were collected during summer (Steunpunt Milieu en Gezondheid, 2020).

Table 1 Characteristics of the study population (n = 423).

		N	%
Gender	Male	197	46.6
	Female	226	53.4
Age (years)	Mean, SD	14.8	0.5
BMI class (kg/m^2)	Underweight (≤ 18)	35	8.3
	Normal weight (18 – 25)	307	72.5
	Overweight (> 25)	85	20.1
School type of adolescent	General school	215	50.8
	Technical school	130	30.7
	Vocational school	78	18.4
Educational level parents[a]	Primary	25	5.9
	Secondary	137	32.4
	Tertiairy	253	59.8
	Missing	8	1.9
Smoking habits	No active or passive smoking in house	363	85.8
	Non-smoker, passive smoking in house	39	9.2
	Smoker	18	4.3
	Missing	3	0.7
Season of sampling	Winter	136	32.1
	Spring	189	44.7
	Summer	0	0
	Autumn	98	23.2

N: number of participants in subgroup; BMI: body mass index. [a]Based on the International Standard Classification of Education (ISCED); SD: standard deviation

3.2. Concentrations of bisphenols in urine

The distribution of bisphenols in the urine of Flemish adolescents is shown in Table 2. All six bisphenols were detected in the study population, indicating that exposure to this group of rapidly excreted chemicals is extensive and very common. The most frequently detected compound is BPF (97%), followed by BPA (86%) and BPS (83%). BPB, BPZ and BPAF were detected in respectively 57%, 37% and 12% of participants. Although BPF was most frequently found, BPA showed the highest concentrations (median 1.05 ng/mL); while medians for BPF (0.14 ng/mL) and BPS (0.12 ng/mL) were substantially lower. However, the highest maximal concentrations were 41.5 and 40.0 ng/mL, detected for BPS and BPF respectively. These values were on the edge or just outside the analytical linear range but were included as they were very close to the upper limit of the calibration (40.0 ng/mL) and these samples were reanalyzed for confirmation. After evaluation of potential determinants of exposure (see 3.4), we found no clear explanation for these high values in the characteristics of the participants. For the other bisphenols that show lower detection frequencies, the maximum measured concentrations were low as well. Statistically significant correlations were found between the measured BPA, BPF and BPS concentrations (Spearman rank, $p < 0.01$). Correlation coefficients ranged from 0.296 to 0.380, expressing weak, but positive associations. This result indicates the occurrence of co-exposure to these three chemicals, potentially through common sources or through certain lifestyle habits. The uncorrected urinary concentrations (in ng/mL) of BPA, BPF and BPS were strongly associated with the specific gravity of the urine, meaning that the concentration of the contaminant decreased significantly with increasing dilution of the urine. This was also illustrated by correction of the aforementioned high maximum concentrations for BPF and BPS for urine dilution using SG (Table 2).

Table 2 Reference values of bisphenols urine of Flemish adolescents (n = 423).

Analyte	LOQ	% > LOQ	10th	25th	Median	75th	Maximum	GM	95% CI
ng/mL, uncorrected									
BPAF	0.02	12	<LOQ	<LOQ	<LOQ	<LOQ	0.13	N/A	
BPF	0.02	97	0.04	0.07	0.14	0.29	40.0	0.14	(0.13, 0.16)
BPA	0.30	86	<LOQ	0.55	1.05	1.79	18.1	0.92	(0.82, 1.02)
BPB	0.02	57	<LOQ	<LOQ	0.03	0.05	0.31	N/A	
BPZ	0.03	37	<LOQ	<LOQ	<LOQ	0.04	2.42	N/A	
BPS	0.04	83	<LOQ	0.06	0.12	0.22	41.5	0.11	(0.10, 0.12)
ng/mL, corrected for SG									
BPF			0.04	0.08	0.15	0.30	33.13	0.17	(0.15, 0.19)
BPA			<LOQ	0.69	1.15	1.91	19.41	1.07	(0.98, 1.18)
BPS			<LOQ	0.07	0.14	0.23	35.58	0.13	(0.11, 0.14)

LOQ: limit of quantification; GM: geometric mean; CI: confidence interval; N/A: not available. GM was only calculated for compounds showing 60% > LOQ.

3.3. Comparison with literature

Urinary bisphenols were measured in several recent international studies. In Table 3, a summary is provided of urinary bisphenol levels reported in other study populations, preferably of similar or overlapping age. Comparisons between the different studies were mainly based on medians for uncorrected concentrations and detection frequencies, keeping in mind that method LOQs and approaches of correcting for urine dilution may differ between studies.

In general, the measured concentration of BPA in Flemish adolescents did not differ substantially from levels reported in children and/or adolescents from U.S.A., Canada and Brazil. Although BPA was not the most frequently detected compound in our study population, it was still the predominant bisphenol in terms of measured concentrations. In all studies, BPA was detected with a high frequency, illustrating that it is still used extensively worldwide (Chen et al., 2018; Lehmler et al., 2018; Rocha et al., 2018; Health Canada, 2019). The detection frequencies and levels of BPF and BPS, however, were more variable and depending on the country, the sampling period and the LOQ of the applied analytical method. Median levels of both compounds were considerably higher in U.S.A. (Lehmler et al., 2018). On the other hand, BPF and BPS were only detected in respectively 9 and 23% of urine samples from Brazil and median concentrations were below the method LOQ, which could be (partially) explained by the higher LOQ for BPF (Rocha et al., 2018). Lower levels for BPA and BPS were measured in Chinese children, but the median concentration of BPF was higher than the median for our study population (Chen et al., 2018). A recent study on young Danish men reported slightly higher median concentrations for BPA, BPF and BPS. In their study population, BPA was the most frequently detected bisphenol (92%) (Frederiksen et al., 2020). In comparison to measured bisphenol levels in 7-year-old Japanese school children (Gys et al., 2020a), concentrations in Flemish adolescents are higher but in the same range for BPA, BPF and BPS. Detection frequencies for BPA and BPS were similar, but BPF was less frequently detected in the Japanese children (83% *versus* 97% in FLEHS), although population size was practically equal (396 *versus* 423) and the employed analytical method was the same (Gys et al., 2020a). In a study comprising data for children (5-12 years old) from six European member states (Belgium, Denmark, Luxembourg, Slovenia, Spain and Sweden), the reported median BPA concentration was 1.96 ng/mL (Covaci et al., 2015). This value is slightly higher than our median urinary BPA level, but it is important to keep in mind that the European children's samples were already collected in 2011 and 2012, from six different countries. Comparing data from large (national) biomonitoring surveys should be done with caution, due to intercountry differences in methodology, legislation and behavior (LaKind et al., 2019).

297 BPA is the only bisphenol that was previously included in the Flemish human biomonitoring program.
298 In the second cycle of the FLEHS (2008-2009), BPA was already measured in urine of 196 adolescents
299 (Geens et al., 2014). Both GM (95% CI) are corrected for gender, age, smoking and SG. A statistically
300 significant decrease in the GM BPA concentration in urine was observed ($p < 0.001$) between FLEHS II
301 (2.56 ng/mL) and the current FLEHS IV (1.07 ng/mL) (displayed in Fig. SI-1). In Belgium, BPA is banned
302 in baby bottles and food contact materials intended for children < 3 years old (European Union, 2011;
303 Moniteur Belge, 2012). Since January 2020, BPA can also not be used in thermal paper in a
304 concentration ≥ 0.02% (European Union, 2016). Because of the age of our study population and the
305 fact that the samples analyzed in this study were collected during 2017 and 2018, it is unlikely that
306 these regulations have influenced the measured concentrations directly. However, it is possible that
307 manufacturers have pro-actively started phasing out BPA in certain consumer applications and are
308 using alternative bisphenols instead, e.g. in thermal paper (Vervliet et al., 2019). A similar decreasing
309 trend in urinary BPA was reported for Canadian and American adolescents (12-19 year-olds) and young
310 Danish men over the past decade as well, while these countries have similar or less strict legislations
311 in place (Centers for Disease Control and Prevention, 2019; Health Canada, 2019; Frederiksen et al.,
312 2020). In our study on Japanese children, we also saw a significant decrease in urinary BPA
313 concentrations between 2012 and 2017 (Gys et al., 2020a).
314
315 **Table 3** Concentrations of urinary bisphenols in Flemish adolescents compared to recent international
316 literature.

Compound	Country	N (age)	Sampling period	Urinary levels (median, ng/mL)	Reference
BPA	BE	423 (14-15 y)	2017-2018	1.05	Present study
	BE	196 (14-15 y)	2008-2009	2.21	(Geens et al., 2014)
	EU[a]	653 (5-12 y)	2011-2012	1.96	(Covaci et al., 2015)
	US	462 (12-19 y)	2013-2014	1.20	(Lehmler et al., 2018)
	BR	300 (6-14 y)	2012-2013	1.66	(Rocha et al., 2018)
	CN	213 (8-11 y)	2015	0.25	(Chen et al., 2018)
	CA	524 (12-19)	2016-2017	0.96	(Health Canada, 2019)
	DE	100 (19-30)	2017	1.24	(Frederiksen et al., 2020)
	JP	396 (7 y)	2012-2017	0.89	(Gys et al., 2020a)
BPF	BE	423 (14-15 y)	2017-2018	0.14	Present study
	US	462 (12-19 y)	2013-2014	0.40	(Lehmler et al., 2018)
	BR	300 (6-14 y)	2012-2013	<LOQ	(Rocha et al., 2018)
	CN	213 (8-11 y)	2015	0.19	(Chen et al., 2018)
	DE	100 (19-30)	2017	0.30	(Frederiksen et al., 2020)
	JP	396 (7 y)	2012-2017	0.07	(Gys et al., 2020a)
BPS	BE	423 (14-15 y)	2017-2018	0.12	Present study
	US	462 (12-19 y)	2013-2014	0.40	(Lehmler et al., 2018)

BR	300 (6-14 y)	2012-2013	<LOQ	(Rocha et al., 2018)
CN	213 (8-11 y)	2015	0.03	(Chen et al., 2018)
DE	100 (19-30)	2017	0.17	(Frederiksen et al., 2020)
JP	396 (7 y)	2012-2017	0.11	(Gys et al., 2020a)

N: number of participants in study population; y: years of age. [a]Data from Belgium, Denmark, Luxembourg, Slovenia, Spain and Sweden.

3.4. Analysis of exposure determinants

We investigated whether demographic, dietary and other variables available from questionnaires were associated with urinary concentrations of BPA, BPF and BPS. Results of the univariate regression analyses of the potential determinants of exposure are shown in Table SI-4. Results of the multiple regression models for BPA and BPF are summarized in Table 4. For BPS, none of the investigated variables remained significant in the multiple model.

Urinary BPF and BPS levels did not differ between male and female participants. Concentrations of BPA were significantly higher in female participants ($p = 0.010$). Contradictory results have been reported in the literature on gender differences in urinary BPA. Most studies report no significant association between gender and BPA levels in children and adolescents (Frederiksen et al., 2013; Geens et al., 2014; Covaci et al., 2015; Hoffman et al., 2018). The relationship between gender and BPA might also depend on the age of the participants, as one American study found slightly higher levels in young girls compared to boys (6-19 years old) as well, but for adults, the relation was reversed and significant (Lehmler et al., 2018). For the other bisphenols, fewer studies investigated the influence of gender on urinary levels. The available data so far suggests no significant association exists between gender and levels of BPA-alternatives (Liao et al., 2012a; Lehmler et al., 2018).

The age of the adolescents was not significantly associated with bisphenol concentrations. In FLEHS II, a positive association was found with age, despite the very narrow range (Geens et al., 2014). In this specific period in life, lifestyle habits, such as food consumption and use of cosmetics and personal care products, may change substantially and quickly, which can add to the variability. Moreover, adolescents' development and habits may have changed over the past ten years. BMI was significantly associated with BPA ($p = 0.027$) and BPF ($p = 0.029$) concentrations in univariate analysis: participants in the overweight/obese group presented higher urinary levels. However, BMI did not remain significant in the multiple regression model (available in Table 4). Urinary BPS was also higher in the overweight/obese group but was not significantly related to BMI class (univariate analyses). During previous analyses of urinary BPA in Flemish adolescents, no association was found with BMI class (Geens et al., 2014). It must be noted that the current FLEHS IV included a relatively higher share of overweight adolescents compared to earlier cycles, which might have had an influence on this

outcome. A case-control study on Indian children found no significant difference in BPA levels between the overweight/obese and non-obese group (Xue et al., 2015), but other studies found significantly higher BPA levels in overweight or obese adults (Geens et al., 2015; Do et al., 2017). For alternative bisphenols, literature on their relation with BMI is scarce. A few studies report higher levels for BPS in obese individuals as well (Liu et al., 2017; Jacobson et al., 2019). Studies in mice indicated that BPS might be obesogenic (Ivry Del Moral et al., 2016; Ahn et al., 2020), but more research is needed to examine the potential association between emerging bisphenols and BMI.

Migrant background of the participants or their parents (univariate analysis, $p = 0.037$) and the home language ($p > 0.05$) appeared to influence BPF concentrations but did not remain significant in the final multiple regression model (Table 4). In American children (6-19 years old), significantly different BPF and BPS concentrations were reported depending on the ethnicity of the participant (Lehmler et al., 2018). School type of the adolescent did not appear to influence urinary bisphenol concentrations significantly. Bisphenol levels were consistently higher in participants following a vocational education, but not significantly. The highest educational level of the parents seemed to have an influence on the bisphenol exposure of the adolescent: BPA levels in the adolescents differed significantly depending on the highest educational level of their mother, while BPF levels were significantly influenced by that of their father. Interestingly, BPA levels were highest in the group with a secondary maternal educational level, but BPF concentrations were highest in the group with a primary paternal educational level. In our study population, a relatively high percentage of parents had a tertiary educational level (even more among mothers), which might have an effect on the outcome. The size of the household income was not significantly associated with any bisphenol levels in our study population. Inconsistent results considering household income have been reported in literature, with some studies reporting statistically significant inverse associations (Lakind and Naiman, 2011; Geens et al., 2014; Gys et al., 2020a) while other studies reporting no association (Kim et al., 2011) and some even a positively significant association (Ye et al., 2008). Additionally, the questionnaire included a question about the level of difficulty experienced by the household in making ends meet. Participants in the category reporting difficulty or great difficulty in making ends meet, showed the highest median BPA levels, but also this association was not statistically significant. It is likely that the socio-economic status (comprising e.g. household income and educational level) has an effect on the purchasing and consumption behavior of a household. Furthermore, these variables might have different implications on lifestyle habits in different countries and categorization of socio-economic status might not be standardized between studies.

Smoking habits and exposure to environmental tobacco smoke (secondhand or passive smoking) were examined as well. Active smokers showed higher levels of all three investigated bisphenols, but not

significantly. This might be due to a lack of sufficient statistical power, as only 18 participants reported being active smokers. Recent smoking (i.e. within the last three days) resulted in significantly higher levels for BPF ($p = 0.013$), but not for the others. Exposure to passive smoking increased BPA and BPF levels significantly and lead to slightly higher BPS concentrations as well (univariate analysis). In FLEHS II, similar relations were reported for BPA (Geens et al., 2014) and in our study on Japanese children, we found a comparable association (Gys et al., 2020a). However, the opposite correlation has also been reported for BPA (Lakind and Naiman, 2011) and it is possible that smoking might be an intermediary for other factors associated with higher exposure to bisphenols (Lehmler et al., 2018). The prevalence of exposure to secondhand smoke in the house was significantly higher (Chi-square; $p < 0.001$) in households with primary (24%) and secondary (29%) educational levels, compared to tertiary (10%) education. The same relation was observed between in-house passive smoke exposure and household income ($p < 0.001$) and between active smoking of the adolescent and household income ($p = 0.031$); the prevalence of passive or active smoking decreased as the income increased. These findings indicate that there is a relation between smoking habits and socio-economic status and that these variables could have a synergistic effect on bisphenol concentrations, or that they are proxies for another, unidentified, variable.

Because food intake is considered the major human exposure route to BPA (European Food Safety Authority, 2015), potential associations of measured concentrations with questionnaire data on food consumption and various other product use parameters were tested. Interestingly, consumption of canned fish within three days before sampling did not have a significant impact on bisphenol levels, despite reports of presence of bisphenols (mostly BPA, also BPB and BPF) in canned foodstuffs and correlations between canned food consumption and higher bisphenol levels (Carwile et al., 2011; Russo et al., 2019; Gonzalez et al., 2020). However, the same absence of association between canned food consumption and BPA levels was reported in a large cross-sectional American study (Lakind and Naiman, 2011). Recent consumption of barbecued or grilled foods was related to a higher urinary BPA concentration. The use of insecticide by the participant three days before sampling resulted in significantly higher levels for BPA and BPF. Consumption of locally caught fish was related to higher BPA concentrations and consuming shellfish in the last year was associated to higher BPF levels. From the univariate analysis, it appeared that recent use of haircoloring was also significantly associated with higher urinary BPF levels, as did the recent consumption of fried food. All these associations are likely related to the packaging of the food or product or the utensils used to cook or apply them, as bisphenols are widely applied in many consumer products (Geens et al., 2011; von Goetz et al., 2017). BPS concentrations seemed to be less influenced by food consumption or product use variables, which might indicate that it is being used as a BPA-alternative in other applications that were not surveyed

in our study. A significant association was also found between dental braces and BPF concentrations in our study population. However, BPF levels were higher in urine of participants who reported not to have braces, which might indicate that this variable is a proxy for other factors and should be investigated further.

The season of sample collection had a significant influence on the measured BPA concentration in univariate analysis ($p = 0.023$) and higher levels were detected in autumn. However, this variable did not remain significant in the final multiple model (Table 4). In a study on Japanese school children, BPA levels were higher in autumn compared to other seasons as well (Gys et al., 2020a). BPF levels were associated with the season of urine collection and appeared to be higher in autumn and spring. Urinary BPS was not subject to significant seasonal variation. Various factors may account for this observation, e.g. seasonal changes in food consumption, amount of time spent indoors/outdoors (Geens et al., 2014). As mentioned, no urine samples were collected during summer, meaning this result should be interpreted with caution. In general, analysis of a single spot urine sample might also not represent exposure accurately due to within-individual variation as a consequence of short half-life of bisphenols (Vernet et al., 2018; Wang et al., 2019; Gys et al., 2020b).

Overall, the proportion of variance in urinary bisphenol concentrations explained by the multiple regression models was low ($R^2 = 0.066$ for BPA and $R^2 = 0.095$ for BPF). For BPS, no significant determinants were retained in the final model. These findings suggest that major predictors of exposure to bisphenols could not be identified and that the questionnaires should be refined for future studies. Additionally, bisphenols are compounds that are quickly metabolized but used in numerous, heterogenous applications. Moreover, in comparison to FLEHS II, the GM of urinary BPA in FLEHS IV is lower, and its variation is smaller, which might also partially explain why certain associations reported in FLEHS II were not confirmed in FLEHS IV.

Table 4 Multiple regression analysis for the assessment of determinants of exposure for bisphenols, normalized for specific gravity.

Compound	(n) variable	ß (95%CI)	p-value
BPA	**Sex**		**0.010**
R^2 = 0.066	(184) boy	0.772 (0.635, 0.939)	**0.010**
	(211) girl	reference	
	Highest education level of the mother		**0.045**
	(37) primary	0.752 (0.539, 1.063)	0.108
	(137) secondary	1.165 (0.948, 1.432)	0.147
	(221) tertiary	reference	
	Consumption of barbecued/grilled food in last 3 days		**0.007**
	(276) no	0.749 (0.607, 0.923)	**0.007**
	(119) yes	reference	
	Consumption of local fish in last year		**0.017**
	(374) no	0.598 (0.392, 0.913)	**0.017**
	(21) yes	reference	
	Use of insecticide by participant		**0.013**
	(384) no	0.480 (0.271, 0.853)	**0.013**
	(11) yes	reference	
BPF	**Highest education level of the father**		**0.010**
R^2 = 0.095	(48) primary	0.564 (0.190, 0.938)	**0.003**
	(149) secondary	0.036 (-0.224, 0.295)	0.788
	(161) tertiary	reference	
	Smoking in last three days		**0.013**
	(350) no	0.356 (0.158, 0.804)	0.013
	(8) yes	reference	
	Season of urine collection		**0.015**
	(116) winter	0.696 (0.497, 0.974)	**0.035**
	(163) spring	1.036 (0.759, 1.414)	0.825
	(0) summer	N/A	N/A
	(179) autumn	reference	
	Consumption of shellfish in last year		**0.008**
	(175) no	0.721 (0.566, 0.919)	**0.008**
	(183) yes	reference	
	Use of insecticide by participant		**0.020**
	(349) no	0.405 (0.189, 0.867)	0.020
	(9) yes	reference	
	Time between urine collection and previous toilet visit		**0.015**
	(40) ≤ 2 h	0.530 (0.342, 0.822)	**0.005**
	(144) 2-4 h	0.966 (0.712, 1.310)	0.823
	(83) 4-6 h	0.769 (0.545, 1.085)	0.134
	(91) > 6 h	reference	

N/A: not available. Only variables showing $p < 0.05$ were retained in the model. For BPS, no significant multiple regressions model could be constructed.

3.5. Comparison with guidance values

A preliminary risk assessment was carried out by 1) by direct comparison of measured urinary bisphenol levels with available HBM-values and 2) calculating estimated daily intakes (EDI) based on

448 the urinary concentrations and comparing them with available reference doses (tolerable daily
449 intakes; TDI). The calculation of estimated daily intakes (EDIs) for BPA, BPF and BPS in this study
450 population was carried out according to the following equation:

451 $$EDI = \frac{C_U \times V_U}{BW} \times 1000$$

452 where the EDI is expressed in ng/kg bw/day, C_U is the measured urinary concentration of the
453 respective bisphenol (in ng/mL), V_U is the daily urine excretion rate (L/day) and BW is the measured
454 body weight of the participant expressed in kg (Lakind and Naiman, 2011; Geens et al., 2015; Chen et
455 al., 2018; Zhang et al., 2020). The urine excretion rate is estimated to be 1.2 L/day for 15-year-olds
456 (Valentin, 2002; Lakind and Naiman, 2008). Calculated EDIs are presented in Table 5. As these EDI
457 values are calculated based on the measured internal exposure and bisphenols are short-lived
458 chemicals (t ½ < 7 h) completely excreted in urine, these numbers represent the intake from all
459 exposure sources (Völkel et al., 2002; Dekant and Völkel, 2008; Thayer et al., 2015). Pharmacokinetic
460 data for alternative bisphenols are scarcer than for BPA, but so far, research indicates that total urinary
461 levels can be considered robust measurements for internal exposure (Koch et al., 2012; Lehmler et al.,
462 2018; Oh et al., 2018). Guidance values such as the HBM-I values define the concentration of a
463 chemical in a biological matrix which is consistent with existing noncancer health-based exposure
464 guidance values such as the TDI calculated by the European Food Safety Authority (EFSA) (Apel et al.,
465 2017). These values allow for a direct comparison of the measured biomonitoring concentration and
466 are intended as a screening tool to assess which contaminants are near or above risk assessment
467 values.
468 A TDI of 4 µg/kg bw/day was established by the EFSA for BPA (European Food Safety Authority, 2015).
469 Other institutions such as USEPA and Health Canada provide higher TDI values for BPA: 50 and 25
470 µg/kg bw/day, respectively (Huang et al., 2017). As expected from the measured urinary levels in
471 adolescents and in accordance to other studies, the EDI is highest for BPA (Zhang et al., 2020).
472 However, even in a high-exposure scenario (95th percentile), the EDI is much lower (factor 45) than
473 any of the established TDI values, indicating that there are no expected health concerns for this study
474 population (Table 5). For other bisphenols, no TDI values are available yet. An HBM-I value was also
475 only available for BPA: 0.1 mg/L in urine for children (Apel et al., 2017). In accordance with the TDI-
476 EDI comparison, no participants showed a urinary concentration (see Table 2) above the HBM-I value,
477 which additionally indicates a low risk potential for the adolescents. However, this preliminary risk
478 assessment is based on the current knowledge on single compounds, which neglects the potential
479 cumulative effects of bisphenols and other environmental chemicals on human health. This implies
480 that continued monitoring is recommended, preferably of multiple classes of contaminants.

Several international studies have calculated EDI values for BPA based on biomonitoring data. Most of these studies report higher EDI values (Lakind and Naiman, 2011; Zhang et al., 2011; Lakind et al., 2012; Huang et al., 2017). Important to note is that EDI values greatly depended on the period of sample collection, since levels of BPA are decreasing during recent years in various countries. The intake of alternative bisphenols is less investigated. A recent study on children from South-China reported lower EDIs for BPA and BPS compared to our results, but a similar value for BPF, which is consistent with their and our reported urinary levels (Chen et al., 2018). EDIs were calculated for Chinese university students and were consistently higher for BPA, BPF and BPS (Zhang et al., 2020). Liao *et al*. calculated EDIs for bisphenols based on measured concentrations in indoor dust. Expectedly, they reported substantially lower values for BPA, BPF and BPS in teenagers compared to our calculations, most probably because the contribution of dust ingestion to the total bisphenol intake is rather small (Liao et al., 2012b). When calculating EDIs based on specific environmental measurements (e.g. bisphenol levels in (canned) food or dust), values will be lower, as bisphenols are used in numerous applications (Geens et al., 2010). Although dietary ingestion is considered the main exposure route for BPA, it is likely that not all non-food sources have been elucidated yet (Geens et al., 2011; European Food Safety Authority, 2015; von Goetz et al., 2017). For alternative bisphenols, the major exposure route has not been established yet, but as they are meant to serve as replacements for BPA, it can be expected that sources are comparable. As the collected urine samples in this study were spot samples rather than 24-h pooled urine, the calculated EDIs of these rapidly excreted chemicals need to be interpreted with caution (Lakind and Naiman, 2008).

Table 5 Selected percentiles for estimated daily bisphenol intake (ng/kg BW/day).

Analyte	25th	Median	75th	95th	TDI[a]	% > TDI	Ratio TDI/95th
			ng/kg BW/day				
BPA	11.6	22.4	39.5	88.8	4000	0	45
BPF	1.4	2.9	6.0	22.7	N/A		
BPS	1.3	2.5	4.8	17.0	N/A		

TDI: tolerable daily intake; N/A: not available; [a]value as provided by EFSA.

3.6. Strengths and limitations

Reliable performance of the analytical method applied in the present study was ensured, both by excellent QA/QC results and by successful participation in various proficiency tests. Our study is, to the best of our knowledge, the first exposure assessment of bisphenol analogues, other than BPA, in a large Flemish study population and the first large European study in adolescents. Because BPA was measured in a previous cycle of this study, comparison between concentrations at the two time points

was possible. Control samples showed good agreement between both cycles, although measured with different analytical methods. A limitation of the study is the lack of multiple urine sampling from the same adolescent because bisphenols are short-lived compounds and can vary considerably within an individual (Gys et al., 2020b). This might have influenced the comparison between FLEHS II and FLEHS IV and the exposure determinant analysis. Given the low proportion of variance in urinary bisphenol concentrations that could be explained by the multiple regression models, it is clear that information on major determinants of exposure was lacking and that questionnaires for future studies should be modified. EDI was calculated based on measured internal exposure, thus accounting for all sources, and measured body weight. The accuracy of this value might have been more accurate if a 24 h pooled urine sample had been collected.

4. Conclusions

In the framework of the 4th Flemish Environment and Health Study (FLEHS IV), BPA and 5 alternative bisphenols were measured in 423 Flemish adolescents. This study was the first to measure other bisphenols in a large Flemish study population and the first in a European study population of this age category. All included compounds were detected in the urine samples of the study population, with BPF, BPA and BPS showing high detection frequencies, indicating extensive and simultaneous exposure. Despite still being the predominant bisphenol, showing highest levels, BPA concentrations had decreased significantly compared to previous measurements during FLEHS II in 2008. Levels of BPA, BPF and BPS were generally in the same range as those reported in literature. Both active and passive smoking were associated with higher bisphenol levels. Some food consumption and product use variables showed significant associations with higher levels of BPA and BPF. EDIs were calculated based on measured internal exposure and were in the same range as or lower than other reported values. Even in a high-exposure scenario, preliminary risk assessment showed that BPA stays below the available health-based guidance values. The exposure data presented in this work are representative for Flanders, Belgium.

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