# Supplementary information

# Determination of the urinary concentrations of six bisphenols in public servants by online solid-phase extraction-liquid chromatography-tandem mass spectrometry

Andrea Estévez-Danta*, Rosario Rodil, José Benito Quintana, Rosa Montes*

Aquatic One Health Research Center (ARCUS) & Department of Analytical Chemistry, Nutrition and Food Chemistry. R. Constantino Candeira S/N, IIAA building, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

**Text S1. Enzymatic hydrolysis optimization**

**Design of experiments (DOE)**

A central composite design (2^2 + star) with 4 center points was created to make an efficient optimization of the enzyme concentration (experimental domain: 250- 850 units) and incubation time (experimental domain: 1.5 – 4.5 h) variables. The obtained experiments were:

Experiment 1. Enzyme concentration = 550 units, incubation time = 0.88 h

Experiment 2. Enzyme concentration = 250 units, incubation time = 1.5 h

Experiment 3. Enzyme concentration = 850 units, incubation time = 1.5 h

Experiment 4. Enzyme concentration = 126 units, incubation time = 3 h

Experiment 5. Enzyme concentration = 550 units, incubation time = 3 h

Experiment 6. Enzyme concentration = 550 units, incubation time = 3 h

Experiment 7. Enzyme concentration = 550 units, incubation time = 3 h

Experiment 8. Enzyme concentration = 550 units, incubation time = 3 h

Experiment 9. Enzyme concentration = 974 units, incubation time = 3 h

Experiment 10. Enzyme concentration = 250 units, incubation time = 4.5 h

Experiment 11. Enzyme concentration = 850 units, incubation time = 4.5 h

Experiment 12. Enzyme concentration = 550 units, incubation time = 5.12 h

**Sample preparation and injection**

375 µL of filtered urine was adjusted at pH 5 with sodium acetate buffer and spiked with 100 ng $mL^{-1}$ of available sulfate and glucuronide metabolites mixture (BPA-S, BPA-DS, BPS-S, BPF-S, BPA-G, BPA-DG, BPS-G and BPF-G) + 20 ng $mL^{-1}$ of ISs BPA-d6 and BPS-d8. Then, each experiment was randomly performed according to the conditions specified in the DOE table and injected into the online SPE-LC-MS/MS system following the optimized protocol.

**Text S2. Analytical methodology for creatinine determination in urine.**

**Sample preparation**

Urine samples were filtered through 0.45 µm PVDF syringe-driven filters. Then, each aliquot was diluted 10,000 times in ultrapure water, spiked with the internal standard (creatinine-d3) at 10 ng $mL^{-1}$ and transferred to an insert for injection in the LC-MS/MS system.

**Liquid chromatography-tandem mass spectrometry parameters**

Instrumental analyses were performed with a Waters Acquity UPLC® H class system (Milford, MA, USA) equipped with a quaternary solvent pump, a thermostated LC column compartment, and a sample manager. The UPLC® system was interfaced to a triple quadrupole mass spectrometer Xevo TQD from Waters.

The chromatographic separation was performed at 30 $^{o}$C on a Luna C18 column (50 x 2.0 mm, I.D., 3 µm particle size) from Phenomenex. A dual eluent system consisting of (A) 0.1% formic acid in ultrapure water and (B) 0.1% formic acid in MeOH was used at a flow rate of 0.3 mL $min^{-1}$ in isocratic mode (50:50) for 3 minutes. Injection volume was set at 3 µL.

The interface between the UPLC® system and the Xevo TQD mass spectrometer was an electrospray ionization (ESI) source operating in positive mode at a fixed capillary voltage of 3 kV and a temperature of 150 ºC. Nitrogen, provided by a nitrogen generator from Peak Scientific Spain (Barcelona, Spain), was used as desolvation gas at 600 L $h^{-1}$ and 450 $^{o}$C (desolvation temperature), and as cone gas at 10 L $h^{-1}$. Analyses were performed by MS/MS in Selected Reaction Monitoring (SRM) mode, where: creatinine SRM transitions 114 > 44 (quantification), 114 > 86 (qualification) and creatinine-d3 SRM transitions 117 > 47 (quantification), 117 > 89 (qualification).

**Validation**

Calibration curves were prepared in ultrapure water and ranged from the method quantification limit (MQL) to 250 ng $mL^{-1}$, with IS level of 10 ng $mL^{-1}$, being the MQL 0.02 ng $mL^{-1}$ and the obtained $R^2$ was 0.9995. Method repeatability was evaluated at 1 and 50 ng $mL^{-1}$ and the RSD were 7 and 5 %, respectively for 5 consecutive injections. Method accuracy was evaluated through spiking 6 different real urine samples at 2 mg $mL^{-1}$ (which corresponds to 200 mg $dL^{-1}$ in sample and 200 ng $mL^{-1}$ in the diluted urine) and the obtained values were 102 ± 6 %.

**Table S1. Chemical structure of target bisphenols**

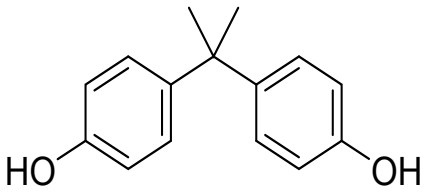
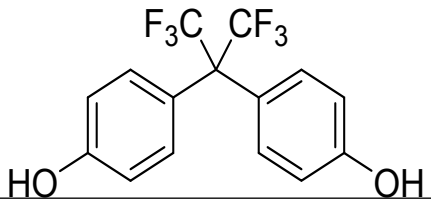
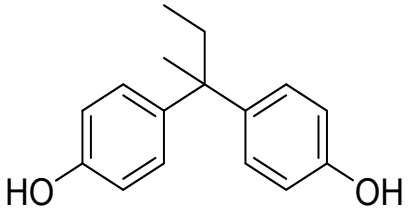
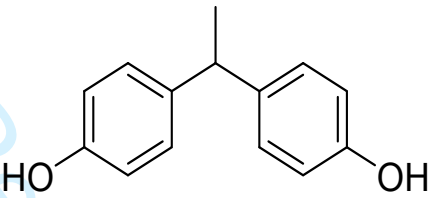
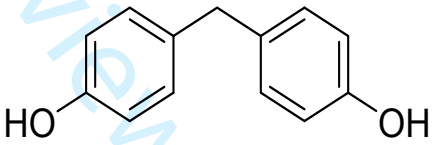
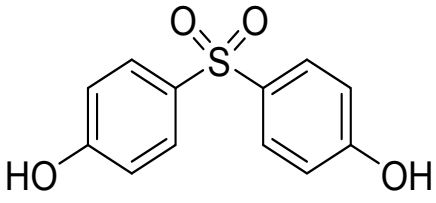
| Name | Structure |
|---|---|
| **Bisphenol A (BPA)** |  |
| **Bisphenol AF (BPAF)** |  |
| **Bisphenol B (BPB)** |  |
| **Bisphenol E (BPE)** |  |
| **Bisphenol F (BPF)** |  |
| **Bisphenol S (BPS)** |  |

**Table S2. Summary of sociodemographic characteristics and creatinine levels for the studied population (full data presented in ZENODO repository (https://doi.org/10.5281/zenodo.10477935)**

| Sociodemographic characteristics | | | | | | | | | | Creatinine (g $L^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gender % | | Tobacco use % | | Residence location % | | | Age | | | | | |
| Female | Male | Yes | No | Urban | Suburban | Rural | Mean | Median | SD | Mean | Median | SD |
| 67.6 | 32.4 | 16.1 | 83.9 | 56.1 | 33.8 | 10.1 | 51.3 | 52 | 7.08 | 0.94 | 0.82 | 0.64 |

**Table S3. Linearity parameters, intercept and slope estimates and standard error.**

| | Intercept estimate | Intercept standard error | Slope estimate | Slope standard error | Stardard error of estimate |
|---|---|---|---|---|---|
| **BPS** | 3.74E-04 | 7.87E-04 | 3.80E-03 | 7.83E-05 | 3.03E-03 |
| **BPF** | 1.43E-03 | 1.27E-03 | 7.82E-03 | 1.31E-04 | 5.19E-03 |
| **BPE** | -8.49E-04 | 1.24E-03 | 1.38E-02 | 1.30E-04 | 5.20E-03 |
| **BPA** | 2.85E-03 | 6.08E-04 | 4.42E-03 | 6.42E-05 | 2.56E-03 |
| **BPAF** | -1.94E-03 | 9.84E-02 | 3.94E-01 | 1.04E-02 | 4.10E-01 |
| **BPB** | -3.84E-03 | 1.87E-03 | 2.07E-02 | 1.98E-04 | 7.87E-03 |

Calculations performed using 3 independent calibration curves with Statgraphics Centurion 19 software

**Table S4.** Comparison of the performance of the method proposed in this work with other online SPE-LC-MS/MS methods published in the literature.

| Reference | Target Bisphenol | Sample preparation | Separation and detection | | %R | MQL |
|---|---|---|---|---|---|---|
| | | Pre-treatment | Extraction | LC-MS/MS | | (ng $mL^{-1}$) |
| This study | BPA, BPAF, BPB, BPE, BPF and BPS | 200 µL of urine<br>Filtration through 0.45 µm PVDF syringe-driven filters<br>Addition of IS + 1mM sodium acetate buffer at pH 5 + 700 units of ß-glucuronidase + 392 µL of ultrapure water<br>Incubation for 5 h at 37 °C | Online SPE on Strata-X 25 µm cartridges<br>Mobile phases: 15 mM of sodium acetate buffer at pH 5 in ultrapure water- MeOH<br>Inj. Vol.: 500 µL | LC-(ESI-)-MS/MS on QqQ (SRM)<br>Luna C18 (150 x 2 mm I.D., 3 µm)<br>Mobile phase: 2 mM of $NH_4F$ in ultrapure water – 2 mM of $NH_4F$ in MeOH | 92-112 % (except BPAF 11-40%) | 0.049-2.2 |
| Ye et al., 2005 | BPA | 100 µL of urine<br>Addition of IS + 50 µL of enzyme/ ammonium acetate Incubation overnight at 37 °C | Online SPE on LiChrosphere RP-18 ADS 25 µm column<br>Mobile phases: ultrapure water- MeOH<br>Inj. Vol.: 1000 µL | LC-(APCI-)-MS/MS on QqQ (MRM)<br>Chromolith Performance RP-18 (100 x 4.6 mm I.D.)<br>Mobile phases: ultrapure water- MeOH | 100 % | 1.3 |
| Koch et al., 2012 | BPA | 300 µL of urine<br>Addition of IS + 1 mM ammonium acetate buffer at pH 5 + 6 µL of ß-glucuronidase<br>Incubation for 4 h at 37 °C | Online SPE on LiChrosphere RP-8 ADS 25 µm column<br>Mobile phases: ultrapure water- Acetonitrile<br>Inj. Vol.: 100 µL | HPLC-(ESI-)-MS/MS on QTRAP (MRM)<br>Waters Atlantis T3 analytical column (150 x 3 mm I.D., 3 µm)<br>Mobile phases: ultrapure water- Acetonitrile | 96.8% | 0.1 |
| Zhou et. al., 2014 | BPA, BPF and BPS | 100 µL of urine<br>Addition of IS + dilution to 1 mL with 0.1 M formic acid + 50 µL of ß-glucuronidase/sulfatase<br>Incubation for 4 h at 37 °C<br>Stop solution: 750 µL 0.1 M formic acid in ultrapure water | Online SPE on LiChrosphere RP-18 ADS 25 µm column<br>Mobile phases: ultrapure water- MeOH<br>Inj. Vol.: 350 µL | LC-(APCI-)-MS/MS on QqQ (MRM)<br>Chromolith High Resolution RP-18e (100 x 4.6 mm I.D.)<br>Mobile phases: ultrapure water- MeOH | 77-106% | 0.03-0.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Heffernan et al., 2016 | BPA, BPAF, BPB, BPF and BPS | 50 µL of urine<br>Addition of IS + 25 µL of ß-glucuronidase (200 units) + 440 µL ultrapure water<br>Incubation for 90 min at 37 °C<br>Stop solution: 400 µL of 0.5 % formic acid in ultrapure water. | Online SPE on Strata-X 25 µm cartridges<br>Mobile phase: 0.05 % of acetic acid in ultrapure water: 0.05 % of acetic acid in MeOH<br>Inj. Vol.: 500 µL | LC-(ESI-)-MS/MS on QTRAP (MRM)<br>Synergi MAX-RP column (150 x 3 mm I.D., 4 µm)<br>Mobile phase: 0.05% of acetic acid in ultrapure water: 0.05 % of acetic acid in MeOH | 101-110% | 0.005-0.39 |
| Jo et al., 2020 | BPA, BPF and BPS | 100 µL of urine<br>Addition of IS + 100 µL of ß-glucuronidase/sulfatase (1000 units)<br>Incubation for 24h at 37 °C<br>Stop solution: 80 µL 1 M formic acid + 670 µL ultrapure water | Online SPE on MAYI-ODS column 50 µm<br>Mobile phases: ultrapure water- MeOH<br>Inj. Vol.: 100 µL | LC-(APCI-)-MS/MS on QqQ (MRM)<br>ACE 5 C18-pentafluorophenyl column (150 x 2.1 mm I.D., 5 µm)<br>Mobile phases: ultrapure water- MeOH | 99.4-108% | 0.13-0.24 |

**References:**

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3. Zhou X, Kramer JP, Calafat AM, Ye X. Automated on-line column-switching high performance liquid chromatography isotope dilution tandem mass spectrometry method for the quantification of bisphenol A, bisphenol F, bisphenol S, and 11 other phenols in urine. Journal of Chromatography B 2014; 944: 152-156.

4. Heffernan AL, Thompson K, Eaglesham G, Vijayasarathy S, Mueller JF, Sly PD, et al. Rapid, automated online SPE-LC-QTRAP-MS/MS method for the simultaneous analysis of 14 phthalate metabolites and 5 bisphenol analogues in human urine. Talanta 2016; 151: 224-233.

5. Jo MJ, Park J-H, An K-A, Choi H, Kang Y-s, Hwang M. Quantification of bisphenols in Korean urine using online solid-phase extraction-high-performance liquid chromatography-tandem mass spectrometry. Environmental Toxicology and Pharmacology 2020; 80: 103491.

**Table S5.** Comparison of the urinary concentrations with other recent published studies.

| Reference | Study period | City | Sub-population type | Target Bisphenol | N | Positive samples | Geometric mean (GSD) concentration ng $mL^{-1}$ [a] | Min-Max ng $mL^{-1}$ | Geometric mean (GSD) adjusted concentration [a] |
|---|---|---|---|---|---|---|---|---|---|
| This study | Sep 2020 | Santiago de Compostela (Spain) | General population (67.6 % women / 36.4% men) | BPA, BPAF, BPB, BPE, BPF and BPS | 435 | **BPA**: 72<br>**BPS**: 364<br>**BPF:** 421<br>**BPAF, BPB, BPE:** 0 | **BPA**: -<br>**BPS**: 0.50 (8.66)<br>**BPF:** 12.4 (3.1) | **BPA**: MQL-103<br>**BPS**: MQL-80<br>**BPF:** MQL-125 | **Creatinine-correction (µg $g^{-1}$):**<br>**BPA:** -<br>**BPS:** 0.67 (6.64)<br>**BPF:** 16.6 (2.6) |
| Peinado et al., 2020 | Jan 2018-Jul 2019 | Granada (Spain) | Women population | BPA, BPS and BPF | 35 | **BPA**: 35<br>**BPS**: 4<br>**BPF:** 10 | **BPA**: 5.5 (1.1)<br>**BPS**: 0.1 (1.1)<br>**BPF**: 0.1 (1.2) | **BPA**: 0.8-18<br>**BPS**: 0.1-1.5<br>**BPF:** 0.1-0.9 | **Creatinine-correction (µg $g^{-1}$) [b]:**<br>**BPA**: 4.7 (1.1)<br>**BPS**: 0.086 (1.100)<br>**BPF**: 0.086 (1.200) |
| Sanchis et al., 2020 | Jun-Nov 2015 | Valencia (Spain) | Breastfeeding mothers | BPA, BPS and BPF | 103 | **BPA**: 78<br>**BPS**: 21<br>**BPF:** 21 | **BPA**: 0.9 (3)<br>**BPS**: 0.06 (0.85)<br>**BPF**: 0.04 (0.34) | **BPA**: LOQ-3<br>**BPS**: LOQ-8.5<br>**BPF:** LOQ-0.34 | **Creatinine-correction (µg $g^{-1}$):**<br>**BPA**: 2.7 (4.7)<br>**BPS**: 0.29 (1.40)<br>**BPF**: 0.19 (0.37) |
| Heffernan et al., 2016 | 2015 | Brisbane (Australia) | Pregnant women | BPA, BPAF, BPB, BPF and BPS | 30 | **BPA**: 30<br>**BPS**: 3<br>**BPF:** 3<br>**BPAF, BPB:** 0 | **BPA**: 5.0<br>**BPS: -**<br>**BPF**: - | **BPA**: 1.7-45<br>**BPS**: MQL-8.1<br>**BPF:** MQL-74 | - |
| Husøy et al., 2019 | Sep 2016-Nov 2019 | Oslo and Akershus (Norway) | General population (69.4 % women / 30.6% men) | BPA, BPS and BPF | 144 | **BPA**: 138<br>**BPS**: 42<br>**BPF:** 6 | - | **BPA**: 0.2-10<br>**BPS**: 0.04-13<br>**BPF:** MDL-9.9 | **Specific gravity-correction (ng $mL^{-1}$):**<br>**BPA**: 1.4<br>**BPS:** 0.2<br>**BPF**: 0.1 |
| Frederisken et al., 2020 | 2009 | Copenhagen (Denmark) | Men population | BPA, BPS, BPF | 100 | **BPA**: 100<br>**BPS**: 68<br>**BPF**: 81 | - | **BPA**: 2.2-24<br>**BPS**: 0.09-5.2<br>**BPF:** 0.27-4.5 | **Osmolality-correction (ng $mL^{-1}$):**<br>**BPA**: 2.27<br>**BPS**: 0.11<br>**BPF:** 0.30 |

| | | | | | |
|---|---|---|---|---|---|
| 2013 | Men population | 100 | **BPA**: 90<br>**BPS**: 65<br>**BPF**: 78 | - | **Osmolality-correction ng mL$^{-1}$:**<br>**BPA**: 1.44<br>**BPS**: 0.06<br>**BPF:** 0.24 |
| 2017 | Men population | 100 | **BPA**: 92<br>**BPS**: 86<br>**BPF**: 87 | - | **Osmolality-correction ng mL$^{-1}$:**<br>**BPA**: 1.33<br>**BPS**: 0.18<br>**BPF:** 0.32 |

[a] Geometric mean and geometric standard deviation were calculated by substituting MQL/2 or MDL/2 for values <MDL/MQL.

[b] Calculated from the data reported in the article

**References:**

1. Peinado FM, Lendínez I, Sotelo R, Iribarne-Durán LM, Fernández-Parra J, Vela-Soria F, et al. Association of Urinary Levels of Bisphenols A, F, and S with Endometriosis Risk: Preliminary Results of the EndEA Study. International Journal of Environmental Research and Public Health 2020; 17: 1194.

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3. Heffernan AL, Thompson K, Eaglesham G, Vijayasarathy S, Mueller JF, Sly PD, et al. Rapid, automated online SPE-LC-QTRAP-MS/MS method for the simultaneous analysis of 14 phthalate metabolites and 5 bisphenol analogues in human urine. Talanta. 2016; 151:224-33

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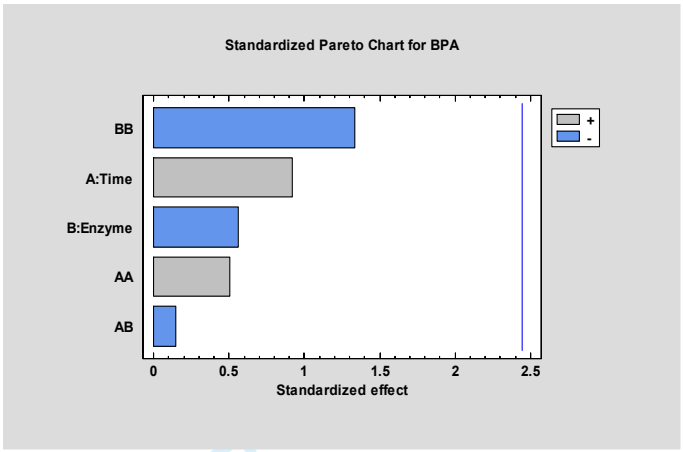
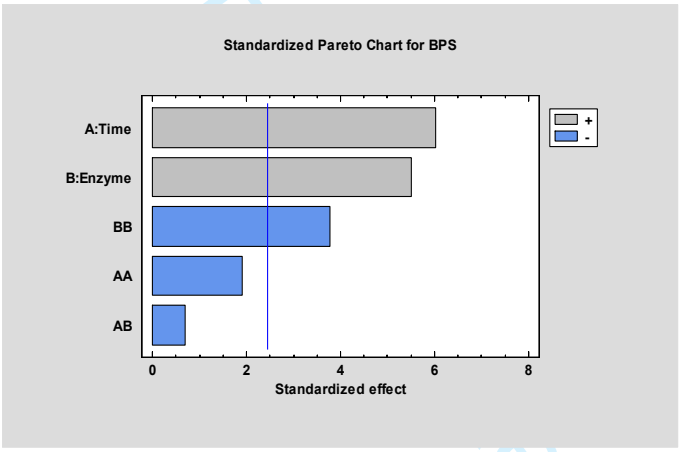
5. Frederiksen H, Nielsen O, Koch HM, Skakkebaek NE, Juul A, Jørgensen N, et al. Changes in urinary excretion of phthalates, phthalate substitutes, bisphenols and other polychlorinated and phenolic substances in young Danish men; 2009–2017. International Journal of Hygiene and Environmental Health. 2020;223(1):93-105.

(a)




(b)

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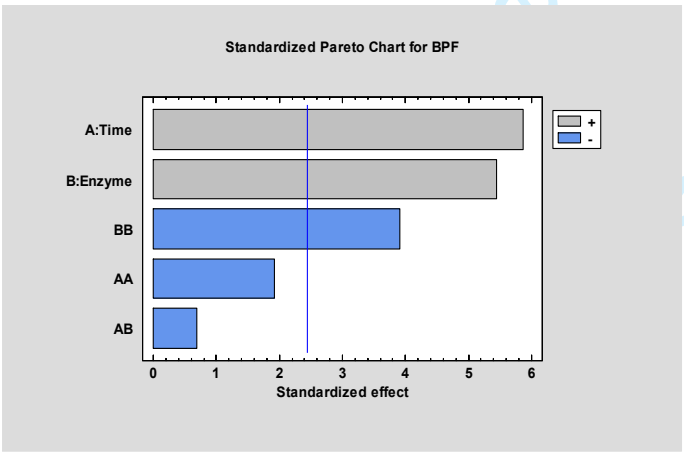


**Fig. S1** Standardized pareto charts for (a) BPA, (b) BPS and (c) BPF, obtained during enzymatic deconjugation DOE optimization

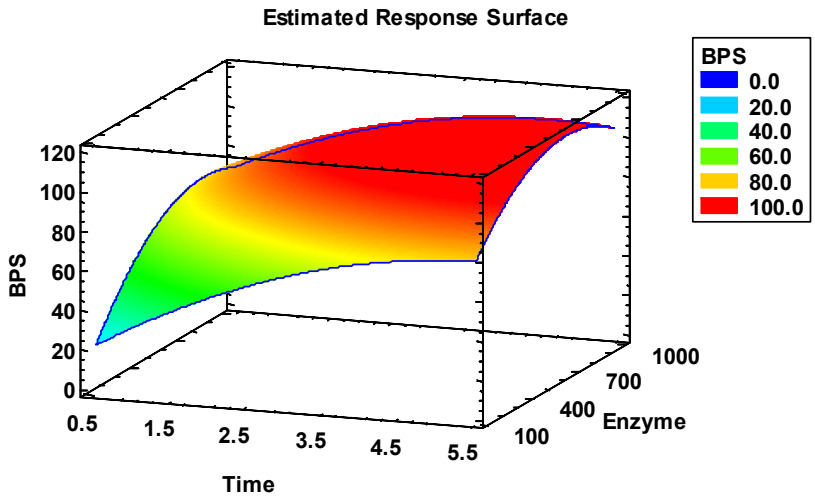


**Fig. S2** Estimated response surface for BPS, obtained during enzymatic deconjugation DOE optimization

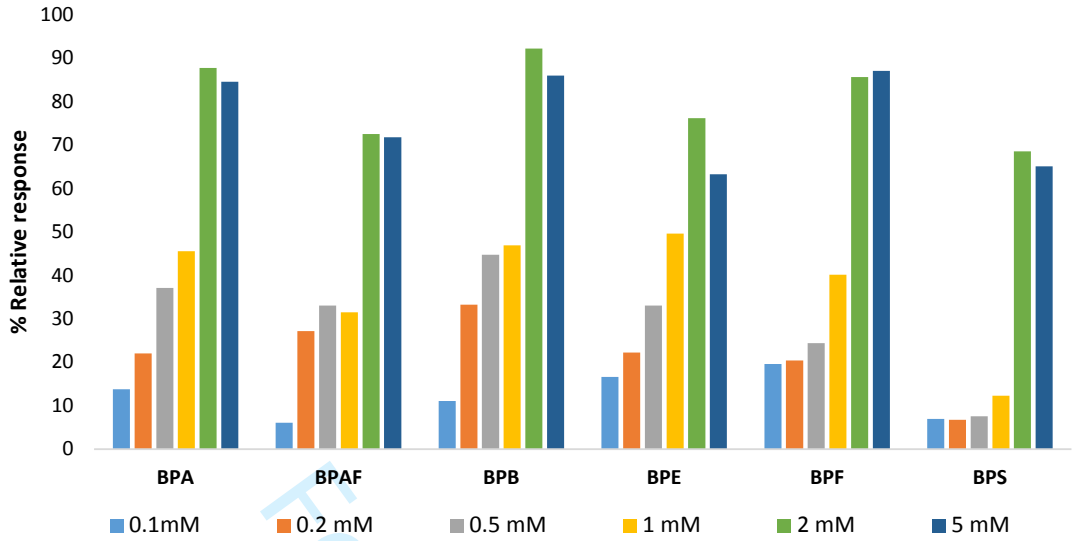


**Fig. S3** Relative response in urine for the target compounds employing different concentrations of $NH_4F$, as modifier for both LC mobile phases (n=2).

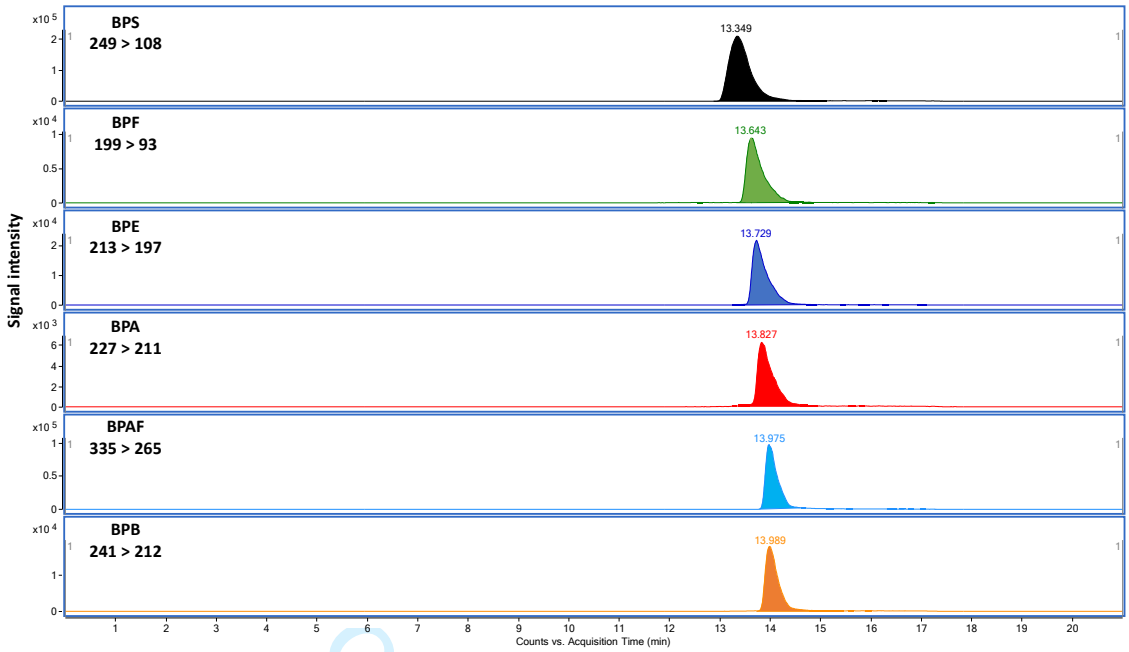


**Fig. S4** Chromatogram of a 100 ng $mL^{-1}$ spiked urine sample under final conditions.

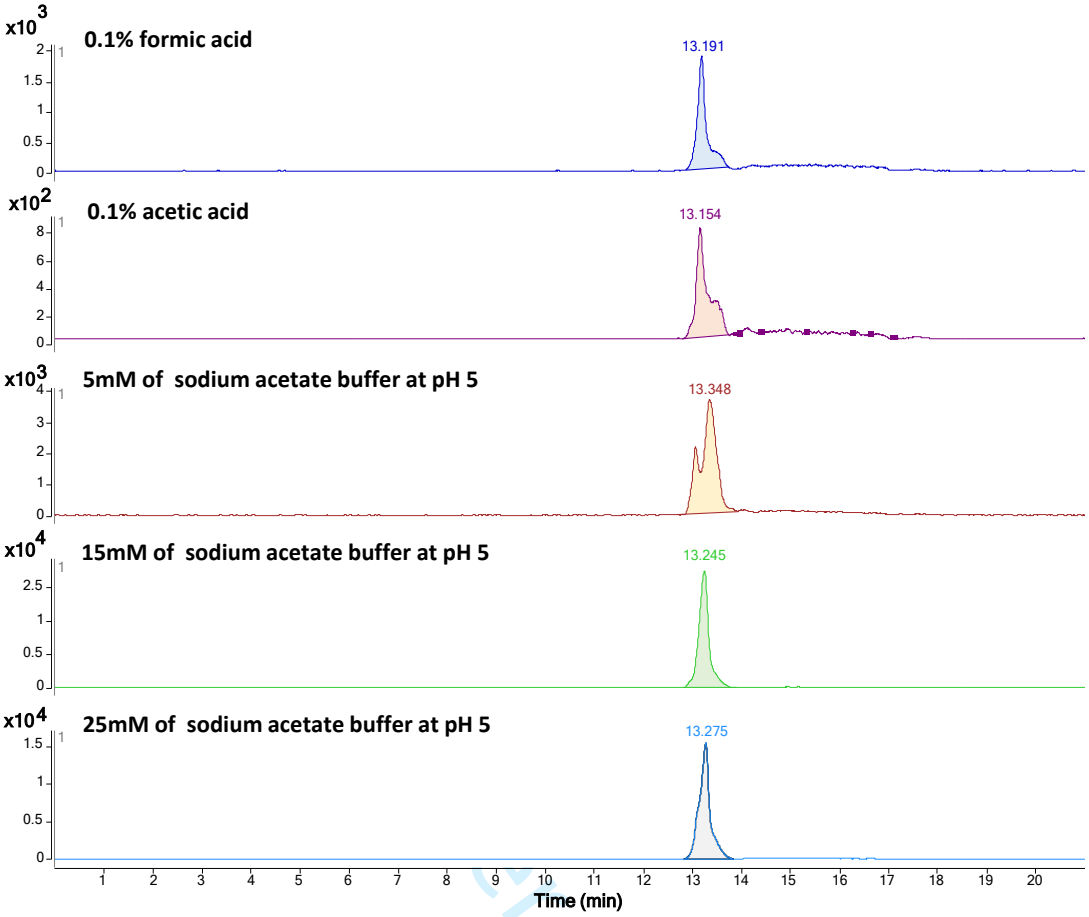


**Fig. S5** Effect of online SPE aqueous phase modifiers (B1) in the peak shape and signal intensity for BPS in urine

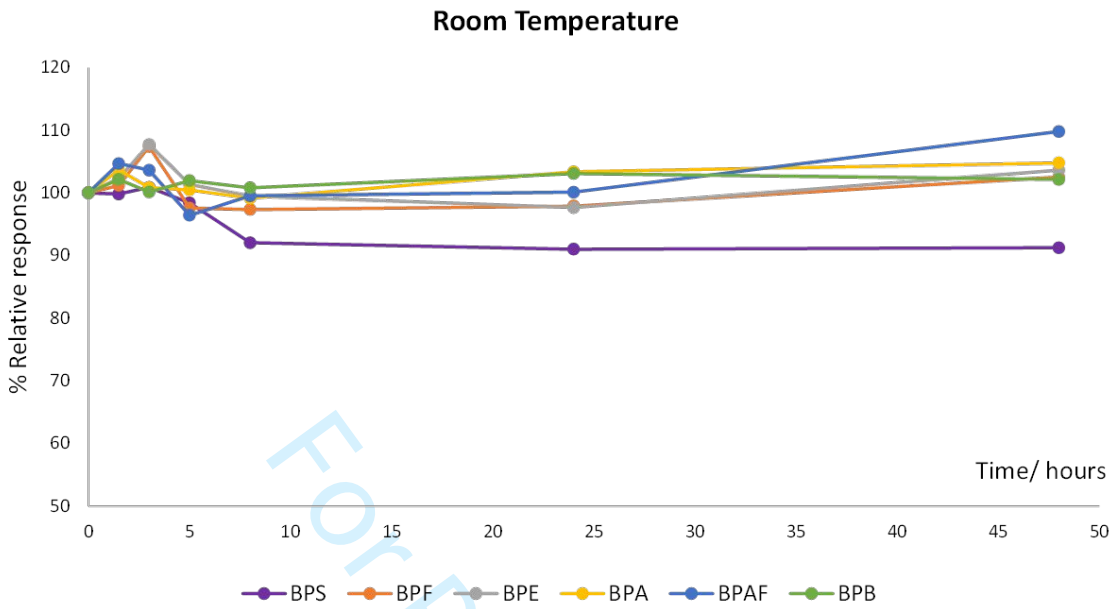


**Fig. S6** Stability of bisphenol in urine stored at room temperature. RSD (n=3) < 10%

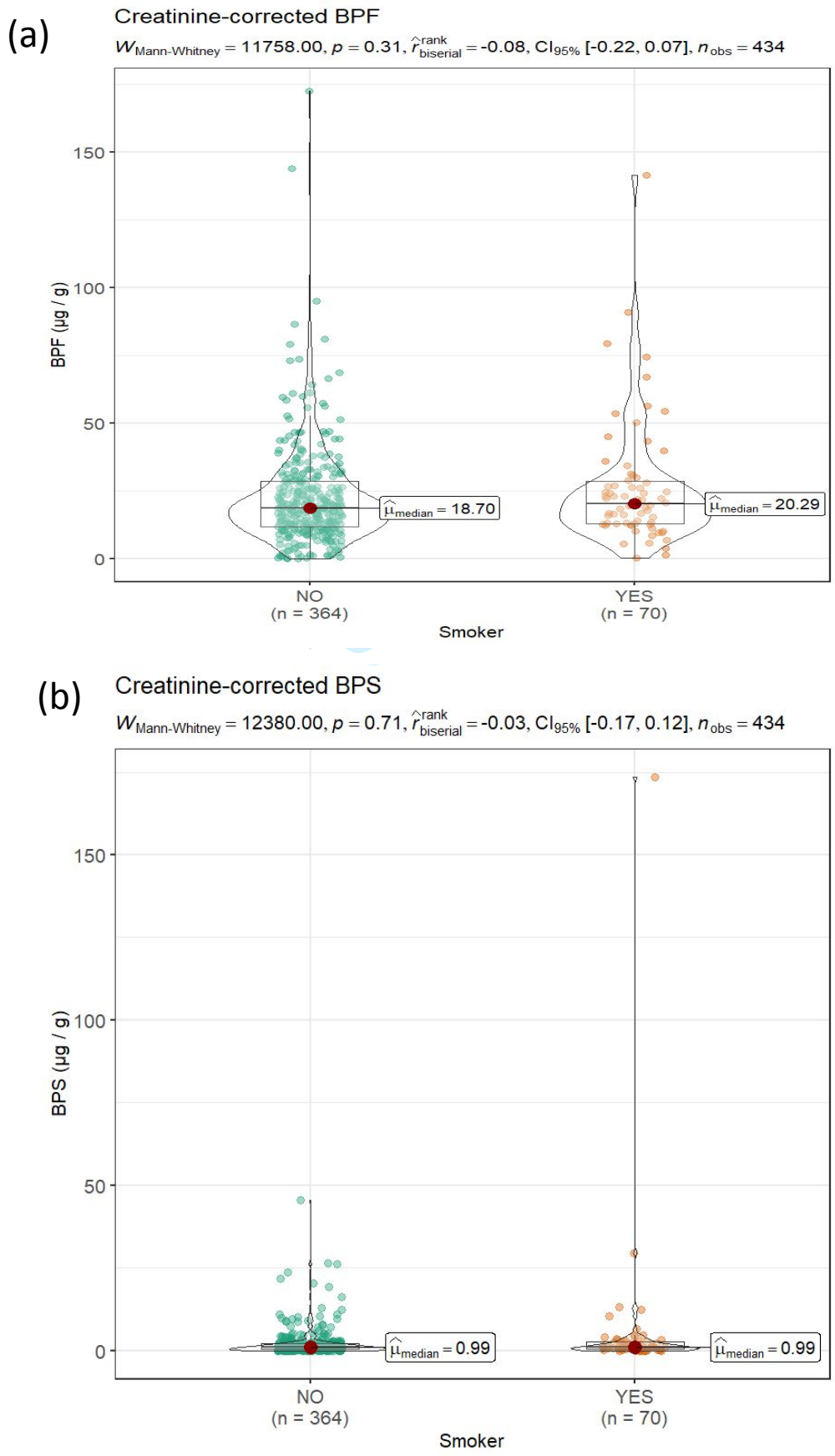


**Fig. S7** Comparison of creatinine corrected concentrations (µg $g^{-1}$) according to tobacco use: (a) BPF and (b) BPS

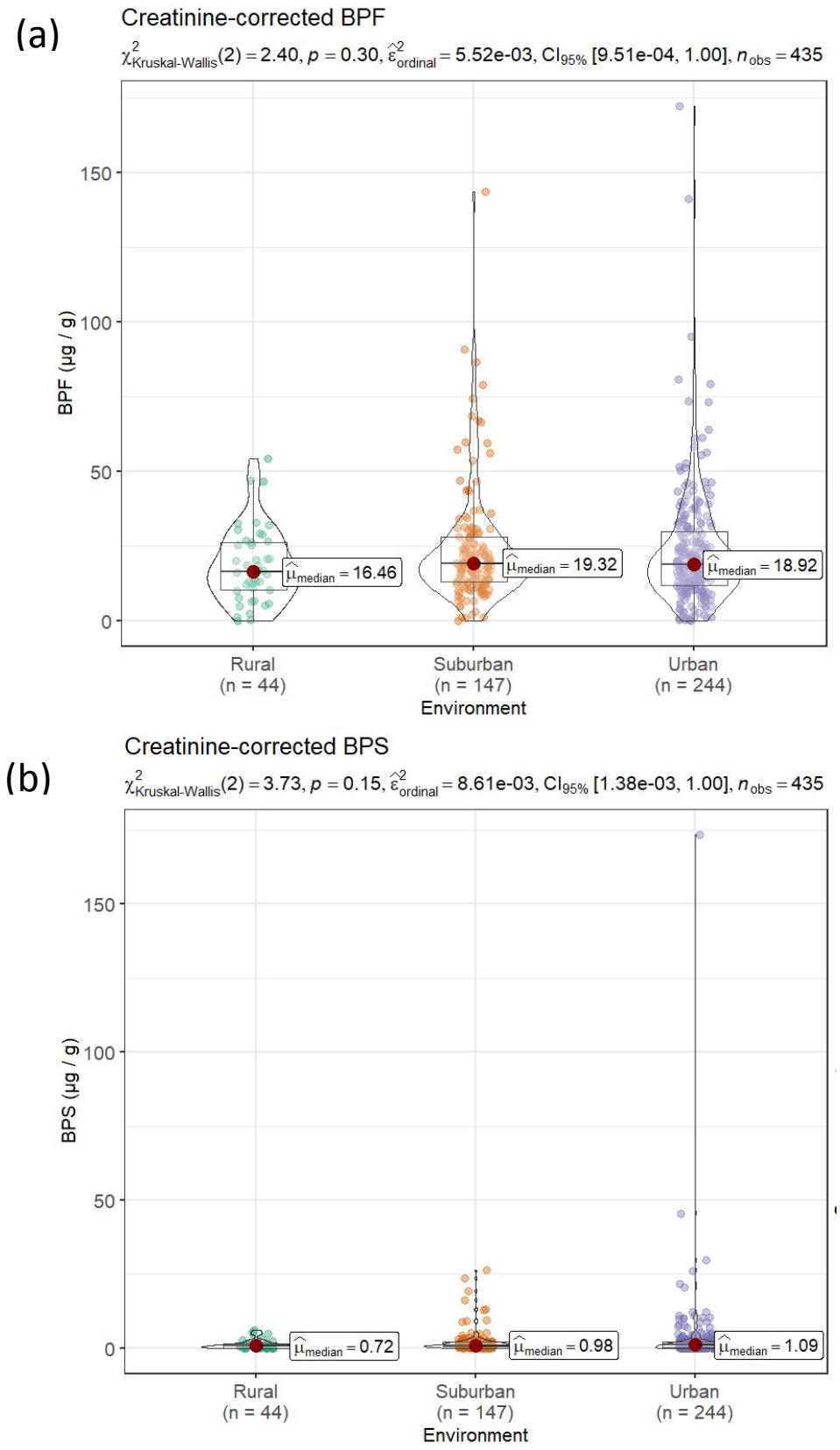


**Fig. S8** Comparison of creatinine corrected concentrations (µg $g^{-1}$) according to the residence environment: (a) BPF and (b) BPS