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► To cite this version:

Guillaume Cambien, Nicolas Venisse, Virginie Migeot, Sylvie Rabouan, Mohamed Belmouaz, et al.. Simultaneous determination of bisphenol A and its chlorinated derivatives in human plasma: Development, validation and application of a UHPLC–MS/MS method. *Chemosphere*, 2020, 242, pp.125236 -. 10.1016/j.chemosphere.2019.125236 . hal-03488543

HAL Id: hal-03488543

<https://hal.science/hal-03488543>

Submitted on 21 Dec 2021

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1 **Simultaneous determination of bisphenol A and its chlorinated derivatives in**
2 **human plasma: development, validation and application of a UHPLC–MS/MS**
3 **method**

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27 **Abstract**

28 Bisphenol A (BPA) is a well-known ubiquitous chemical found in polycarbonate, polysulfone
29 and epoxy resins, used in mass production for many consumer products. BPA exhibits
30 endocrine disruptor properties that can potentially induce adverse health effects. In aquatic
31 environments, it can react with chlorine to produce chlorinated derivatives (ClxBPAs).
32 ClxBPAs exhibit oestrogenic activity 10 to 105 times higher than BPA itself. Assessing
33 human exposure to endocrine disrupting chemicals is mandatory to assess health risk.
34 Blood, as well as urine matrix, are commonly used to perform human biomonitoring. We
35 therefore developed, fully validated and applied a method based on Ultra High Performance
36 Liquid Chromatography coupled to a Triple Quad Mass Spectrometer to determine BPA,
37 monochlorobisphenol A (MCBPA), dichlorobisphenol A (DCBPA), trichlorobisphenol A
38 (TCBPA) and tetrachlorobisphenol A (TTCBPA) in human blood plasma. The European
39 Medicines Agency guidelines for bioanalytical method validation have been applied.
40 Precision and trueness of the method were <15% at medium and high levels of quality
41 control and <20% at the limits of quantification (LOQs). The LOQs were settled at 0.1 ng/mL
42 for BPA, 0.02 ng/mL for TTCBPA and 0.005 ng/mL for MCBPA, DCBPA, and TCBPA. The
43 analytical method was applied to ten patients suffering from end stage renal disease. BPA
44 was quantified in all ten patients while MCBPA, DCBPA and TTCBPA were determined in
45 three and TCBPA in four. In conclusion, we have successfully developed a highly sensitive
46 method to determine BPA and ClxBPAs in human plasma. Thanks to this method, for the first
47 time, we could demonstrate ClxBPAs occurrence in human blood.

48

49 Keywords: bisphenol A; chlorinated bisphenol A; endocrine disrupting chemicals; human
50 plasma biomonitoring; UHPLC-MS/MS;

51

52 1. Introduction

53 In 2017, the member state committee of the European CHemicals Agency (ECHA)
54 unanimously agreed that bisphenol A (BPA), exhibits endocrine disruptor properties (ECHA,
55 2017). Epidemiological studies have demonstrated that BPA exposure was related to
56 diabetes (Kim and Park, 2013), obesity (Carwile and Michels, 2011), cardiovascular diseases
57 (Gao and Wang, 2014) and disorders on reproductive systems (Lee et al., 2015). These
58 adverse health effects combined with the fact that BPA is a ubiquitous compound render this
59 problem a priority to human health (Bergman et al., 2013). BPA is produced in one of the
60 highest volumes of chemicals worldwide. BPA is widely used in the production of
61 polycarbonate, polysulfone and epoxy resins (ANSES, 2013; FAO/WHO Expert, 2010) that
62 are applied in many common consumer products such as flasks, paints, varnishes,
63 manufacturing sheets and thermal paper (Dodson et al., 2012; Vandenberg et al., 2007). It
64 can also be found in aquatic environments such as surface waters (Fromme et al., 2002),
65 and tap water (Dupuis et al., 2012). During drinking water treatment, BPA is in contact with
66 chlorine used as a disinfectant to produce tap water. This may result in the addition of
67 chlorine atoms to the phenolic rings leading to production of BPA chlorinated derivatives
68 (ClxBPAs) (Fan et al., 2013).

69 Most human biomonitoring research has focused on BPA only, but it is known that humans
70 may also be exposed to ClxBPAs *via* drinking water (Dupuis et al., 2012) as mentioned
71 above, and *via* flame retarded products (Kitamura et al., 2002). Furthermore, ClxBPAs
72 exhibit oestrogenic activity 10 to 105 times higher than BPA itself (Hu et al., 2002; Mutou et
73 al., 2006). *In vitro* studies have revealed the toxicity of ClxBPAs such as genotoxicity,
74 regulation of adipogenesis (Riu et al., 2011), cancer (Rivas et al., 2002) and endocrine
75 disruption (Viñas et al., 2013). Few epidemiological studies have demonstrated that ClxBPAs
76 exposure is related to metabolic disorders such as obesity and type II diabetes (Andra et al.,
77 2015; Andra and Makris, 2015).

78 Assessing human exposure to environmental pollutants is a key-point for health risk
79 assessment. It is essential to determine human exposure to BPA and ClxBPAs using human
80 biomonitoring. Determination of environmental pollutants in human body fluids, most
81 commonly in urine or blood, is considered as the gold standard method (Zidek et al., 2017).
82 Regarding polar or easily metabolizable endocrine disrupting chemicals (EDCs), studies
83 have focused on urine matrix with the advantage of non-invasive sampling method and to the
84 large volume available. However, to our knowledge ClxBPAs toxicokinetics has not yet been
85 characterized. Furthermore, urinary data may require creatinine or specific gravity
86 adjustment in order to take into account urine dilution. Moreover, urine collection may be
87 compromised in newborns or anuric patients. In contrast, blood is the major body fluid
88 distributing exogenous compounds throughout the body. Therefore, micropollutants
89 determination in blood is highly relevant for human biomonitoring and for health risk
90 assessment. Hence, in this study we have focused on human plasma, a matrix available in
91 all populations and commonly used in large-scale human biomonitoring even if lower
92 micropollutants concentrations may be found in this matrix (Calafat et al., 2013).
93 Regarding BPA, most authors have included a deconjugation step in their analytical method
94 leading to determination of "total" BPA without distinguishing conjugated from unconjugated
95 form (La Rocca et al., 2014; Rönn et al., 2014; Savastano et al., 2015). On the other hand,
96 our work has focused on parent compounds, BPA and ClxBPAs, since ClxBPAs metabolism
97 is still unknown. Moreover, BPA glucuronide does not competitively displace [3H]-17beta-
98 estradiol from any of the estrogen receptors (Cho et al., 2017; FAO/WHO Expert, 2010;
99 Matthews et al., 2001), and unconjugated BPA is consequently assumed to be the form
100 leading to toxic effects.
101 To our knowledge, only one study has described simultaneous quantification of BPA and
102 ClxBPAs in human serum, but the validation of the reported analytical method does not
103 comply with international guidelines (Liao and Kannan, 2012).

104 Therefore, the aim of this work was to develop, validate and apply Ultra High Performance
105 Liquid Chromatography coupled to tandem Mass Spectrometry (UHPLC-MS/MS) method for
106 simultaneous determination of BPA and ClxBPAs in human plasma.

107 **2. Materials and methods**

108 2.1. Chemicals and standards

109 Bisphenol A and deuterated BPA (BPA-d₁₆) were purchased from Sigma-Aldrich® (St Louis,
110 USA). Monochlorobisphenol A (MCBPA), 2,2'-dichlorobisphenol A and 2,6'-dichlorobisphenol
111 A (DCBPA), trichlorobisphenol A (TCBPA), tetrachlorobisphenol A (TTCBPA) and deuterated
112 DCBPA standard (DCBPA-d₁₂) were obtained from @rtMolecule® (Poitiers, France).
113 Methanol (MeOH) and water were purchased from Carlo Erba (Val de Reuil, France). All
114 solvents and reagents were of UHPLC grade and free of BPA and ClxBPAs. Polypropylene
115 or glass materials were used to avoid BPA contamination.

116 2.2. Preparation of stock solutions, working standard solutions and
117 calibration standards

118 Stock solutions were prepared at 10 µg/mL for BPA and for BPA-d₁₆, and at 2 µg/mL for
119 ClxBPAs and for DCBPA-d₁₂ in MeOH. These solutions were stored at -20°C. For each
120 experiment, two working solutions were prepared extemporaneously in MeOH: the analytical
121 standard solution (AS) at 32 ng/mL and 6.4 ng/mL, for BPA and ClxBPAs respectively, the
122 internal standard solution (IS) at 16 ng/mL and 3.2 ng/mL for BPA-d₁₆ and for DCBPA-d₁₂,
123 respectively. The AS solution was then diluted in MeOH to obtain calibration standards from
124 32 ng/mL to 1 ng/mL for BPA, from 6.4 to 0.05 ng/mL for MCBPA, DCBPA and TCBPA, and
125 from 6.4 to 0.2 ng/mL for TTCBPA. These solutions were used to prepare calibration
126 standards in plasma. Human plasma, collected from anonymous donors was used for the
127 preparation of calibration standards in plasma and quality controls (QCs). Three standard
128 solutions (SS) were carried out for each analyte in MeOH (16, 2 and 1 ng/mL for BPA; 3.2,
129 0.4, 0.05 for MCBPA, DCBPA, TCBPA; 3.2, 0.4 and 0.2 for TTCBPA) to prepare three levels
130 of quality controls.

131 2.3. Samples

132 Human plasma, collected using lithium heparin tubes free of BPA, from patients suffering
133 from end stage renal disease and hospitalized at the University Hospital of Poitiers were
134 used to apply our analytical method. Informed and written consent was obtained from all
135 subjects enrolled in the study. All samples were stored at -20 °C.

136 2.4. Extraction and optimization

137 Each plasma sample (500 µL) was spiked with 50 µL of IS. Then, 50 µL of calibration
138 standard were added to obtain final concentrations ranging from 3.2 ng/mL to 0.1 ng/mL for
139 BPA, from 0.64 to 0.02 ng/mL for TTCBPA, and from 0.64 to 0.005 ng/mL, for MCBPA,
140 DCBPA, and TCBPA. For blank and patient samples, AS was replaced by 50 µL of MeOH.
141 Then, 2.5 mL of water were added. Samples were shaken for one minute before performing
142 solid phase extraction (SPE). The SPE procedure for clean-up samples was performed using
143 glass Oasis HLB® Cartridges (200 mg/5 mL Waters®, Milford, USA). Cartridges were
144 conditioned with MeOH and then equilibrated with water. Samples were loaded on the wet
145 cartridges, and then washed (MeOH/water, 30/70, v/v, 4 mL) and dried. Elution was
146 performed with MeOH (4 mL). Extracts were evaporated to dryness at 40°C under a gentle
147 nitrogen stream. Residues were dissolved ((MeOH/water 30/70, v/v, 500 µL) and vortexed for
148 10 s. Finally, 20 µL of extract were injected into the UHPLC–MS/MS apparatus.

149 2.5. Liquid chromatography

150 The UHPLC Shimadzu® Nexera X2 system (Kyoto, Japan) was equipped with an Acquity
151 CSH C18 (1.7 µm particle size, 2.1 x 100 mm, Waters®, Milford, USA) column maintained at
152 40°C, and a binary mobile phase was delivered in the gradient mode, at a flow rate of 350
153 µL.min⁻¹. Water (A) and MeOH (B) were used as mobile phase solvents. Gradient started at
154 30% of B and linearly increased to 90% of B (0.5 to 7 min) and then to 99% of B (7 to 7.5
155 min); maintained at 99% of B (7.5 to 10 min); decreased linearly to 30% of B (10 to 10.5 min)
156 and then maintained at 30% of B (10.5 to 13 min) to re-equilibrate the column.

157 2.6. Mass spectrometry

158 Quantification was performed using an API 6500+ mass spectrometer (ABSciex®, Concord,
159 Canada) equipped with an electrospray ionization (ESI) interface, operating in negative
160 ionization mode. Ion source and MS/MS conditions were optimized by direct infusion of 1
161 µg/mL solutions of the individual analytes into the mass spectrometer at 10 µL/min. Ions
162 were analyzed in multiple reaction monitoring mode (MRM) using two specific transitions of
163 each precursor ion. The first transition was used for quantification while the second was used
164 for confirmation. Dwell and cycle times were optimized using the Scheduled MRM®
165 algorithm. Data were acquired using Analyst® software, and processed using MultiQuant®
166 software.

167 2.7. Method validation

168 Analytical method was validated according to EMA guidelines (European Medicines Agency
169 Science Medicines Health, 2011). Validation parameters included selectivity, precision,
170 trueness, linearity, Matrix Effect (ME), and REcovery (RE).

171 Five calibration curves containing analytes and internal standards and three levels of quality
172 controls (QC) were prepared as described above. Linear regression was performed with 1/x
173 weighting factor.

174 To determine selectivity, for each analyte and deuterated standard we analyzed
175 chromatograms at their corresponding retention time in background plasma samples. High
176 selectivity was defined by the absence of any detectable peak.

177 Calibration curves ranged from 0.1 to 3.2 (0.1, 0.2, 0.4, 0.8, 1.6, 3.2) ng/mL for BPA, from
178 0.02 to 0.64 (0.02, 0.04, 0.08, 0.16, 0.32, 0.64) ng/mL for TTCBPA and from 0.005 to 0.64
179 (0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64) ng/mL for MCBPA, DCBPA and TCBPA. In
180 addition, quantification of QC (0.1, 0.2, 1.6 ng/mL for BPA, 0.02, 0.04, 0.32 ng/mL for
181 TTCBPA and 0.005, 0.04, 0.32 ng/mL for MCBPA, DCBPA and TCBPA), was systematically
182 performed during sample analyses. Precision (expressed as relative standard deviation) and

183 trueness (expressed as percentage of target concentration) were assessed using five
184 replicates of QC assayed the same day (intraday) and over five different days (inter-day).

185 Relative standard deviation (RSD) was supposed to be lower than 15 % except at the limit of
186 quantification (LOQ) of which the value was supposed to be lower than 20 %. Trueness was
187 supposed to range from 85 to 115 % except at the LOQ, of which the value was supposed to
188 range from 80 to 120 % (European Medicines Agency Science Medicines Health, 2011).

189 The limit of detection (LOD) was defined for each batch of analyses as three times the
190 standard deviation of the mean peak area (n=5) obtained using blank plasma samples
191 ("Network of reference laboratories and related organisations for monitoring and bio-
192 monitoring of emerging environmental pollutants (NORMAN) - Protocol for the validation of
193 chemical and biological monitoring methods," 2009). A compound was considered to be
194 detectable in a sample if the peak area was greater than LOD.

195 In accordance with guidelines, the limit of quantification (LOQ), was set at the lowest level of
196 calibration standard (0.1 ng/mL for BPA, 0.02 ng/mL for TTCBPA, and 0.005 ng/mL for
197 MCBPA, DCBPA and TCBPA), and fully validated using QC prepared with plasma spiked at
198 this concentration.

199 Linearity of the calibration curve was assessed using correlation coefficients (r^2) and
200 contamination was estimated using intercept value. Linearity was demonstrated when the
201 correlation coefficient was greater than 0.99.

202 To determine ME and RE of the extraction step (Matuszewski et al., 2003), several levels of
203 QCs were prepared according to the following procedures:

- 204 - 100 μ L of IS and 100 μ L of SS were spiked to 800 μ L of mobile phase to obtain
205 Matrix effect solution (MES) solution. Then, 20 μ L were injected into the UHPLC-
206 MS/MS system to obtain MS/MS response A, expressed using the AS peak area and
207 the ratio AS peak area / IS peak area (area ratio A).
- 208 - 500 μ L of MES were used to dissolve blank plasma extract obtained following the
209 extraction procedure described above. Then, 20 μ L were injected into the UHPLC-

210 MS/MS system to obtain MS/MS response B, expressed using the AS peak area and
211 the ratio AS peak area / IS peak area (area ratio B).

212 - 50 μ L of IS and 50 μ L of SS were spiked to 400 μ L of plasma and then extracted
213 following the procedure described above. Then, 20 μ L were injected into the UHPLC-
214 MS/MS system to obtain MS/MS response C, expressed using the ratio AS peak area
215 / IS peak area (area ratio C).

216 The percentages of ME and RE were assessed as follow:

$$217 \text{ME (\%)} = (\text{Area B} / \text{Area A}) \times 100$$

$$218 \text{IS-corrected ME (\%)} = (\text{Area ratio B} / \text{Area ratio A}) \times 100$$

$$219 \text{RE (\%)} = (\text{Area ratio C} / \text{Area ratio B}) \times 100$$

220 2.8. Application to patients suffering from end stage renal disease

221 We have randomly chosen ten plasma samples from patients suffering from end stage renal
222 disease and hospitalized at the University Hospital of Poitiers. Indeed, for these anuric
223 patients, urine is not available therefore it is mandatory to determine EDCs in another matrix
224 such as blood. The study was approved by the French Ethics Committee (2016-A00475-48),
225 and patients gave their informed consent after being fully informed. After centrifugation
226 plasma samples were stored at -80°C up to 6 months before analysis. Plasma was collected
227 using lithium heparin tubes free of BPA.

228 **3. Results**

229 3.1. Ultra High Performance Liquid Chromatography and MS/MS conditions

230 The optimal conditions for the electrospray ionization were as follows: ion spray voltage set
 231 at -4500V, temperature set at 650°C, curtain gas set at 30.0 psi, collision gas set as high, ion
 232 source gas 1 set at 40.0 psi and ion source gas 2 set at 60.0 psi. Ion source and specific
 233 MS/MS conditions were optimized and results are listed in Table 1.

234 Table 1. Descriptive table of retention times, precursor ions, fragment ions and MS/MS
 235 optimization conditions (declustering potential, DP; entrance potential, EP; collision energy,
 236 CE; collision cell exit potential, CXP) for all analytes. For CE and CXP, first and second
 237 values are for quantification and confirmation ions, respectively.

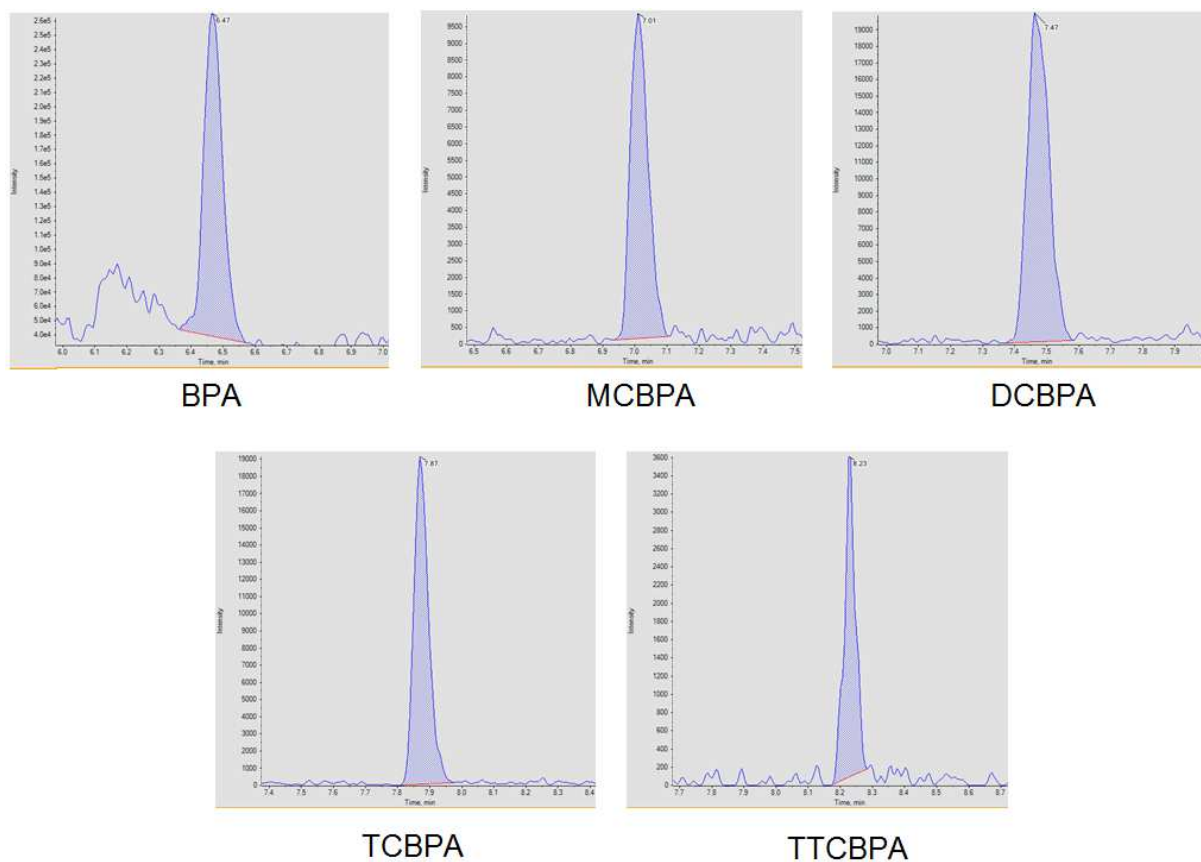
Analyte	Retention time (min)	Precursor ion (m/z)	Quantification ion (m/z)	Confirmation ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
BPA	6.5	226.967	211.800	132.800	-65	-10	-24 / -34	-19 / -11
BPA-d ₁₆	6.4	241.078	222.900	141.900	-90	-10	-28 / -34	-25 / -15
MCBPA	7.0	260.896	181.700	209.700	-120	-10	-38 / -30	-19 / -21
DCBPA	7.5	294.869	243.700	215.800	-100	-10	-32 / -42	-25 / -23
TCBPA	7.9	328.713	249.700	277.700	-85	-10	-42 / -34	-15 / -29
TTCBPA	8.2	364.794	313.700	285.600	-110	-10	-36 / -44	-33 / -29
DCBPA-d ₁₂	7.4	306.988	224.900	252.800	-120	-10	-42 / -34	-23 / -25

238

239 Chromatographic conditions were optimized and for each target analyte we obtained high
 240 peak resolution (Figure 1). Retention times for these five EDCs are reported in Table 1.

241

242 Figure 1. Examples of chromatograms obtained for medium quality control (0.2 ng/ml for
243 bisphenol A and 0.04 ng/ml for chlorinated derivatives).



244

245

3.2. Method validation

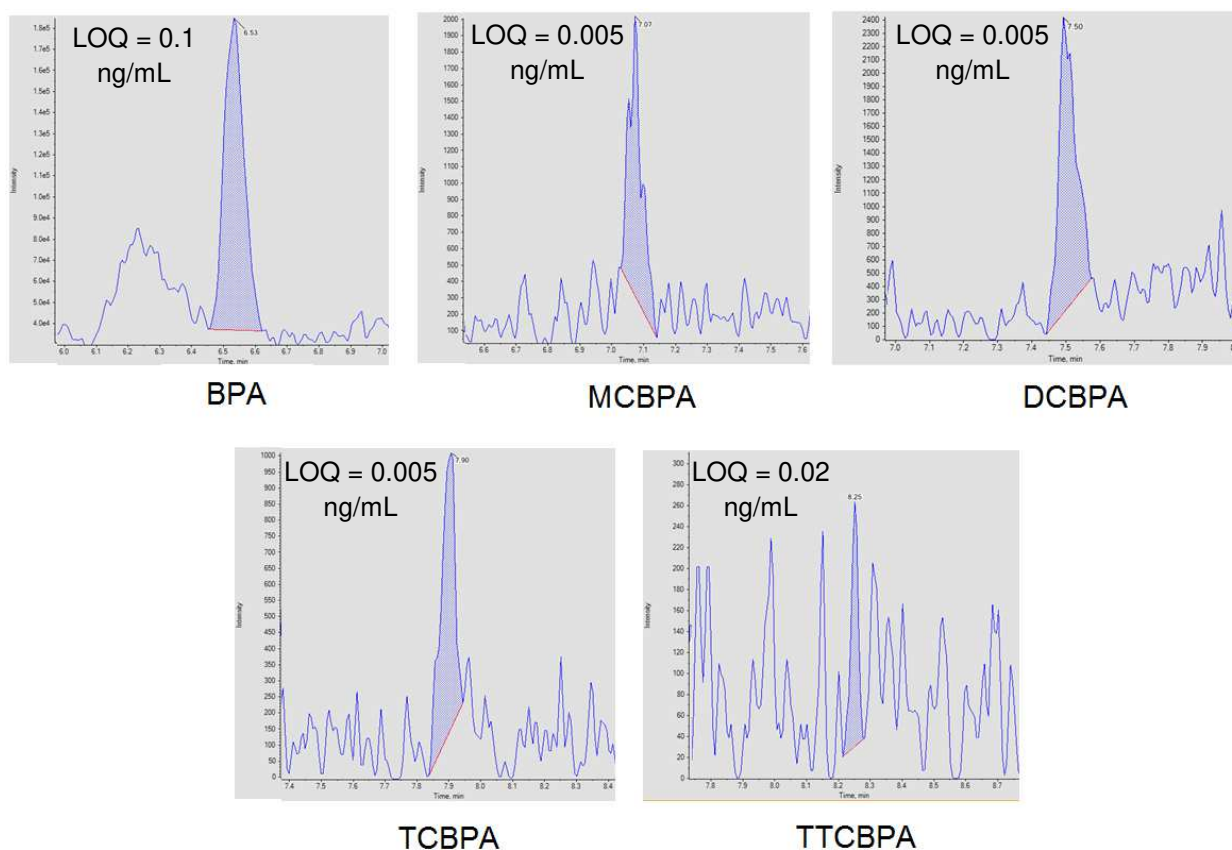
246

3.2.1. Selectivity

247 For each analyte, in blank plasma samples (AS free), no significant peak of ClxBPAs was
248 detected at the corresponding retention time (Figure 2), in accordance with high selectivity of
249 the method. Due to BPA contamination of human plasma, a non-significant peak of BPA was
250 observed in blank samples (Figure 2).

251

252 Figure 2. Examples of chromatograms obtained for blank plasma.



253

254

3.2.2. Precision and trueness

255 The accuracy of the method was fully demonstrated. Indeed, RSD did not exceed 17% at
256 LOQ, and 12% at the other levels for intra and inter-day analysis. Trueness ranged from 83
257 to 115 at LOQ, and from 85 to 107 % at the other levels for intra and inter-day analysis
258 (Table 2).

259 Table 2. Intra and inter-day precision and trueness for each analyte at each level of QC
 260 (n=5), Matrix Effect (ME) and Recovery (RE) data for high level quality control (n=3) in
 261 human plasma for all analytes.

Compound	Concentration of QC (ng/mL)	Precision (%)		Trueness (%)		Matrix effect (%)	IS-corrected Matrix effect (%)	Recovery (%)
		Intra-day	Inter-day	Intra-day	Inter-day			
BPA	0.1 (LOQ)	11	11	94	101	56	86	
	0.2	9	5	107	99			
	1.6	3	4	100	97	54	84	92
MCBPA	0.005 (LOQ)	12	16	92	104	69	92	
	0.04	12	7	104	99			
	0.32	9	12	102	94	56	94	90
DCBPA	0.005 (LOQ)	12	14	92	100	81	110	
	0.04	9	11	100	100			
	0.32	3	7	89	105	61	105	48
TCBPA	0.005 (LOQ)	12	17	92	83	99	133	
	0.04	12	9	101	91			
	0.32	11	6	91	100	79	134	75
TTCBPA	0.02 (LOQ)	11	8	115	108	77	103	
	0.04	10	11	99	98			
	0.32	10	12	85	95	67	115	31

262

263

264 3.2.3. Linearity and intercept

265 BPA and ClxBPAs calibration curves showed appropriate linearity ($r^2 > 0,99$) (Table 3).
266 Furthermore, due to contamination of human plasma, an intercept value of 0.320 ± 0.113
267 was obtained for BPA calibration curves (Table 3). Indeed, BPA was detected in plasma from
268 anonymous donors used for standards and quality controls (Figure 2), however it was always
269 at a level markedly lower than the limit of quantification (0.1 ng/mL) and has been taken into
270 account in the calculation method. To summarize, the BPA/IS peak area ratio of blank
271 plasma was daily subtracted from the BPA/IS ratio of each standard used to construct the
272 calibration curve.

273

274 Table 3. Mean (+/- SD) correlation coefficient (r^2), slope, and y-intercept of calibration curves
 275 prepared with plasma (n=5).

	BPA	MCBPA	DCBPA	TCBPA	TTCBPA
Correlation coefficients	0.996 ±	0.997 ±	0.998 ±	0.997 ±	0.990 ±
r^2 (mean +/- SD)	0.003	0.003	0.001	0.002	0.007
Slope (mean +/- SD)	1.283 ±	1.922 ±	4.975 ±	3.091 ±	0.302 ±
	0.235	0.487	0.353	0.160	0.066
Intercept (mean area	0.320 ±	0.007 ±	0.003 ±	0.005 ±	0.002 ±
ratio +/- SD)	0.113	0.004	0.002	0.001	0.004

276

277 3.2.4. Recovery and matrix effect

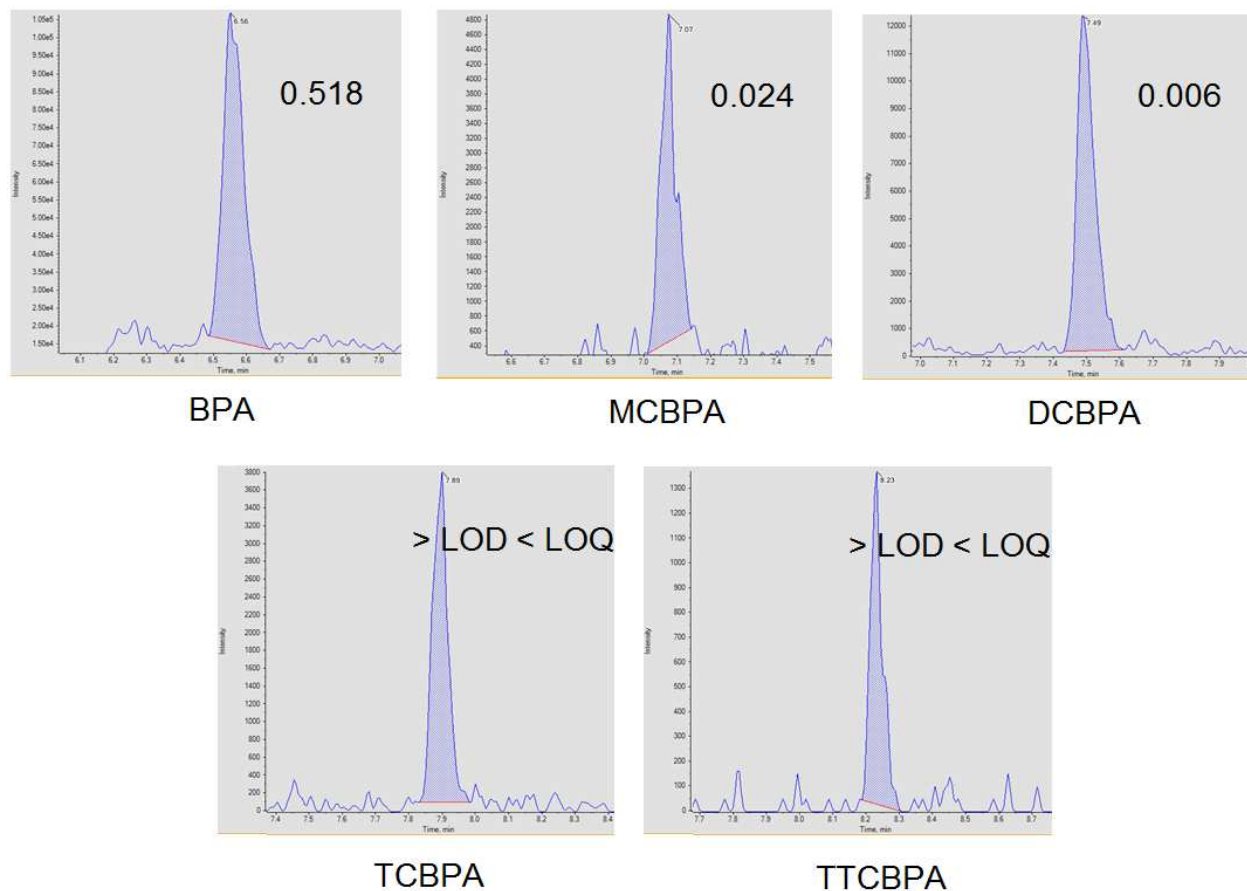
278 Results concerning ME and RE are reported in Table 2. ME were low to moderate from 56 to
 279 99% and from 54 to 79 % for low and high levels of QC, respectively. However, they were
 280 satisfactorily corrected with BPA-d₁₆ and DCBPA-d₁₂. RE were good to moderate from 92 to
 281 31% (Table 2).

282 3.3. Application to human plasma samples

283 The method was applied to human blood samples from patients suffering from end stage
 284 renal disease. Figure 3 shows a chromatogram obtained from a human plasma sample (n°
 285 10) in which BPA and ClxBPAs were found.

286

287 Figure 3 Examples of chromatograms and concentrations obtained for BPA and ClxBPAs in
288 human plasma sample (ng/mL) (detected but not quantified, > LOD < LOQ).



289

290 BPA was quantified in all ten patients with concentrations ranging from 0.266 to 86.831
291 ng/mL while MCBPA, DCBPA and TCBPA were quantified in two, one and one patient,
292 respectively. TTCBPA was detected in three out of the ten patients (Table 4). Some patients
293 exhibit multi exposure to BPA and ClxBPAs while others are only exposed to BPA (Table 4).

294

295 Table 4. Quantification of unconjugated bisphenol A and its chlorinated derivatives in human
 296 patient plasma (ng/mL) (not detected, ND; detected but not quantified, > LOD < LOQ).

Sample	BPA	MCBPA	DCBPA	TCBPA	TTCBPA
1	0.329	ND	ND	ND	> LOD < LOQ
2	0.428	> LOD < LOQ	> LOD < LOQ	> LOD < LOQ	ND
3	0.458	0.040	> LOD < LOQ	0.006	> LOD < LOQ
4	0.266	ND	ND	> LOD < LOQ	ND
5	5.625	ND	ND	ND	ND
6	41.007	ND	ND	ND	ND
7	0.379	ND	ND	ND	ND
8	9.622	ND	ND	ND	ND
9	86.831	ND	ND	ND	ND
10	0.518	0.024	0.006	> LOD < LOQ	> LOD < LOQ

297 4. Discussion

298 We have developed and validated (according to EMA guidelines (European Medicines
299 Agency Science Medicines Health, 2011)) an original UHPLC–MS/MS method for
300 simultaneous determination of BPA and ClxBPAs in human plasma. According to the results
301 the method was ultrasensitive and reliable. Indeed, although moderate matrix effects have
302 been observed, using deuterated internal standards, high-accuracy of the method has been
303 demonstrated. Several reliable methods for simultaneous determination of BPA and ClxBPAs
304 have been already reported by our team but due to the specific properties of the plasma
305 matrix we had to develop entirely original extraction and separation procedures (Cariot et al.,
306 2012; Grignon et al., 2016; Venisse et al., 2019).

307 So far, the toxicokinetics of ClxBPAs remains unknown, even though adverse health effects
308 have been suggested. Moreover, the few published studies have essentially provided data in
309 urine samples (Andra and Makris, 2015; Kalyvas et al., 2014; Vela-Soria et al., 2014;
310 Venisse et al., 2014). This may be due to a lack of analytical method, particularly regarding
311 human plasma matrix. On the other hand, our method offers reliable determination of
312 ClxBPAs in human plasma, which is mandatory in toxicokinetic investigative studies. Like
313 most of EDCs, BPA and ClxBPAs are found at a trace level and exhibit their oestrogenic
314 activity at a very low concentration, acting according to non-monotonic dose-response
315 relationships (Bergman et al., 2013; Lagarde et al., 2015). To comply with this requirement,
316 our method provides very low LOQs (0.1 ng/mL for BPA, 0.02 ng/mL for TTCBPA and 0.005
317 ng/mL for MCBPA, DCBPA, and TCBPA).

318 Cocktail effects are key points of EDCs toxicology since synergistic or antagonistic
319 mechanisms have been described for these micropollutants (Gaudriault et al., 2017). Hence,
320 using our method, simultaneous determination of five EDCs in human plasma may be
321 performed. This “cocktail-exposure” assessment better reflects real-life situation and
322 provides more relevant data, thereby heightening the statistical power of epidemiologic
323 studies. Future HBM studies for EDCs should take into account their cocktail effects using

324 analytical methods simultaneously assessing several EDCs as does the method developed
325 in this work. Finally, simultaneous determination of EDCs is mandatory in order to assess the
326 relationship between EDCs exposure and health outcomes.

327 To our knowledge, only Liao *et al.* have reported simultaneous determination of BPA and
328 ClxBPAs in human blood samples (Liao and Kannan, 2012). However, TTCBPA
329 determinations were not included and the method requires two solid phase extractions,
330 thereby increasing duration and analysis cost. In addition, the method developed by Liao *et*
331 *al.* is ten times less sensitive than our method, since their LOQs have been settled at 0.05
332 ng/mL for MCBPA DCBPA and TCBPA and therefore did not allow to detect ClxBPAs in
333 human samples.

334 Another method has been developed to determine ClxBPAs in human plasma but without
335 including BPA. This method is based on gas chromatography technology, requiring a
336 derivatization step (del Olmo *et al.*, 2005). Moreover, the method requires a large blood
337 sample volume (about 10 mL) while providing a poor sensitivity; indeed, their LOQs were 160
338 to 900 times higher than LOQs obtained using our method. Last but not least, this method
339 has never been applied to determine ClxBPAs in human blood samples.

340 The method developed in our study is suitable to assess ultratrace levels of BPA and
341 ClxBPAs in human plasma. Indeed, our method enabled us to determine BPA as well as, for
342 the first time, ClxBPAs concentrations, in several human blood samples.

343

344 **5. Conclusion**

345 In conclusion, we have successfully developed a highly sensitive method to determine BPA
346 and ClxBPAs in human plasma. Thanks to this method, for the first time, we could
347 demonstrate ClxBPAs occurrence in human blood. Finally, this method is suitable to perform
348 large-scale human biomonitoring of these EDCs.

349

350 **Acknowledgment**

351 We wish to thank Jeffrey Arsham an American scientific translator for his highly helpful
352 reading of our original text.

353

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