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8

9 **Determination of trace levels of eleven bisphenol A analogues in human blood serum by**  
10 **high performance liquid chromatography – tandem mass spectrometry.**

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19

## 20 **Abstract**

21 Chemicals showing structural or functional similarity to bisphenol A (BPA), commonly  
22 called BPA analogues, have recently drawn scientific attention due to their common industrial  
23 and commercial application as a substitutes for BPA. In European Union, the use of BPA has  
24 been severely restricted by law due to its endocrine disrupting properties. Unfortunately, it

25 seems that all BPA analogues show comparable biological activity, including hormonal  
26 disruption, toxicity and genotoxicity. Until now the knowledge about human exposure to BPA  
27 analogues is scarce, mainly due to lack of the data concerning their occurrence in human derived  
28 biological samples. This study presents the development of an analytical method for  
29 determination of trace levels of eleven BPA analogues in human blood serum samples.  
30 The method involves fast and simple liquid-liquid extraction, consuming small volumes of the  
31 sample and organic solvents.

32 Method of chromatographic separation was optimized by performing planned series of  
33 trial and errors tests (including e.g. gradient, chromatographic column selection, software  
34 optimization of ESI and MS/MS working parameters). The method allows for effective  
35 separation of the analytes, even in the case of configurational isomers (bisphenol M and  
36 bisphenol P). The calibration curves for all analytes were linear in the range tested. The limits  
37 of detection and quantitation were in the range of 0,0079–0,039 ng/mL and 0,024–0,12 ng/mL  
38 respectively. Compound-dependent recovery values were in the range of 87,6–138,2%. Matrix  
39 effects were mitigated with the help of matrix-matched calibration curves prepared for every  
40 batch of samples. Results obtained after analysis of 245 real serum samples indicates that  
41 human beings are exposed to different BPA analogues, that are present in the environment and  
42 in common, daily use products.

43  
44 **keywords: bisphenol A, BPA analogues, blood serum analysis, liquid chromatography,**  
45 **tandem mass spectrometry, endocrine disrupters**

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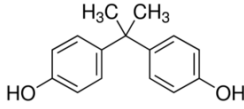
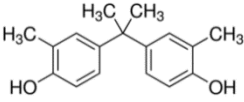
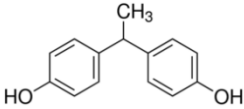
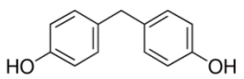
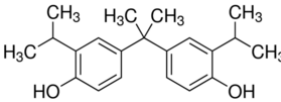
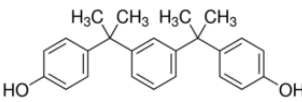
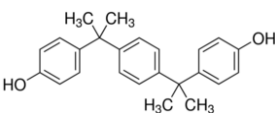
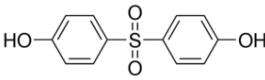
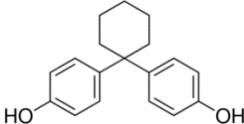
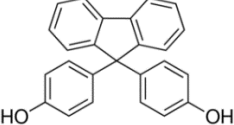
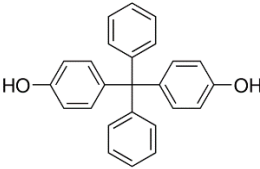
## 48 1. Introduction

49 In the last years endocrine disrupting compounds (EDCs) have become the chemical group  
50 of special concern due to their ability to interfere with hormonal system and ubiquitous presence  
51 (Rissma and Adli, 2014). Bisphenols (BPs) are the chemicals that have recently been the subject  
52 of growing interest due to their endocrine disrupting properties. BPs contain two *p*-  
53 hydroxyphenyl functionalities in their molecular structure and include several analogues, of  
54 which bisphenol A (BPA) is the most commonly used and known . Since early 1950s, BPA has  
55 been used in plastic industry for the production of epoxy and polycarbonate resins (Vogel,  
56 2009) being commonly used as raw materials in the manufacturing of a broad spectrum of  
57 everyday use products (tin linings and other food contact materials, water pipes, powder paints,  
58 toys etc.). Nowadays, BPA annual production reaches over 6 million tons and the demand for  
59 this compound is predicted to increase over next years (Kadasala et al., 2016).

60 The effects exerted by BPA on human health have been extensively studied and its  
61 estrogenic activity is one of the best known upshots. Besides that, the vast number of other  
62 adverse effects have been proven, including neural and developmental disorders (Arbuckle et  
63 al., 2016, Kundakovic et al., 2013), alternation of thyroid function (Ahmed, 2016), metabolic  
64 disorders (Stojanoska et al., 2017) and suspicion of increasing the risk of Parkinson disease  
65 (Huanga et al., 2014). Hazardous implications of BPA presence are not only limited to humans.  
66 Especially, the homeostasis of aquatic ecosystems can be disrupted in various ways such as  
67 feminization of many wildlife species or developmental and behavioral alternations (Bhandari  
68 et al., 2015). Detailed information including chemical structure and IUPAC names of bisphenol  
69 A analogues that are subjected to present study are given in Table 1.

70

Table 1. Basic information on bisphenol A analogues.

Compound/ molecular weight [g/mol]	CAS number	Structure	IUPAC name
<b>BPA</b> 228,29	80-05-7		2,2-Bis(4-hydroxyphenyl)propane
<b>BPC</b> 256,34	79-97-0		2,2-Bis(4-hydroxy-3-methylphenyl)propane
<b>BPE</b> 214,26	2081-08-5		1,1-Bis(4-hydroxyphenyl)ethane
<b>BPF</b> 200,23	620-92-8		4,4'-Methylenediphenol
<b>BPG</b> 312,45	127-54-8		2,2-Bis(4-hydroxy-3-isopropylphenyl)propane
<b>BPM</b> 346,46	13595-25-0		4,4'-(1,3-Phenylenediisopropylidene)bisphenol
<b>BPP</b> 346,46	2167-51-3		4,4'-(1,4-Phenylenediisopropylidene)bisphenol
<b>BPS</b> 250,27	80-09-1		4,4'-Sulfonyldiphenol
<b>BPZ</b> 268,35	843-55-0		4,4'-Cyclohexylidenebisphenol
<b>BPFL</b> 350,41	3236-71-3		4,4'-(9-Fluorenylidene)diphenol
<b>BPBP</b> 352,43	1844-01-5		1,1-Bis(4-hydroxyphenyl)-1,1-diphenylmethane

72 Due to the growing doubts concerning ecological and long-term health implications,  
73 new BPA-related chemicals were considered to be safer alternatives for industrial applications.  
74 The total number of 16 bisphenols have been documented to be commercially applied (Chen et  
75 al., 2016). Bisphenol S and bisphenol F are nowadays the most commonly used BPA  
76 substitutes, predominantly in the manufacturing of epoxy resins, polyesters and polycarbonate  
77 plastics. Other analogues are also used in plastic industry to produce dental sealants, pesticides,  
78 thermal papers, food container's inner coatings, toys, lacquers, powder paints, flame retardants,  
79 personal care products, thermosensitive materials and others (Hada et al., 2010, Hsieh and Hsu,  
80 2015, Ochiai and Masuda, 2009, Teichert et al., 2014, Wagner et al., 2015, Zouta et al., 2014).  
81 Currently, only the bisphenol A applications are regulated by legislative standards in European  
82 Union, United States and Canada (Yang et al., 2014a). BPA, BPE, BPF, BPS, BPP, BPZ,  
83 TBBPA, TCBPA, and BPAF were detected in sludge, surface water and indoor dust (Bhandari  
84 et al. 2015, Lee et al., 2015, Song et al., 2014, Yamazaki et al., 2015). BPA, BPB, BPE, BPF,  
85 BPP, BPS, BPZ BPAF, and BPAP were detected in foodstuffs (Liao and Kannan, 2013, Yang  
86 et al., 2014). Unfortunately, the understanding of the environmental, biological and health  
87 impact of BPA analogues is still very scarce. The environmental abundance of BPs undoubtedly  
88 indicates that humans are constantly exposed to the wide spectrum of these chemicals, but the  
89 data concerning the presence of BPA analogues in human-derived samples is still very limited.

90 Human exposure to mentioned chemicals include dietary (as a most probable) and non-  
91 dietary (inhalation, dermal) routes. Human exposure to bisphenol analogues other than BPA is  
92 not well characterized. Available data concerning bisphenols levels in body fluids and tissues  
93 is very limited so far. Majority of studies has been conducted on the urine where BPA, BPAF,  
94 BPB, BPF, BPS, BPP and BPZ have been measured.

95

96           Among sampling material of human origin, blood (and its derivatives - plasma and  
97 serum) is a matrix that carries most valuable information about short-term exposure due to its  
98 contact with all body cells and tissues. Because of lack of scientific data concerning analytical  
99 methods for determination of wide spectrum of BPA analogues, the aim of this research was to  
100 develop easy, fast, highly sensitive and robust method for human biomonitoring of these  
101 chemicals. To encompass the range of bisphenols that are commonly present in environment  
102 constituents and may pose health risk, the total number of 11 bisphenols were determined in  
103 human serum by high-performance liquid chromatography tandem mass spectrometry (HPLC-  
104 MS/MS). Analytes have been selected on the basis of the probability of their occurrence in both  
105 environment and common goods and previous research concerning their genotoxicity,  
106 estrogenicity or toxicity (Chen et al., 2002, Rivas et al., 2002). Taking into consideration that  
107 most of bisphenols other than BPA exhibit similar biological activity (Chen et al., 2002, Rivas  
108 et al., 2002, Rosenmai et al., 2014, Sui et al., 2012) and number of their industrial and  
109 commercial applications is increasing (Bhandari et al. 2015, Lee et al., 2015, Song et al., 2014,  
110 Yamazaki et al., 2015), introducing new methods for BPs biomonitoring seems to be justified  
111 due to constant human exposure to them. In addition, the data concerning occurrence and  
112 concentration levels of BPA analogues in human derived samples is very limited or absent. To  
113 the best of authors' knowledge, this is the first report on determination of eleven BPA analogues  
114 in human blood serum samples.

115

## 116       **2. Experimental**

### 117       **2.1 Materials and standards**

118       Analytical standards of BPA, BPC, BPE, BPF, BPG, BPM, BPP, BPS, BPZ, BPFL and  
119 BPBP were purchased from Sigma-Aldrich (St. Louis, USA), 99% purity. Acetic acid, formic

120 acid and ammonia were purchased from Sigma-Aldrich (St. Louis, USA). MgSO<sub>4</sub> was obtained  
121 from Eurochem BGD (Tarnów, Poland). Internal standard <sup>13</sup>C-labeled BPA (ring-<sup>13</sup>C<sub>12</sub>) was  
122 supplied by Cambridge Isotope Laboratories Inc. (UK). Blank bisphenol-free normal human  
123 serum reference material was obtained from Sigma-Aldrich (St. Louis, USA). Acetonitrile  
124 (ACN) and methanol (MeOH), used during the sample preparation procedure and as a mobile  
125 phase components, were LC-MS grade, obtained from Merck KGaA (Darmstadt, Germany).  
126 Ultrapure water was produced by the Milli-Q Gradient A10 system equipped with an EDS-Pak  
127 cartridge for removing endocrine disrupting compounds (Merck-Millipore).

128

## 129 **2.2 Samples**

130 A total of 245 serum samples were collected by personnel of Medical University of Gdansk  
131 from adult female patients suffering from various disorders of endocrine nature. Informed  
132 consent has been obtained from all patients who participated in clinical investigations. Samples  
133 were collected in glass vials and stored in -80°C.

134

## 135 **2.3 Preparation of standards and calibration**

136 Individual stock solutions (0,5 mg/mL) of all analytes were prepared by dissolving  
137 accurately weighted amounts of analytical standards in ACN. Working solution was obtained  
138 by mixing the stock solutions and diluting the mixture with ACN. All solutions were stored in  
139 a freezer (-20°C). All glassware was pre-washed with methanol. Seven-point (0,05, 0,1, 0,25,  
140 0,5, 1, 2,5 and 5 ng/mL) matrix-matched calibration curves were prepared using bisphenol-free  
141 blood serum. Internal standard (IS) concentration was kept at 25 ng/mL in all calibration  
142 samples. Fresh calibration solutions were prepared for every batch of samples. Purchased blank

143 normal human serum, used for preparation of the calibration curves, was analyzed and  
144 confirmed to be free of bisphenols.

145

#### 146 **2.4 Sample preparation**

147 Sample preparation step was conducted with precautions intended to minimize sample  
148 contamination. All used glassware was previously flushed with MeOH and all plastics were  
149 made of high quality polypropylene to avoid bisphenols passing into the samples. In order to  
150 extract the analytes from serum samples, the modified liquid-liquid extraction method  
151 described was used. The 500  $\mu\text{L}$  of serum was mixed with 1,5 mL of ACN and 10  $\mu\text{L}$  of IS  
152 solution (2,5  $\mu\text{g}/\text{mL}$ ) was added. Samples were shaken for 30 seconds and left for 10 minutes  
153 in room temperature for complete protein precipitation. After that, 250 mg of anhydrous  $\text{MgSO}_4$   
154 was added to each sample that were then vortexed to remove water, followed by centrifugation  
155 (6000 rpm, 2 minutes). Supernatants were transferred to clean glass tubes and evaporated under  
156 the gentle stream of nitrogen in water bath (42°C), to the final volume of about 150  $\mu\text{L}$ . The  
157 residue was diluted with 250  $\mu\text{L}$  of mobile phase (MeOH:H<sub>2</sub>O, 50:50, 0,01% v/v NH<sub>3</sub>),  
158 vortexed again and transferred to chromatographic vials for analysis. Procedural blanks spiked  
159 with IS were prepared in the same way as other samples for every batch in triplicate along with  
160 system blanks. Chromatograms of procedural blanks are given in Supplementary Figure 1. in  
161 Supplementary Materials.

162

#### 163 **2.5 MS/MS and separation conditions**

164 All analyses were performed with Shimadzu triple quadrupole LC-MS/MS system (LCMS-  
165 8060, Shimadzu, Japan) equipped with an electrospray ionisation source (ESI) working in the





166 negative multiple reaction mode (MRM). Conditions of ion fragmentation were optimized for  
167 all analytes with the help of LabSolutions v.5.85 Software. Detailed information on ion  
168 transitions, MS/MS operational parameters and ion source parameters are given in  
169 Supplementary Material (Supp. Tab.1). **Analytes standard solutions including IS were first**  
170 **separately injected directly into MS/MS instrument to determine pseudomolecular ions.**  
171 **Then product ion scan was performed to find and identify specific molecular fragments.**  
172 **Finally, software automatic optimization of voltages for specific MRM transition was**  
173 **performed. Ion source parameters was adjusted manually to obtain the best signal**  
174 **intensity for all analytes.**

175

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177

## 178 **2.6 Separation conditions**

179 Chromatographic separation was carried out using UPLC Nexera X2 system (Shimadzu,  
180 Japan) consisting of degasser DGU-20A5R, controller CBM-20A, binary pump LC-30AD,  
181 autosampler SIL-30AC and column oven CTO-20AC. Two chromatographic methods were  
182 applied to determine analytes. BPC, BPE, BPF, BPG, BPM, BPP, BPZ, BPFL, BPBP were  
183 separated using gradient of H<sub>2</sub>O (mobile phase A) and MeOH (mobile phase B), both modified  
184 with 0,01% v/v of ammonia. Initial conditions of 5% B were kept for 1.5 minutes, before  
185 increasing to 75% over 10,5 minutes and further **gradient** increased to 100% over 4 minutes.  
186 Following this, mobile phase composition was set-back to starting conditions and maintained  
187 for 5 minutes for column re-equilibration.

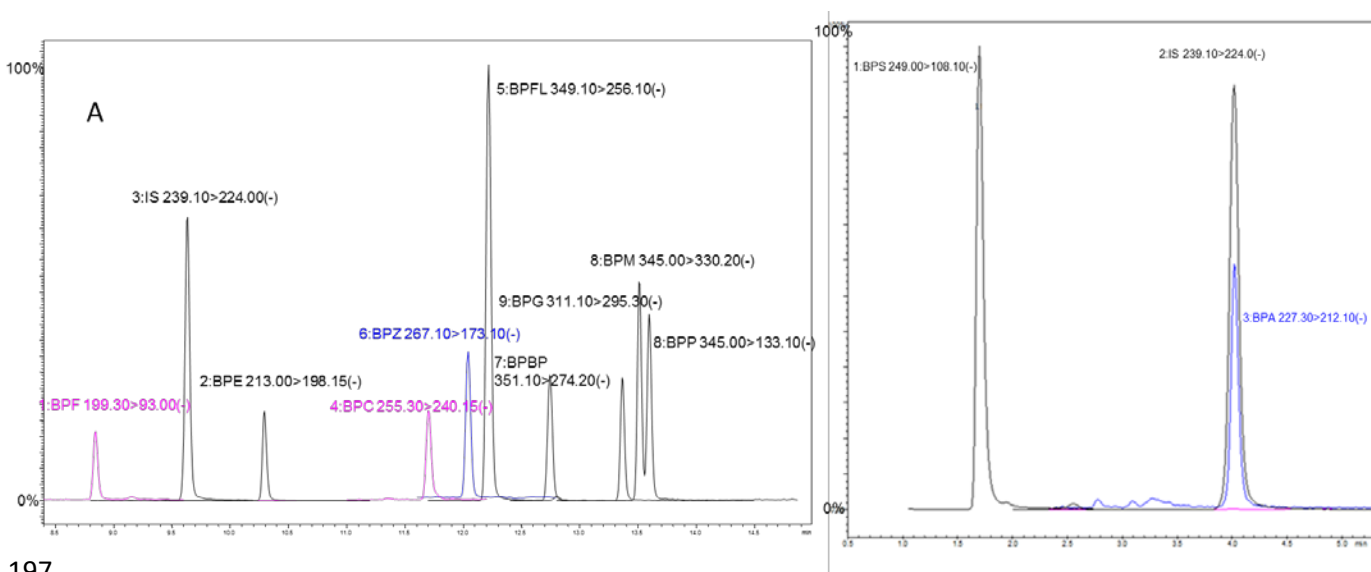


188 For the determination of BPA and BPS the mobile phase consisted of H<sub>2</sub>O (component A)  
189 and MeOH (component B) without additives. Isocratic elution of 50% B was used.

190 Ascentis® Express (C18 15cm x 2.1mm, 2.7µm) with guard column (0,5cm x 2,1mm,  
191 2,7µm), mobile phase flow of 0,55 mL/min, 50°C of thermostated column compartment and  
192 injection volume of 5µL were applied for separation of analytes in case of both methods.

193 Examples of chromatograms obtained after analysis of calibration solutions using both  
194 methods are given in the Figure 1. More detailed information on separation conditions will be  
195 discussed in the next section.

196



197

198 **Figure 1.** Chromatograms obtained after analysis of calibration solutions; (A) mixture of 9 bisphenols, (B) mixture  
199 of BPA and BPS.

200

201

202

### 203 3. Results and discussion

#### 204 3.1 Extraction conditions

205 General problem in bisphenols analysis is ion suppression, resulting from the presence  
206 of matrix components. In case of such complex biological matrix as blood serum, procedure  
207 was focused on removal of its elements. ACN and MeOH were tested as the extraction solvents  
208 that are also needed for precipitation of endogenous proteins. Eventually, ACN was chosen due  
209 to significantly higher recovery of analytes. Modification of liquid-liquid extraction with  
210 anhydrous MgSO<sub>4</sub> enhanced peaks intensity, due to precipitation of water soluble interferences,  
211 which caused signal suppression. Smaller amount of water in the liquid phase could also  
212 improve transport of analytes into the extractant.

213

#### 214 3.2 Separation and detection of the analytes

215 The goal of the conducted research was to develop analytical method for separation and  
216 determination of 11 bisphenols in blood serum samples. Ascentis® Express C18 (15cm x  
217 2,1mm, 2,7µm) column with guard column (0,5cm x 2,1mm, 2,7µm), packed with core-shell  
218 technology particles, was chosen due to its high separation efficiency and relatively short  
219 analysis time.

220 MeOH and ACN were tested as main organic components of the mobile phase, however,  
221 in the case of ACN, peak broadening, peak shape deterioration along with the strong signal  
222 suppression were noted. The probable cause of response decrease in the presence of ACN is  
223 lower surface tension of the MeOH, that is conducive to desolvation of electrospray droplets  
224 (Caballero-Casero et al., 2002, Regueiro et al., 2015), hence methanol was selected as the  
225 mobile phase component. 5, 10, 20 and 25 mM ammonium acetate, 0,01, 0,05 and 0.1% v/v

226 formic acid, acetic acid and ammonia were tested separately as the mobile phase additives to A  
227 and B components. The most promising results (in terms of response and peak shapes) were  
228 obtained when applying the latter. Buffer and acid solutions caused signal suppression.

229 All analytes are similar in molecular structure and two of them – namely BPM and BPP,  
230 are configurational isomers (Tab.1). In order to gain enhanced chromatographic separation of  
231 mentioned analytes, the initial content of the organic mobile phase component was kept at 5%  
232 for 1,5 minutes and was slowly increased up to 75%. Optimum column oven temperature was  
233 set to 50°C (range from 30 – 50°C), providing best peak shapes and separation.

234 Addition of ammonia to the mobile phase resulted in sensitivity decrease and shifting  
235 the bisphenol S signal towards system void time. Moreover, low initial content of methanol and  
236 long analysis and conditioning time caused the enrichment of bisphenol A, derived from system  
237 elements, on the front of separation column (Wilczewska et al., 2016). Therefore BPA and BPS  
238 were determined with separate method using a mobile phase consisting of MeOH:H<sub>2</sub>O without  
239 additives. Isocratic flow of relatively high elution strength (50% v/v MeOH) mobile phase  
240 provided accurate results and good linearity of calibration curves.

### 241 **3.3 Method validation**

242 The performance of both analytical methods was evaluated in terms of linearity, limits  
243 of detection (LODs) and quantification (LOQs) and recoveries. Obtained results are presented  
244 in Table 2 and in Suppl. Table 2 of Supplementary Material. For both methods the linear  
245 calibration equations were obtained from 7-point calibration curves, that were made by plotting  
246 the ratios of analyte peak area to IS peak area versus corresponding concentrations. Calibration  
247 curves were linear in the tested concentration range from 0,05 to 5 ng/mL. To increase accuracy  
248 of lowest concentration points, the weighting factor 1/x was applied to every calibration curve.



249 The LOD values were evaluated on the basis of matrix-matched calibration curves **analyzed in**  
 250 **triplicates**, using equation  $LOD=(3,3S_b)/a$ , where:  $S_b$  – standard deviation of intercept of the  
 251 calibration curve,  $a$  – slope of the calibration curve.

252 In further calculations of LOQ values it was assumed that  $LOQ = 3xLOD$ . LOD values were in  
 253 the range from 0,0079 ng/mL for BPG to 0.039 ng/mL for BPBP, which indicate that proposed  
 254 analytical method is highly sensitive towards BPA analogues. More detailed information on  
 255 validation parameters are given in Suppl. Table 2 of Supplementary Material.

256 To evaluate the recoveries, spiked samples were prepared according to the described  
 257 procedure using serum free from bisphenols. Six independent chromatographic runs were  
 258 carried out for each of three concentration levels. Obtained recoveries vary from 87,6% for BPC  
 259 up to 138,2% for BPZ. All relative standard deviations are below 10% and are within the range  
 260 of 1,2% to 7,8%. Recovery discrepancies observed for some bisphenol analogues confirm, that  
 261 preparing matrix-matched calibration curves during analysis of real samples is highly justified  
 262 in order to minimize matrix effects.

263 Matrix effects for each analyte were also evaluated and are given in Suppl. Table 2  
 264 (Supplementary Material). The enhancement of signal in the range of 5% for BPA and BPZ up  
 265 to 29% for BPP were observed, which is another significant premise for use of matrix-matched  
 266 calibration curves.

Tab. 2 Recovery values obtained for three independent concentrations of spiked quality control (QC) samples

Analyte	Recovery (RSD) [%] of analyte (n=6)			Detection and quantification limits (n=3)	
	0,05 ng/mL	0,5 ng/mL	1 ng/mL	LOD [ng/mL]	LOQ [ng/mL]
<b>BPC</b>	89,8 (3,4)	87,6 (1,9)	88,7 (2,3)	0,021	0,061
<b>BPE</b>	96,0 (3,7)	95,5 (3,0)	106,1 (2,9)	0,011	0,032
<b>BPF</b>	123,4 (4,2)	120,7(3,6)	118,7 (2,8)	0,012	0,037
<b>BPG</b>	103,7 (1,4)	104,4 (1,7)	103,9 (1,2)	0,008	0,024

<b>BPM</b>	90,3 (2,3)	90,7 (3,2)	93,4 (4,8)	0,018	0,054
<b>BPP</b>	105,1 (6,5)	103,5 (7,8)	105,8 (2,6)	0,019	0,056
<b>BPZ</b>	132,4 (1,5)	138,2 (1,9)	134,6 (1,9)	0,017	0,051
<b>BPFL</b>	98,3 (3,3)	99,1 (2,7)	99,6 (2,8)	0,014	0,041
<b>BPBP</b>	99,2 (2,7)	98,9 (1,9)	99,0 (1,7)	0,039	0,12
<b>BPA</b>	103,2 (12)	106,0 (2,9)	101,0 (2,6)	0,009	0,028
<b>BPS</b>	96,0 (15)	96,5 (3,7)	101,6 (3,0)	0,022	0,067

267

#### 268 4. Analysis of real samples

269 Proposed methods were successfully applied to analyse 245 real human blood serum  
 270 samples in order to determine the analytes' content and to assess the human exposure to 11  
 271 BPA analogues for the first time. The results are summarized in Table 3 and examples of real  
 272 sample chromatograms are given in the Figure 2. Analytes were found in over 50% of serum  
 273 samples except for BPC, BPZ, BPFL and BPBP. Bisphenol A, bisphenol G and bisphenol S  
 274 were the most often occurring analogues. Beyond the problem of constant human exposure, the  
 275 presence of bisphenols in blood is important in the terms of possible health issues. There are  
 276 scientific proofs that these compounds have the ability to induce eryptosis (suicidal death of  
 277 erythrocytes) (Maćczak et al., 2016) or biochemical and morphological alternations in  
 278 mononuclear cells of peripheral blood (Michałowicz et al., 2015).

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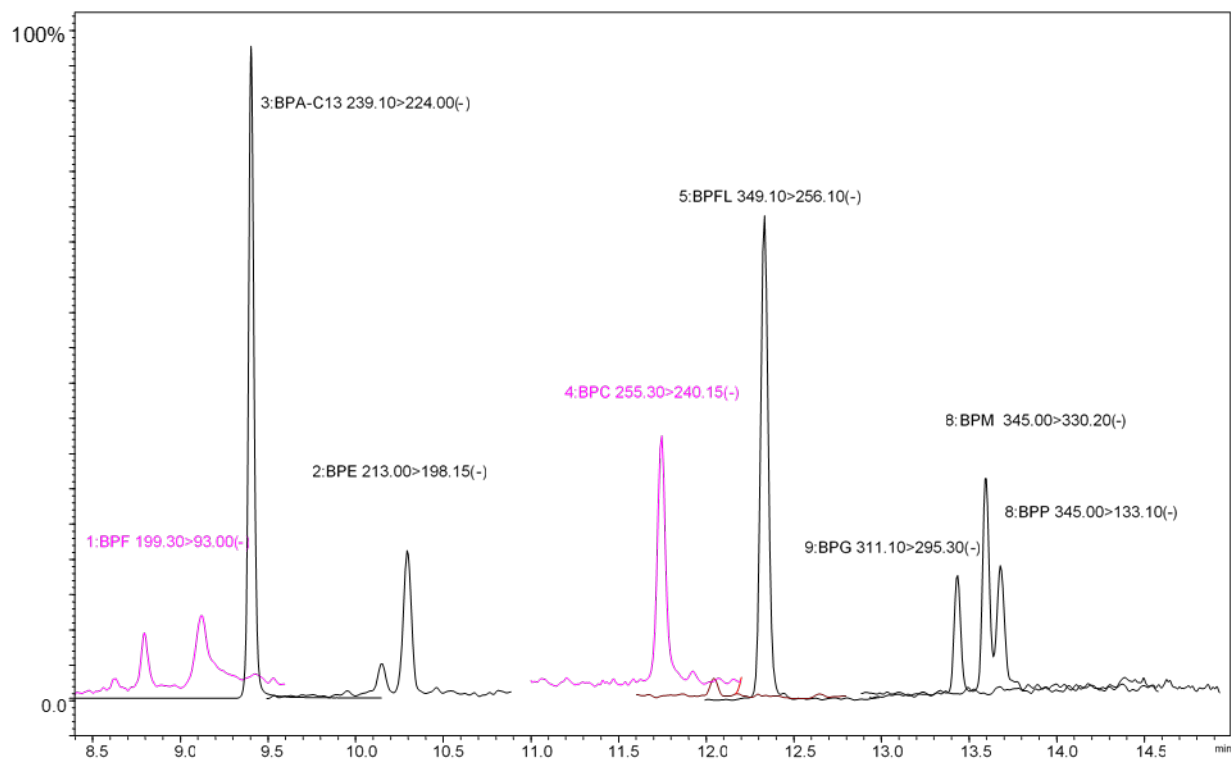
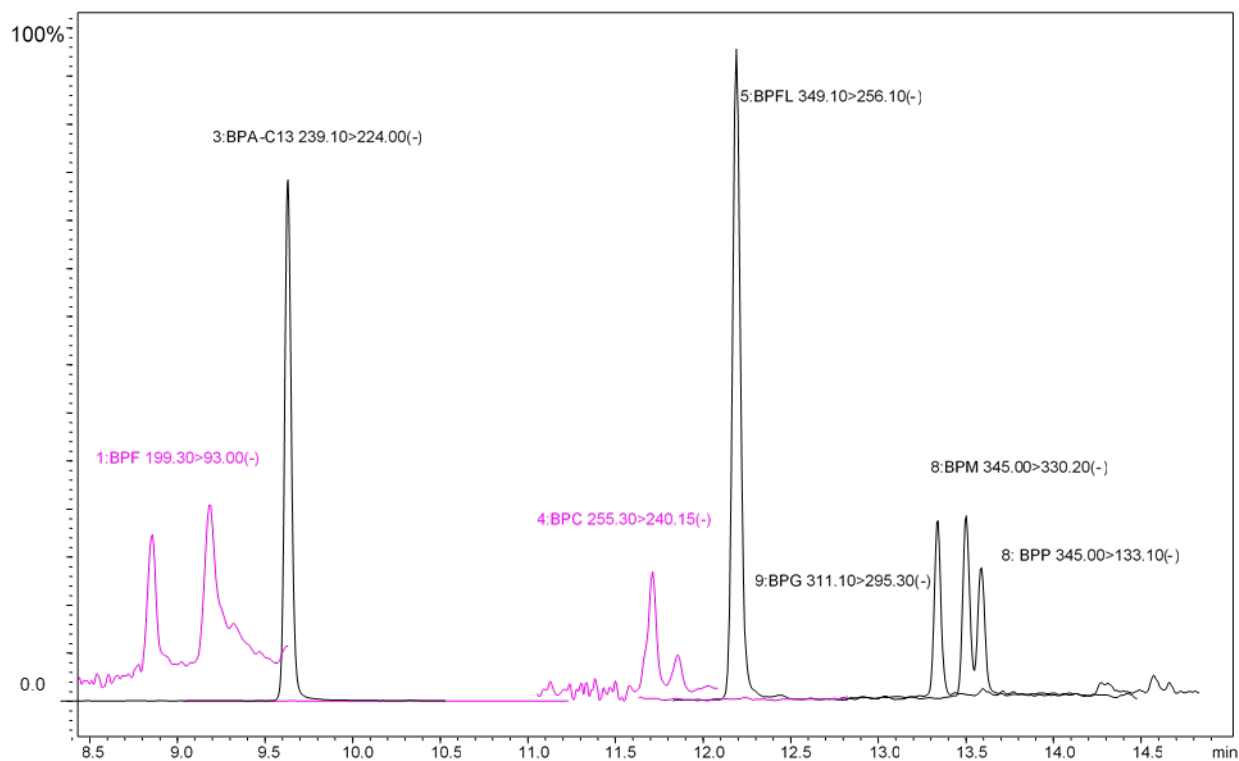
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Tab. 3 Information on the results obtained for the real samples analysis

<b>Analyte</b>	<b>Quantification rate [%of all samples]</b>	<b>Detection rate &gt;LOD [%of all samples]</b>	<b>Median [ng/mL]</b>	<b>Concentration range [ng/mL]</b>
BPC	27,2	40,7	0,18	0,071-3,8
BPE	55,1	59,7	0,15	0,053-0,828
BPF	49,8	65,0	0,12	0,052-0,845
BPG	60,5	70,4	0,19	0,050-1,190
BPM	58,8	65,8	0,21	0,057-1,104
BPP	52,7	66,3	0,14	0,057-0,917
BPZ	37,5	45,3	0,24	0,053-1,415
BPFL	7,8	23,5	0,070	0,050-1,597
BPBP	32,9	39,5	0,40	0,13-2,846
BPA	86,4	91,4	0,12	0,050-4,1
BPS	68,7	72,0	1,1	0,073-4,8

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286

287 Fig.2 Examples of chromatograms obtained after the analysis of a real blood serum samples





288

## 289 **5. Conclusions**

290 Presence of BPA and its analogues in different environmental matrices has been well  
291 studied by now, but there is still an insufficient scientific data on the occurrence of bisphenol  
292 analogues in the human and animal tissues/fluids. Although bisphenols other than BPA do  
293 not seem to be safer alternative, the interest in this research area has increased in the last  
294 decade. In the field of biomonitoring of BPs in human fluids and tissues the scientific data  
295 is still scarce although already noticeable. In 2015 Asimakopoulos et al. determined 8  
296 bisphenols in human urine samples along with 49 other xenobiotics. Mean analytes  
297 concentrations varied from 0,05 ng/mL (for BPB and BPAF) up to 13,3 ng/mL (for BPS)  
298 (Asimakopoulos et al., 2015). In 2016 BPA and six other analogues (BPF, BPS, BPAP,  
299 BPAF, BPP, BPZ) were determined in human urine by applying novel DLLME technique  
300 coupled to LC-MS/MS. Mean BPA concentration was 2,8 ng/mL, while other analogues  
301 were found at much rates (2-10% of samples) (Rocha et al., 2016). In case of serum, plasma  
302 and blood, scientific data is even more limited. In 2009 BPA and BPB were determined in  
303 human serum at 0,79-7,12 ng/mL (BPA) and 0,88-11,94 ng/mL (BPB) concentration ranges  
304 (Cobellis et al., 2009). A broader range of analogues (BPA, BPB, BPC, BPE, BPF, BPPH,  
305 BPS, BPFL, BPP, BPM and BPZ) was determined in human breast milk in 2015  
306 Deceuninck et al. Only BPA and BPS were found in concentrations 0,002-1,16 ng/g and  
307 0,23 ng/g respectively. More information on the comparison of the results obtained in this  
308 study to other studies are given in Supplementary Table 3. Because of very limited data on  
309 analogues selected for this research, this comparison has been extended to bisphenol  
310 derivatives and other biological matrices. Most of analytes have been found in  
311 concentrations corresponding to the results presented.

312 In this paper the development of a novel analytical LC-MS/MS method for determination  
313 of a broad spectrum of bisphenol A analogues was described. Sample preparation procedure  
314 consisting of liquid-liquid extraction is a routine and inexpensive approach that consumes low  
315 volumes of the sample (500 $\mu$ L) and relatively low volumes of organic solvent. An addition of  
316 anhydrous MgSO<sub>4</sub> provided better peak shapes and response, due to removing water soluble  
317 interfering matrix compounds. The method is suitable to identify and effectively separate  
318 compounds of interest, even constitutional isomers (BPM and BPP), as well as to obtain very  
319 low detection and quantification limits. The developed method was successfully applied for the  
320 analysis of real human blood serum samples. To the best of authors' knowledge, this study is  
321 the first attempt to determine selected 11 bisphenol A analogues in human derived serum  
322 samples. Results indicate that the problem of the bisphenols occurrence in body fluids is still  
323 underestimated, and may lead to some adverse health issues. For this reason, the development  
324 and application of novel analytical procedures focused on bisphenols' human biomonitoring  
325 are of high scientific importance.

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## ELECTRONIC SUPPLEMENTARY MATERIALS

### Determination of trace levels of eleven bisphenol A analogues in human blood serum by high performance liquid chromatography – tandem mass spectrometry.

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Suppl. Tab. 1 Optimized MS/MS conditions for negative mode MRM analysis for target analytes

<b>Compound</b>	Precursor ion [m/z]	M-H	Quantitation ion [m/z]	Confirmation [m/z]	Q1 PreRod bias* [V]	Collision energy [V]*	Q3 PreRod bias* [V]
<b>BPA</b>	227.3		212.1	133.1	11	20	13
<b>BPS</b>	249.0		108.1	92.0	12	27	10
<b>BPC</b>	255.3		240.15	147.1	13	20	10
<b>BPE</b>	213.0		198.15	119.1	10	18	18
<b>BPF</b>	199.3		93.0	105.1	13	23	13
<b>BPG</b>	311.1		295.3	175.2	22	35	29
<b>BPM</b>	345.0		330.2	133.1	17	29	14
<b>BPP</b>	345.0		330.2	133.1	17	29	14
<b>BPZ</b>	267.1		173.1	145.1	12	27	10
<b>BPFL</b>	349.1		256.1	215.1	12	27	11
<b>BPBP</b>	351.1		274.2	258.2	12	25	25
<b><sup>13</sup>C-BPA</b>	239.1		224.0	138.0	11	20	13
<b>Nebulizing gas flow [L/min]</b>	<b>Heating gas flow [L/min]</b>		<b>Interface temperature [°C]</b>		<b>DL temperature [°C]</b>	<b>Heat block temperature [°C]</b>	<b>Drying gas flow [L/min]</b>
3	10		300		250	400	10

\* presented values refer to quantitation ion only



Suppl. Tab. 2 Regression equations, LOD and LOQ for each analyte

Analyte	Calibration curve equation (7 points, n=3)	R <sup>2</sup>	LOD [ng/mL]	LOQ [ng/mL]	Matrix effect[%]
<b>BPC</b>	y=0.0052x+0.00035	0.9975	0.021	0.061	27
<b>BPE</b>	y= 0.015x+0.00077	0.9992	0.011	0.032	7
<b>BPF</b>	y=0.0035x+0.00051	0.9996	0.012	0.037	17
<b>BPG</b>	y= 0.0046x+0.00034	0.9991	0.008	0.024	15
<b>BPM</b>	y=0.010x+0.00023	0.9986	0.018	0.054	15
<b>BPP</b>	y=0.0088x+0.000070	0.9997	0.019	0.056	29
<b>BPZ</b>	y=0.0082x+0.00097	0.9993	0.017	0.051	6
<b>BPFL</b>	y=0.024x+0.00079	0.9988	0.014	0.041	13
<b>BPBP</b>	y=0.0068x+0.0035	0.9968	0.039	0.120	19
<b>BPA</b>	y=0.092x+0.0015	0.9997	0.0093	0.028	6
<b>BPS</b>	y=0.065x+0.017	0.9999	0.022	0.067	12

$$ME = \left( \frac{a_m}{a_s} - 1 \right) \times 100\%$$

where:

$a_m$  is the slope of the extracts (matrix) spiked with analytes,

$a_s$  is the slope of the solvent.

Suppl. Tab. 3 Comparison of LOD and LOQ values for different studies concerning determination of bisphenol analogues and derivatives in biological samples of human origin.

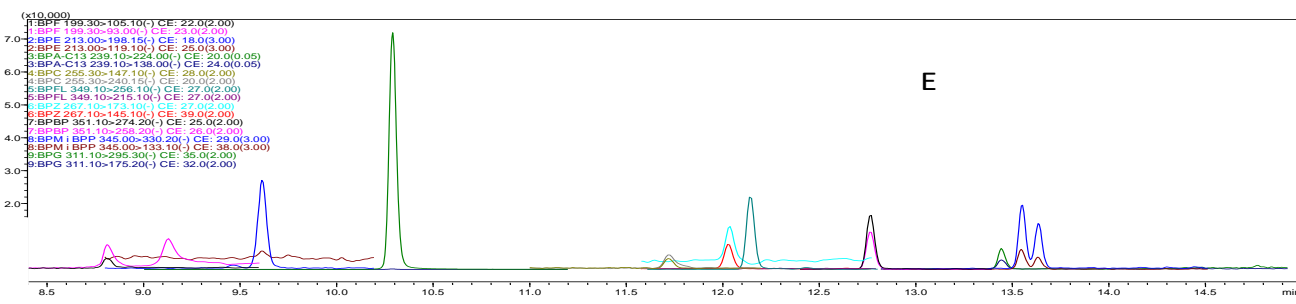
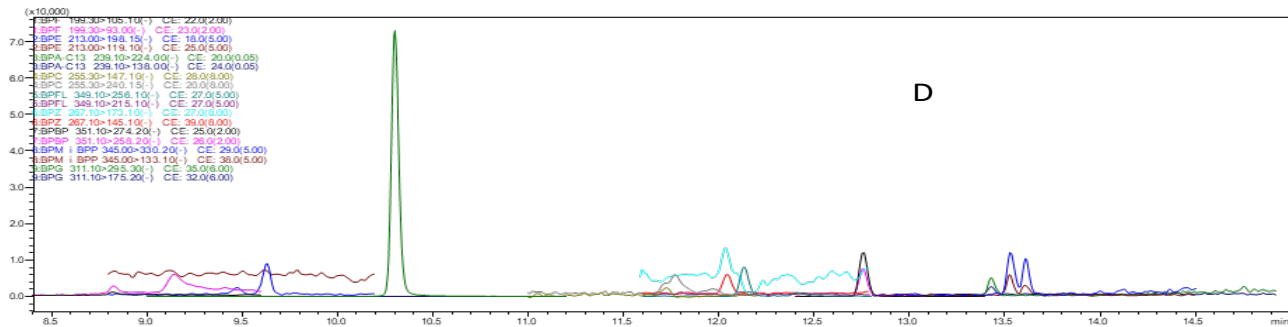
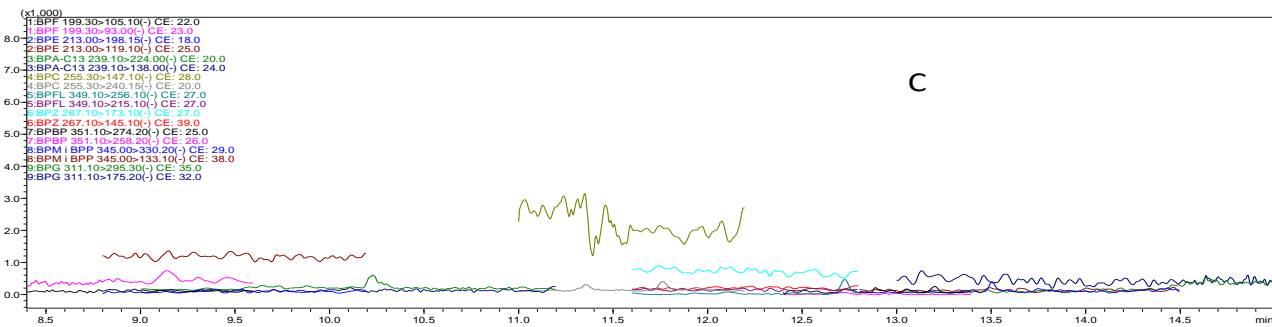
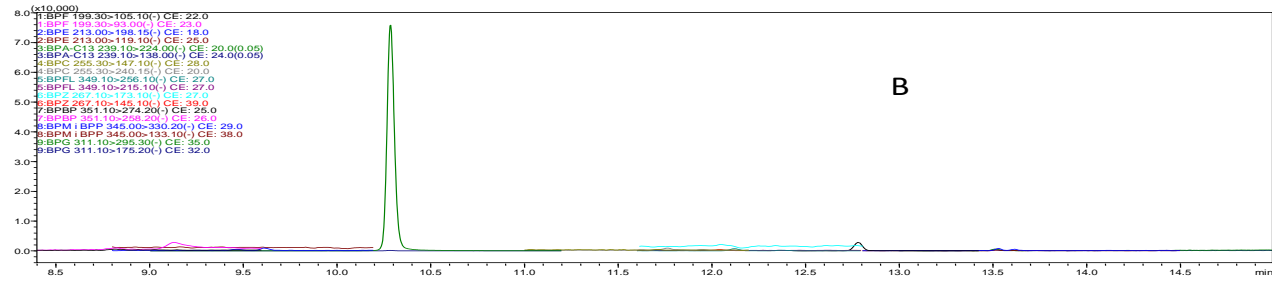
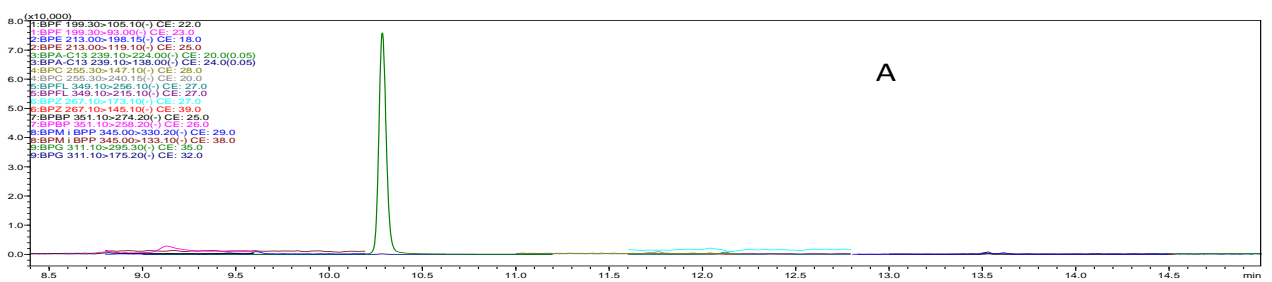
Analytes	Matrix	Sample volume [μL]	Determination method	LOD and LOQ [ng/mL]	Measured concentrations [ng/mL]	Reference
BPA, BPC, BPE, BPF, BPG, BPM, BPP, BPS, BPZ, BPFL, BPBP	Serum	500	HPLC-ESI-MS/MS	LOD = 0,0079 - 0,039 LOQ = 0,024 - 0,12	0,05- 4,8*	This study
BPA, BPADS, BPAG, BPAMC, DCBPA, TCBPA,	Serum	500	LC-ESI-MS/MS	LOD = 0,003 – 0,02 LOQ = 0,01 – 0,05	BPA <LOQ- 0,588 BPAG<LOQ – 11,9 BPADS <LOQ- 1,77	Liao et al., 2012
BPA, BPB	Serum	300	LC-FD	LOD = 0,15 – 0,18 LOQ = 0,5 – 0,6	BPA 0,79-7,12 BPB 0,88-11,94	Cobellis et al., 2009
BPA, BPB, BPAP, BPAF, BPBP, BPC, BPE, BPPH, BPS, BPF, BPFL, BPZ, BPM, BPP	Breast milk	3 [g]	GC-ESI-MS	MDL = 0,001 – 0,03 MQL = 0,003 – 0,1	BPA 0,02-1,16 BPS 0,23	Deceuninck et al., 2015
BPA	Breast milk	1000	LC-MS/MS	LOD = 0,22	0,22-10,8	Zimmers et al., 2014
BPA, BPB, BPF, BPS, BPAF,TCBPA	Urine	-	LC-MS/MS	LOQ = 0,024-0,310	BPA <LOQ – 8,07 BPAF<LOQ – 0,217	Yang et al., 2014b
BPA, BPF, BPP, BPS, BPZ, BPAF, BPAP	Urine	5000	LC-MS/MS	LOD = 0,005 – 0,2 LOQ = 0,02 – 0,5	BPA (mean) 2,8 Other analytes were found in very limited range of samples	Rocha et al., 2016

More details on analytes concentration ranges are given in Table 3.

MC – BPA monochloride, BPADC – BPA dichloride, BPATC – BPA trichloride, BPADG – BPA glucuronide, BPADS – BPA disulfate,

PA – tetrachloro- BPA





pplementary Figure 1. Chromatogram examples for procedural blanks (A and B), system blank (C), samples of human serum spiked with low concentration of analytes 0,05 ng/mL (D) and 0,5 ng/mL (E)

