



**HAL**  
open science

## Simultaneous determination of bisphenol A and its chlorinated derivatives in human plasma: Development, validation and application of a UHPLC–MS/MS method

Guillaume Cambien, Nicolas Venisse, Virginie Migeot, Sylvie Rabouan, Mohamed Belmouaz, Guillaume Binson, Marion Albouy-Llaty, Sarah Ayraud-Thevenot, Antoine Dupuis

### ► 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

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

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**

4 Guillaume CAMBIEN<sup>a,b</sup>, Nicolas VENISSE<sup>a,b</sup>, Virginie MIGEOT<sup>a,b,c</sup>, Sylvie RABOUAN<sup>a,c</sup>,  
5 Mohamed BELMOUAZ<sup>d</sup>, Guillaume BINSON<sup>a,b,c</sup>, Marion ALBOUY-LLATY<sup>a,b,c</sup>, Sarah  
6 AYRAUD-THEVENOT<sup>a,b,c</sup>, Antoine DUPUIS<sup>\*a,b,c</sup>.

7 <sup>a</sup>INSERM, University Hospital of Poitiers, University of Poitiers, CIC1402, HEDEX research  
8 group, 86021 Poitiers CEDEX, France.

9 <sup>b</sup>Biology-Pharmacy-Public Health Department, University Hospital of Poitiers, 2 rue de la  
10 Milétrie, 86021, Poitiers CEDEX, France.

11 <sup>c</sup>Faculty of Medicine and Pharmacy, University of Poitiers, TSA 51115, 86073 Poitiers  
12 Cedex.

13 <sup>d</sup>Digestiv, Urology, Nephrology, Endocrinology Department, University Hospital of Poitiers, 2  
14 rue de la Milétrie, 86021, Poitiers CEDEX, France.

15 e-mail addresses: guillaume.cambien@univ-poitiers.fr; nicolas.venisse@chu-poitiers.fr;  
16 virginie.migeot@univ-poitiers.fr; sylvie.rabouan@univ-poitiers.fr; mohamed.belmouaz@chu-  
17 poitiers.fr; guillaume.binson@univ-poitiers.fr; marion.albouy.llaty@univ-poitiers.fr;  
18 sarah.ayraud@univ-poitiers.fr; antoine.dupuis@univ-poitiers.fr

19

20 \*Corresponding author:

21 Antoine DUPUIS

22 INSERM CIC 1402

23 HEDEX research group

24 University Hospital of Poitiers, 86021 POITIERS Cedex, France

25 Tel: 33 5.49.44.37.68

26 e-mail address: antoine.dupuis@univ-poitiers.fr

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 couples 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-d<sub>16</sub>) 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-d<sub>12</sub>) 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-d<sub>16</sub>, and at 2 µg/mL for  
119 ClxBPAs and for DCBPA-d<sub>12</sub> 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-d<sub>16</sub> and for DCBPA-d<sub>12</sub>,  
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<sup>-1</sup>. 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  $\mu$ L of IS and 100  $\mu$ L of SS were spiked to 800  $\mu$ L of mobile phase to obtain  
205 Matrix effect solution (MES) solution. Then, 20  $\mu$ 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  $\mu$ L of MES were used to dissolve blank plasma extract obtained following the  
209 extraction procedure described above. Then, 20  $\mu$ 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  $\mu$ L of IS and 50  $\mu$ L of SS were spiked to 400  $\mu$ L of plasma and then extracted  
213 following the procedure described above. Then, 20  $\mu$ 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.

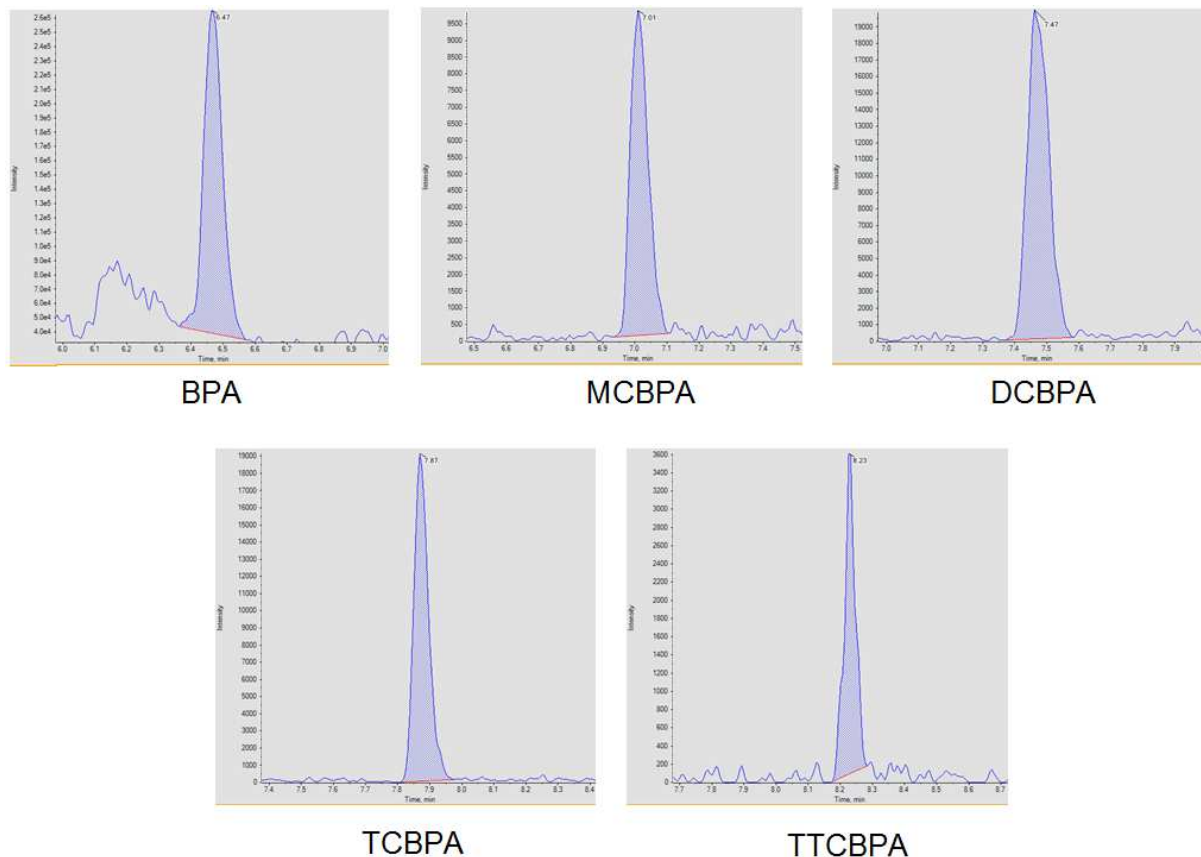
Analyte	Retention time (min)	Precursor ion (m/z)	Quantification ion (m/z)	Confirmation ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
BPA	6.5	226.967	211.800	132.800	-65	-10	-24 / -34	-19 / -11
BPA-d <sub>16</sub>	6.4	241.078	222.900	141.900	-90	-10	-28 / -34	-25 / -15
MCBPA	7.0	260.896	181.700	209.700	-120	-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 <sub>12</sub>	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

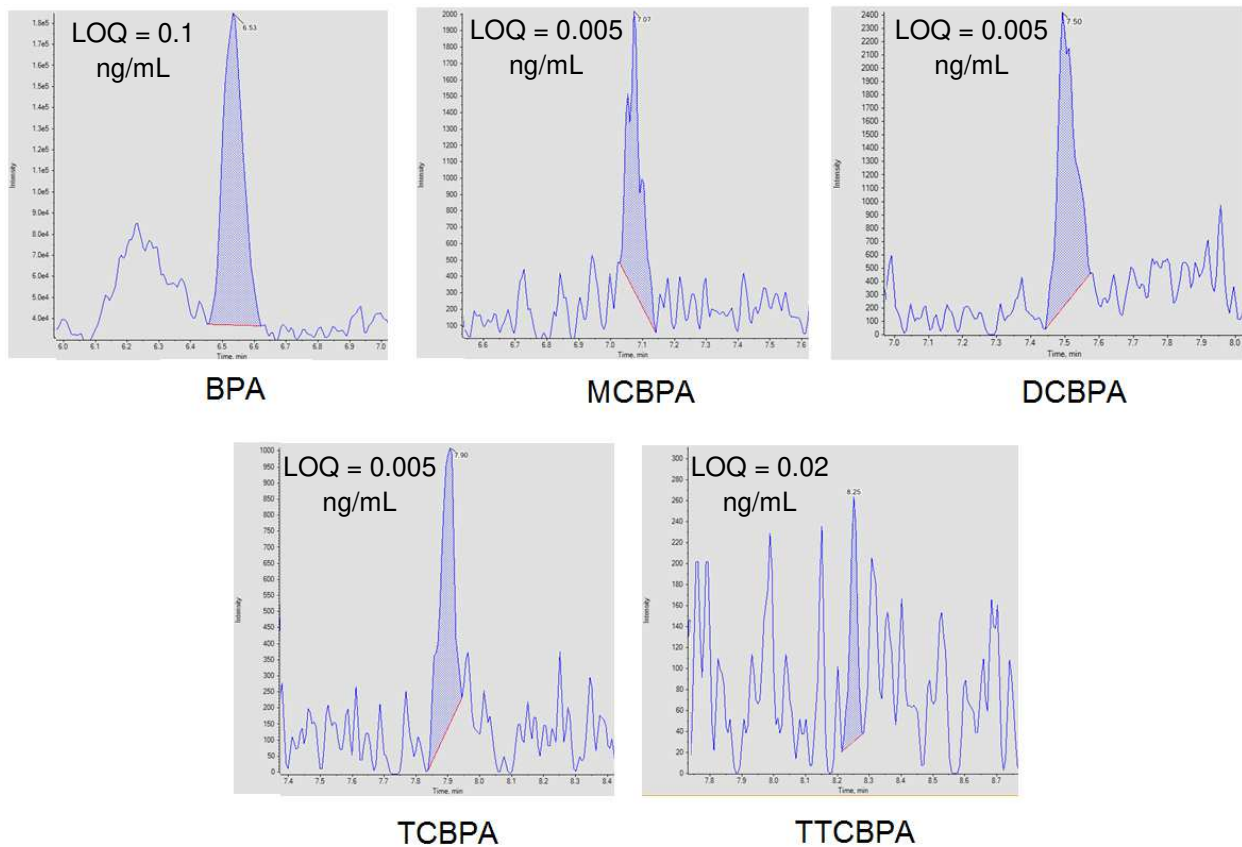
246

#### 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  $0.320 \pm 0.113$   
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

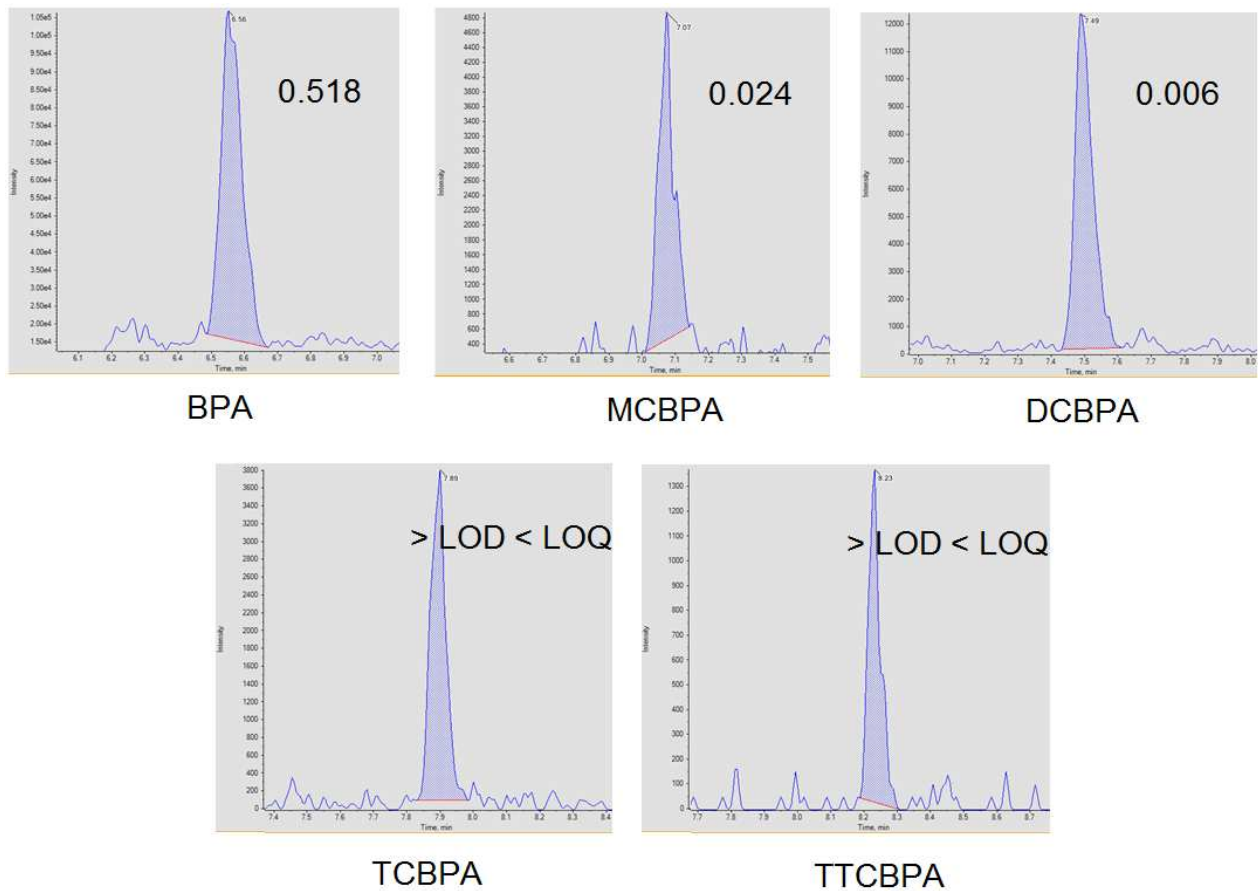
278 Results concerning ME and RE are reported in Table 2. ME were low to moderate from 56 to  
 279 99% and from 54 to 79 % for low and high levels of QC, respectively. However, they were  
 280 satisfactorily corrected with BPA-d<sub>16</sub> and DCBPA-d<sub>12</sub>. RE were good to moderate from 92 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

354 **References**

- 355 Andra, S.S., Kalyvas, H., Andrianou, X.D., Charisiadis, P., Christophi, C.A., Makris, K.C.,  
356 2015. Preliminary evidence of the association between monochlorinated bisphenol A  
357 exposure and type II diabetes mellitus: A pilot study. *Journal of Environmental*  
358 *Science and Health, Part A* 50, 243–259.  
359 <https://doi.org/10.1080/10934529.2015.981111>
- 360 Andra, S.S., Makris, K.C., 2015. Association between urinary levels of bisphenol A and its  
361 monochlorinated derivative and obesity. *Journal of Environmental Science and Health,*  
362 *Part A* 50, 1169–1179. <https://doi.org/10.1080/10934529.2015.1047674>
- 363 ANSES, 2013. Évaluation des risques du bisphénol A (BPA) pour la santé humaine.
- 364 Bergman, Å., United Nations Environment Programme, World Health Organization, 2013.  
365 State of the science of endocrine disrupting chemicals - 2012 an assessment of the  
366 state of the science of endocrine disruptors. WHO : UNEP, Geneva.
- 367 Calafat, A.M., Koch, H.M., Swan, S.H., Hauser, R., Goldman, L.R., Lanphear, B.P.,  
368 Longnecker, M.P., Rudel, R.A., Teitelbaum, S.L., Whyatt, R.M., Wolff, M.S., 2013.  
369 Misuse of blood serum to assess exposure to bisphenol A and phthalates. *Breast*  
370 *Cancer Res* 15, 403. <https://doi.org/10.1186/bcr3494>
- 371 Cariot, A., Dupuis, A., Albouy-Llaty, M., Legube, B., Rabouan, S., Migeot, V., 2012.  
372 Reliable quantification of bisphenol A and its chlorinated derivatives in human breast  
373 milk using UPLC–MS/MS method. *Talanta* 100, 175–182.  
374 <https://doi.org/10.1016/j.talanta.2012.08.034>
- 375 Carwile, J.L., Michels, K.B., 2011. Urinary bisphenol A and obesity: NHANES 2003–2006.  
376 *Environ. Res.* 111, 825–830. <https://doi.org/10.1016/j.envres.2011.05.014>
- 377 Cho, S.H., Choi, Y., Kim, S.H., Kim, S.J., Chang, J., 2017. Urinary bisphenol A versus serum  
378 bisphenol A concentration and ovarian reproductive outcomes among IVF patients:  
379 Which is a better biomarker of BPA exposure? *Mol. Cell. Toxicol.* 13, 351–359.  
380 <https://doi.org/10.1007/s13273-017-0039-0>
- 381 del Olmo, M., Zafra, A., Suárez, B., Gonzalez-Casado, A., Taoufik, J., Vélchez, J.L., 2005.  
382 Use of solid-phase microextraction followed by on-column silylation for determining

383 chlorinated bisphenol A in human plasma by gas chromatography-mass spectrometry.  
384 *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 817, 167–172.  
385 <https://doi.org/10.1016/j.jchromb.2004.11.059>

386 Dodson, R.E., Nishioka, M., Standley, L.J., Perovich, L.J., Brody, J.G., Rudel, R.A., 2012.  
387 Endocrine Disruptors and Asthma-Associated Chemicals in Consumer Products.  
388 *Environ Health Perspect* 120, 935–943. <https://doi.org/10.1289/ehp.1104052>

389 Dupuis, A., Migeot, V., Cariot, A., Albouy-Llaty, M., Legube, B., Rabouan, S., 2012.  
390 Quantification of bisphenol A, 353-nonylphenol and their chlorinated derivatives in  
391 drinking water treatment plants. *Environ Sci Pollut Res Int* 19, 4193–4205.  
392 <https://doi.org/10.1007/s11356-012-0972-3>

393 ECHA, 2017. The Member State Committee (MSC) unanimously agrees that Bisphenol A is  
394 an endocrine disruptor.

395 European Medicines Agency Science Medicines Health, 2011. Guideline on bioanalytical  
396 method validation.

397 Fan, Z., Hu, J., An, W., Yang, M., 2013. Detection and Occurrence of Chlorinated Byproducts  
398 of Bisphenol A, Nonylphenol, and Estrogens in Drinking Water of China: Comparison  
399 to the Parent Compounds. *Environ. Sci. Technol.* 47, 10841–10850.  
400 <https://doi.org/10.1021/es401504a>

401 FAO/WHO Expert, 2010. Toxicological and Health Aspects of Bisphenol A.

402 Fromme, H., Küchler, T., Otto, T., Pilz, K., Müller, J., Wenzel, A., 2002. Occurrence of  
403 phthalates and bisphenol A and F in the environment. *Water Research* 36, 1429–1438.  
404 [https://doi.org/10.1016/S0043-1354\(01\)00367-0](https://doi.org/10.1016/S0043-1354(01)00367-0)

405 Gao, X., Wang, H.-S., 2014. Impact of Bisphenol A on the Cardiovascular System —  
406 Epidemiological and Experimental Evidence and Molecular Mechanisms. *IJERPH* 11,  
407 8399–8413. <https://doi.org/10.3390/ijerph110808399>

408 Gaudriault, P., Mazaud-Guittot, S., Lavoué, V., Coiffec, I., Lesné, L., Dejuq-Rainsford, N.,  
409 Scholze, M., Kortenkamp, A., Jégou, B., 2017. Endocrine Disruption in Human Fetal  
410 Testis Explants by Individual and Combined Exposures to Selected Pharmaceuticals,  
411 Pesticides, and Environmental Pollutants. *Environ. Health Perspect.* 125, 087004.  
412 <https://doi.org/10.1289/EHP1014>

413 Grignon, C., Venisse, N., Rouillon, S., Brunet, B., Bacle, A., Thevenot, S., Migeot, V.,  
414 Dupuis, A., 2016. Ultrasensitive determination of bisphenol A and its chlorinated  
415 derivatives in urine using a high-throughput UPLC-MS/MS method. *Anal Bioanal*  
416 *Chem* 408, 2255–2263. <https://doi.org/10.1007/s00216-015-9288-8>

417 Hu, J., Aizawa, T., Ookubo, S., 2002. Products of Aqueous Chlorination of Bisphenol A and  
418 Their Estrogenic Activity. *Environ. Sci. Technol.* 36, 1980–1987.  
419 <https://doi.org/10.1021/es011177b>

420 Kalyvas, H., Andra, S.S., Charisiadis, P., Karaolis, C., Makris, K.C., 2014. Influence of  
421 household cleaning practices on the magnitude and variability of urinary  
422 monochlorinated bisphenol A. *Sci. Total Environ.* 490, 254–261.  
423 <https://doi.org/10.1016/j.scitotenv.2014.04.072>

424 Kim, K., Park, H., 2013. Association between urinary concentrations of bisphenol A and type  
425 2 diabetes in Korean adults: A population-based cross-sectional study. *International*  
426 *Journal of Hygiene and Environmental Health* 216, 467–471.  
427 <https://doi.org/10.1016/j.ijheh.2012.07.007>

428 Kitamura, S., Jinno, N., Ohta, S., Kuroki, H., Fujimoto, N., 2002. Thyroid hormonal activity  
429 of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. *Biochem.*  
430 *Biophys. Res. Commun.* 293, 554–559. [https://doi.org/10.1016/S0006-](https://doi.org/10.1016/S0006-291X(02)00262-0)  
431 [291X\(02\)00262-0](https://doi.org/10.1016/S0006-291X(02)00262-0)

432 La Rocca, C., Tait, S., Guerranti, C., Busani, L., Ciardo, F., Bergamasco, B., Stecca, L., Perra,  
433 G., Mancini, F., Marci, R., Bordi, G., Caserta, D., Focardi, S., Moscarini, M.,  
434 Mantovani, A., 2014. Exposure to Endocrine Disrupters and Nuclear Receptor Gene  
435 Expression in Infertile and Fertile Women from Different Italian Areas. *International*  
436 *Journal of Environmental Research and Public Health* 11, 10146–10164.  
437 <https://doi.org/10.3390/ijerph111010146>

438 Lagarde, F., Beausoleil, C., Belcher, S.M., Belzunces, L.P., Emond, C., Guerbet, M.,  
439 Rousselle, C., 2015. Non-monotonic dose-response relationships and endocrine  
440 disruptors: a qualitative method of assessment. *Environ Health* 14, 13.  
441 <https://doi.org/10.1186/1476-069X-14-13>

442 Lee, M.-R., Kim, J.H., Choi, Y.-H., Bae, S., Park, C., Hong, Y.-C., 2015. Association of  
443 bisphenol A exposure with overweight in the elderly: a panel study. *Environ Sci Pollut*  
444 *Res* 22, 9370–9377. <https://doi.org/10.1007/s11356-015-4087-5>

445 Liao, C., Kannan, K., 2012. Determination of Free and Conjugated Forms of Bisphenol A in  
446 Human Urine and Serum by Liquid Chromatography–Tandem Mass Spectrometry.  
447 *Environ. Sci. Technol.* 46, 5003–5009. <https://doi.org/10.1021/es300115a>

448 Matthews, J.B., Twomey, K., Zacharewski, T.R., 2001. In Vitro and in Vivo Interactions of  
449 Bisphenol A and Its Metabolite, Bisphenol A Glucuronide, with Estrogen Receptors  $\alpha$   
450 and  $\beta$ . *Chemical Research in Toxicology* 14, 149–157.  
451 <https://doi.org/10.1021/tx0001833>

452 Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M., 2003. Strategies for the assessment  
453 of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal.*  
454 *Chem.* 75, 3019–3030. <https://doi.org/10.1021/ac020361s>

455 Mutou, Y., Ibuki, Y., Terao, Y., Kojima, S., Goto, R., 2006. Change of estrogenic activity and  
456 release of chloride ion in chlorinated bisphenol a after exposure to ultraviolet B. *Biol.*  
457 *Pharm. Bull.* 29, 2116–2119. <https://doi.org/10.1248/bpb.29.2116>

458 Network of reference laboratories and related organisations for monitoring and bio-  
459 monitoring of emerging environmental pollutants (NORMAN) - Protocol for the  
460 validation of chemical and biological monitoring methods, 2009. 99.

461 Riu, A., Grimaldi, M., le Maire, A., Bey, G., Phillips, K., Boulahtouf, A., Perdu, E., Zalko,  
462 D., Bourguet, W., Balaguer, P., 2011. Peroxisome Proliferator-Activated Receptor  $\gamma$  Is  
463 a Target for Halogenated Analogs of Bisphenol A. *Environmental Health Perspectives*  
464 119, 1227–1232. <https://doi.org/10.1289/ehp.1003328>

465 Rivas, A., Lacroix, M., Olea-Serrano, F., Laños, I., Leclercq, G., Olea, N., 2002. Estrogenic  
466 effect of a series of bisphenol analogues on gene and protein expression in MCF-7  
467 breast cancer cells. *The Journal of Steroid Biochemistry and Molecular Biology* 82,  
468 45–53. [https://doi.org/10.1016/S0960-0760\(02\)00146-2](https://doi.org/10.1016/S0960-0760(02)00146-2)

469 Rönn, M., Lind, L., Örberg, J., Kullberg, J., Söderberg, S., Larsson, A., Johansson, L.,  
470 Ahlström, H., Lind, P.M., 2014. Bisphenol A is related to circulating levels of  
471 adiponectin, leptin and ghrelin, but not to fat mass or fat distribution in humans.  
472 *Chemosphere* 112, 42–48. <https://doi.org/10.1016/j.chemosphere.2014.03.042>

473 Savastano, S., Tarantino, G., D’Esposito, V., Passaretti, F., Cabaro, S., Liotti, A., Liguoro, D.,  
474 Perruolo, G., Ariemma, F., Finelli, C., Beguinot, F., Formisano, P., Valentino, R.,  
475 2015. Bisphenol-A plasma levels are related to inflammatory markers, visceral obesity  
476 and insulin-resistance: a cross-sectional study on adult male population. *Journal of*  
477 *Translational Medicine* 13. <https://doi.org/10.1186/s12967-015-0532-y>

478 Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W.V., 2007. Human  
479 exposure to bisphenol A (BPA). *Reprod. Toxicol.* 24, 139–177.  
480 <https://doi.org/10.1016/j.reprotox.2007.07.010>



481 Vela-Soria, F., Ballesteros, O., Zafra-Gómez, A., Ballesteros, L., Navalón, A., 2014.  
482 UHPLC–MS/MS method for the determination of bisphenol A and its chlorinated  
483 derivatives, bisphenol S, parabens, and benzophenones in human urine samples. *Anal*  
484 *Bioanal Chem* 406, 3773–3785. <https://doi.org/10.1007/s00216-014-7785-9>  
485 Venisse, N., Cambien, G., Robin, J., Rouillon, S., Nadeau, C., Charles, T., Rabouan, S.,  
486 Migeot, V., Dupuis, A., 2019. Development and validation of an LC–MS/MS method  
487 for the simultaneous determination of bisphenol A and its chlorinated derivatives in  
488 adipose tissue. *Talanta* 204, 145–152. <https://doi.org/10.1016/j.talanta.2019.05.103>  
489 Venisse, N., Grignon, C., Brunet, B., Thévenot, S., Bacle, A., Migeot, V., Dupuis, A., 2014.  
490 Reliable quantification of bisphenol A and its chlorinated derivatives in human urine  
491 using UPLC-MS/MS method. *Talanta* 125, 284–292.  
492 <https://doi.org/10.1016/j.talanta.2014.02.064>  
493 Viñas, R., Goldblum, R.M., Watson, C.S., 2013. Rapid estrogenic signaling activities of the  
494 modified (chlorinated, sulfonated, and glucuronidated) endocrine disruptor bisphenol  
495 A. *Endocrine Disruptors* 1, e25411. <https://doi.org/10.4161/endo.25411>  
496 Zidek, A., Macey, K., MacKinnon, L., Patel, M., Poddalgoda, D., Zhang, Y., 2017. A review  
497 of human biomonitoring data used in regulatory risk assessment under Canada’s  
498 Chemicals Management Program. *International Journal of Hygiene and Environmental*  
499 *Health* 220, 167–178. <https://doi.org/10.1016/j.ijheh.2016.10.007>  
500

