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Recent advances in simultaneous analysis of bisphenol A and its conjugates in human matrices: exposure biomarker perspectives

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Abstract

Human exposures to bisphenol A (BPA) has attained considerable global health attention and represents one of the leading environmental contaminants with potential adverse health effects including endocrine disruption. Current practice of measuring of exposure to BPA includes the measurement of unconjugated BPA (aglycone) and total (both conjugated and unconjugated) BPA; the difference between the two measurements leads to estimation of conjugated forms. However, the measurement of BPA as the end analyte leads to inaccurate estimates from potential interferences from background sources during sample collection and analysis. BPA glucuronides (BPAG) and sulfates (BPAS) represent better candidates for biomarkers of BPA exposure, since they require in vivo metabolism and are not prone to external contamination. In this work, the primary focus was to review the current state of the art in analytical methods available to quantitate BPA conjugates. The entire analytical procedure for the simultaneous extraction and detection of aglycone BPA and conjugates is covered, from sample pre-treatment, extraction, separation, ionization, and detection. Solid phase extraction coupled with liquid chromatograph and tandem mass spectrometer analysis provides the most sensitive detection and quantification of BPA conjugates. Discussed herein are the applications of BPA conjugates analysis in human exposure assessment studies. Measuring these potential biomarkers of BPA exposure has only recently become analytically feasible and there are limitations and challenges to overcome in biomonitoring studies.

Graphical Abstract

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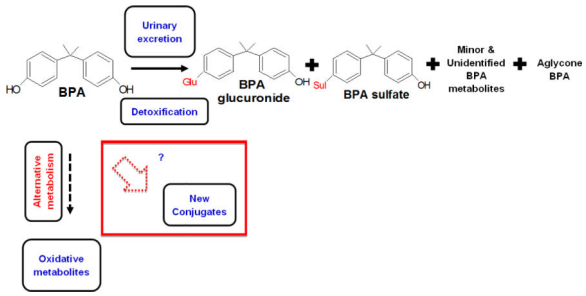
Conflicts of interest

The authors have no conflicts of interest.



Keywords

Biomarkers of exposure; Bisphenol conjugates; Chromatography; Human biomonitoring; Mass spectrometry

1. Background

Bisphenol A (BPA) is a phenolic compound in wide use with innumerable industrial, commercial, consumer, and domestic applications. It is a monomer used in polycarbonate and epoxy resins that are used in the production of food, water and beverage packaging material [1–3]. Human exposure to BPA primarily occurs from oral ingestion and dietary sources such as canned foods, water bottles and other food contact material [4–17], and through inhalation and dermal routes as well [2, 18–24]. The bioavailability of BPA is dependent on the exposure route and is therefore an important factor for assessing BPA exposure risks in humans [25, 26]. A schematic for the general exposure sources and routes in humans are presented in Figure SI–1. Upon oral ingestion in humans, BPA is mostly absorbed and undergoes fast and almost complete conversion to conjugates by uridine diphosphate and glucuronosyltransferase (UGT) isoforms, and sulfotransferase in the gastrointestinal track and liver [27]. Within 24h BPA is almost completely eliminated via urine with 84–97% of absorbed BPA excreted there within the first 5–7h following ingestion [27, 28]. The major fraction of BPA gets excreted in conjugated forms namely bisphenol A glucuronide (BPAG) and bisphenol A sulfate (BPAS). A minor fraction, usually less than 1% of total (aglycone and conjugated forms) BPA measured, circulates as the aglycone [27–29], and absolute bioavailability is less than 0.1–0.2% [30]. BPA pharmacokinetics and bioavailability vary by route of exposure in humans [31]. For example, BPA exposure via dermal absorption [19] resulted in a longer half-life and increased bioavailability [32, 33]. Associations between human exposures to BPA and several health outcomes were reviewed [34–45].

2. Conventional practice of BPA exposure assessment: measuring total and aglycone BPA

Biomonitoring of BPA in various human matrices was recently reviewed [46]. Urine has traditionally been the most preferred matrix to study because BPA is extensively conjugated via glucuronidation and excreted in urine and sampling is minimally invasive. However given the short half-life of BPA in humans (~6 hr), the observed levels in urine can only

reflect recent exposure to BPA, limiting its scope as a biomarker [47–49]. An overview of the common analytical workflow for the determination of total and aglycone BPA in human matrices is outlined in Figure SI–2. Details of the analytical steps followed for the determination of total BPA, and BPA structural analogs and chlorinated derivatives in human matrices were recently reviewed [50]. Current analytical methods determine the total concentration of BPA after enzymatic hydrolysis preferably at 37°C for a few hours and in some cases overnight. Typically only the β-glucuronidase enzyme is used for the deconjugation step because of the predominant presence of the glucuronide conjugate form of BPA, while few have additionally used sulfatase enzyme for the release of BPA from the sulfate conjugate that occurs as a very minor fraction. In addition to the glucuronide form, sulfatase is also deconjugated if the source of β-glucuronidase is from *Helix pomatia-H1* compared to *Escherichia coli*-K12 [51]. Some issues that lead to inaccurate measurement or underestimation due to suboptimal conditions of total BPA arises from insufficient enzyme concentrations, inappropriate choice of enzymes, incomplete deconjugation, unfavorable hydrolysis conditions and overall suboptimal deconjugation protocol. In cases where only aglycone BPA was measured the enzymatic deconjugation step was skipped. Representative sample preparation, analyte separation and detection approaches for the analysis of total and aglycone BPA in human matrices are presented in Table SI–1.

3. Rationale to study BPA conjugates

A major concern in using BPA concentrations in bio-matrices for assessing human exposure is the potential post-exposure specimen contamination from external sources. Such contaminations result in elevation of aglycone BPA that subsequently contributes to inflated total BPA concentrations. High blood levels of aglycone BPA in some studies [52–54] was questioned because (i) deconjugation can happen during the sample collection, storage, or analysis, and (ii) measuring aglycone BPA but not its conjugated forms could reflect external contamination rather than blood levels [55]. However, a recent review overrules the occurrence of external contamination in the current studies [31]. Unlike aglycone BPA, conjugates are not prone to external contamination [56].

Aglycone BPA was shown to passively cross the placenta in a bi-directional fashion between maternal and fetal compartments, while BPAG has limited permeability in either direction [57]. Moreover, the authors concluded that given the limited clearance of BPAG via placenta, it is likely that BPA conjugates form and accumulate in fetal compartments. If this is the case, then the presence of BPA conjugates in maternal-fetal matrices such as amniotic fluid, meconium and first urine at birth is likely attributed to fetal, not maternal, metabolism. Though conjugated forms of BPA are widely considered as biologically inactive and lacking estrogenicity, [58] it is speculated that they could interfere with certain physiological and metabolic pathways during windows of susceptibility in certain life stages such as fetal and early infancy. In support of this are the findings from recent *in vitro* studies that showed BPA conjugates altered mechanisms in prolactemia cells [59] and induced adipocyte differentiation in human and 3T3L1 murine pre-adipocytes [60]. The authors concluded that BPA conjugates might interact with membrane estrogen receptor alpha (ER-α) that mediates cellular signaling [59] and BPAG was biologically active. BPAG has no estrogen receptor transcriptional activation function but induced adipogenesis via a different biological

pathway yet to be determined [60]. In addition, other minor and secondary conjugated forms such as hydroxylated- and carboxylated-BPA [61–64] have shown some estrogenic activity [65, 66].

A growing concern is the reversion of glucuronide (BPAG) and sulfate forms (bisphenol A monosulfate – BPAS and bisphenol A disulfate – BPADS) to BPA by deconjugation with β-glucuronidase [67] and arylsulfatase C [68], which occur at high levels in human tissues such as liver, kidney, intestine and placenta. This phenomenon is particularly important for pregnant women and their fetuses because of the presence of de-conjugating enzymes in the placenta and fetal tissues during pregnancy [68–70]. However, the deconjugation of BPAG to BPA was not observed in population-based human studies [71]. This is because circulating BPA levels are very low in human tissues [67] and not applicable to whole body exposure scenario in humans [71]. Moreover, the inter-species differences in BPA metabolism observed in human versus non-human primates due to differences in exposure routes could lead to differential interpretation on fate of BPA conjugates [71]. Lower uridine 5'-diphospho glucurontosyltransferase and higher sulfotransferase activities in fetal placenta and fetal liver could result in differential conjugation efficiency and outcomes of BPA exposure [72–75]. Moreover difference in BPA exposure levels will dictate the amount of conjugation depending on saturation of BPA metabolism [69, 76, 77].

In summary, the additional monitoring of BPA conjugates is recommended to (i) avoid misinterpretation due to external contamination and/or biological sample degradation during handling in the field or laboratory [78] contributing to aglycone BPA concentrations, (ii) determine the exposure route with higher levels of conjugates arising from oral versus dermal exposure [79], and (iii) understand the inter-individual, inter-life stage and excretion compartment (e.g. breast milk vs urine) variability in BPA metabolism [67, 80]. As emphasized, it is very important to measure both aglycone and conjugated forms of BPA for assessing human exposures and to reduce external contamination issues. However, to date, the most commonly practiced approach is to measure total BPA and aglycone and infer the conjugated levels based on these measurements. More recently advances in analytical methods and commercial availability of analytical standards for BPA conjugates has enabled quantitation of specific BPA conjugates feasible.

4. Novel approach for BPA exposure assessment: simultaneous measurement of aglycone BPA and conjugates

Glucuronide and sulfate conjugates are the most common metabolites of xenobiotics and exogenous chemicals that undergo phase II metabolism in humans as a detoxification measure. Such conjugates are detected and measured preferentially with LC-MS based methods. The following section presents the analytical approaches taken by studies for the simultaneous analysis of BPA and its conjugates.

Only ten studies are available as of April 2016 that have developed and reported analytical methods for the simultaneous determination of aglycone BPA and its primary conjugates BPAG, BPAS, and BPADS. Research was limited by the lack of both commercial or custom-synthesized BPA conjugates and respective labelled internal standards until very recently.

Though some studies reported analyzing conjugated BPA in bio-specimens, the information was derived from taking the difference between total and free BPA but not a measurement of BPAG and BPAS directly (for example, see reported concentrations of conjugated BPA in adult and fetal liver tissues [81]). Studies that made direct measurement of individual BPA conjugates in bio-matrices used (i) urine [28, 82–88], (ii) plasma [28, 88, 89], (iii) serum[82, 90], and (iv) cord serum [91]. More than 50% of these studies were reported in the last couple of years (Figure SI–3A). The majority of these studies used human matrices (84%) (Figure SI℃3B) with almost equal preference for urine (44%) and blood serum (37%) (Figure SI–3C). All the reported assays have used LC coupled with MS measurement. Extraction and satisfactory recovery of BPA and its conjugates from the biological sample is critical, followed by their best separation from matrix ions that yields optimal ionization, detection and quantitation by the MS platform. Analytical steps at each stage of the sample preparation and analysis for BPAG in human serum by the four laboratories that participated in the round robin trial was presented to the finest detail [90]. However, the rest of the studies that analyzed BPAG and BPAS do not provide information to comparable detail (Table SI–2). We attempt to present and discuss the analytical steps reported in literature in the following sub-sections.

4.1. Sample preparation

Sample preparation is a key step of bio-analysis that impacts the later steps during analysis and results. Sample volume for extraction was lab-dependent and for urine ranged between 0.1mL [28, 88] and 1.5 mL [86], and serum or plasma between 0.1 mL [28, 88, 89] and 1.0 mL [90]. Enzymatic deconjugation was not performed to recover glucuronide and sulfate forms along with aglycone BPA from the sample. Protein precipitation was performed as a pre-cleanup step using acetonitrile or methanol [85, 88, 90]. This step helps to minimize matrix effects and interferences on both hydrophilic and hydrophobic molecules but analyte loss induced by co-precipitation is possible. Recoveries of BPA and its conjugates were above 90% in studies that used a protein removal step [85, 88]. Derivatization of BPA and conjugates was performed only in a minor fraction of the studies (14%) (Figure SI–3D). Derivatizations with dansyl chloride was proposed to overcome the need for any further sample extraction steps and yet achieve desirable recoveries and detection limits for free and total BPA [56]. This approach was extended to analyze BPAG in addition to measuring free BPA in neonates' urine [83, 84]. No reports were found that used gas chromatography after derivatization of BPA conjugates. Further, isolation and pre-concentration of BPA and its conjugates in biological samples was achieved with either liquid-liquid extraction [28, 85, 86, 88, 90] or solid phase extraction [82, 86, 89–91]. Application of SPE was a popular choice (57%) compared to LLE (36%) in the reported studies (Figure SI–3E). LLE or SPE steps not only enhance analyte recovery but also minimize matrix effects and prolong the life of the chromatography column and minimize the matrix residues deposit in the ionization source and on the mass spectrometer detector. Though SPE offers greater selectivity and cleaner extracts than LLE procedures, BPA aglycone and conjugates recoveries were similar and typically in an acceptable range of 80%-120% (Table SI–2).

Use of different SPE phase materials was reported for the extraction of BPA and conjugates in various biological matrices. The most common SPE materials were a conventional C18

[89] and polymeric reversed-phase sorbent such as Oasis HLB [82, 91]. Due the differences in polarity of BPA and its conjugates, some studies used a combination of extraction techniques such as (i) SPE with reversed-phase (C18) for aglycone BPA, and amino (NH_2) phase for BPAG and BPADS in human urine [82], (ii) sequential use of SPE phases such as amino and a mixed-mode (reversed-phase / strong cation-exchange) for human serum [82], and (iii) LLE for aglycone BPA and SPE with weak anion-exchange polymeric sorbent for BPG, BPAS, and BPADS in human urine [86]. Details of the extraction solutions used in LLE and elution mixtures for SPE are presented in Table SI–2.

4.2. Chromatographic separation and mass spectrometry detection

Separation of the aglycone BPA and its conjugates has been performed using liquid chromatography in all the available studies (Figure SI–3F). Reversed-phase LC was applied in all the studies (Table 1). A C18 column was the most popular choice (67%). Other columns included C8 [84, 89], PFP [85], HSS T3 [86], and BEH amide [87] (Figure SI–3G). All the reported methods used a gradient elution of concentrated and unaltered solvent made of acetonitrile [28, 88, 89] or methanol [82] or either one with some modifiers [84, 86, 87, 91] as the mobile phase. Run length and retention times of aglycone BPA and its conjugates are dependent on the chromatographic conditions used in the individual study. The run times ranged between <5.0 min [88] and 22.0 min [28]. BPA conjugates eluted prior to aglycone BPA and hence had shorter retention times compared to the latter (Table SI–2).

Electrospray ionization (ESI) mode was the only ionization mode used. The presence of a phenolic hydroxyl functional group in aglycone BPA and its conjugates promotes efficient ionization in the negative electrospray ionization mode and hence was preferred in 86% of the studies listed in this review (Figure SI–3H). In studies where a derivatization step was used, the polarity was switched so that positive mode was more effective for ionization [84, 86, 91]. In the ESI negative mode, most studies used the following multiple reaction monitoring mass transitions for the accurate detection and quantification of BPA: m/z 227→212; BPAG: m/z 403→113; BPAS: m/z 307→227; and BPADS: m/z 387→307. Use of isotope-labeled internal standards in the very beginning of sample preparation ensures quantification is not affected significantly by matrix effects. Both deuterated and carbon isotope labeled BPA conjugate standards were used such as BPAG-$d1_6$ [28, 88], BPAG-d_6 [56, 86], BPAG-$^{13}C1_2$ [87, 90], and BPAS- d_6 and BPADS-d_6 [86].

Limits of detection (LOD) and quantitation (LOQ) are used to define the analytical sensitivity of a method. These are usually different between the laboratories because of the differences in definitions and procedures to estimating them. Though different, the intention of the definitions remains the same. LOD is the smallest concentration of an analyte that can be detected beyond a doubt, and LOQ is the lowest concentration that can be quantified without a bias. Sample volume or injection volume was not an indicator of the reported LOD and LOQ in the study matrices (Table 1, Table SI–2). The sensitivity of analytical methods was dependent on the matrix, sample extraction, chromatography and detector features and parameters (Table SI–2). The lowest reported LOD values are (i) 0.003 ng/mL for aglycone BPA in human urine (using 0.5 mL sample for SPE with Oasis HLB, 10 μL injection volume onto a Betasil C18 LC column with methanol mobile phase, and an API 5500 triple

quadrupole mass spectrometer) [82], (ii) 0.002 ng/mL for BPAG in human serum (using 0.2 mL sample for LLE with protein precipitation, 5 μL injection volume onto a Shimpack XR-ODS III LC column with acetonitrile mobile phase, and a Shimadzu LCMS-8080 triple quadrupole mass spectrometer) (Lab 1 in [90]), (iii) 0.011 ng/mL for BPADS in human urine (using 1.5 mL sample for SPE with Strata X-AW, 10 μL injection volume onto a Acquity UPLC HSS T3 LC column with ammonium hydroxide in methanol mobile phase, and a Xevo TQ-S tandem mass spectrometer) [86], and (iv) 0.02 ng/mL for BPADS in human urine (using 0.5 mL sample for SPE with Strata NH_2, 10 μL injection volume onto a Betasil C18 LC column with methanol mobile phase, and an API 5500 triple quadrupole mass spectrometer) [82]. It is very important to distinguish the LOD and LOQ estimation procedures and capabilities between the studies while studying BPA conjugates at such trace concentrations. Nevertheless, it is essential to determine the “fit for purpose” of a method for its use in quantifying BPA conjugates in human exposure assessment studies.

Individual study details in regards to LOD and LOQ definitions and values that are dependent on the respective sample preparation protocols, and chromatography and mass spectrometry method features are presented in Table SI–2.

5. Perspectives: Inclusion of BPAG and BPAS conjugates as additional biomarkers of BPA exposure

Total BPA measurements in urine are a reliable measure only when external contamination is controlled. Because the introduction of BPA from external sources not only elevates the aglycone but also total BPA levels, measuring either one is not necessarily a reliable biomarker of exposure to BPA. However, BPAG and BPAS quantification are gaining consideration and value because the levels are unaffected by the general sample handling procedures either during collection or analysis. BPA and its conjugates in human matrices were quantified primarily as a component of method development [28, 56, 82, 85–91], while very few studies have applied this approach for biomonitoring of human exposures to BPA [84, 92–94]. Specificity of BPAG is similar to aglycone BPA in exposure assessment studies; however, frequency of detection of BPAG in urine is almost always greater than aglycone BPA. In the studied populations, BPAG was measured in almost all human matrices and at a higher detection frequency and concentration compared to the aglycone BPA (Table 2). The first report on BPAG levels in humans was published in 2005 [28]. Since then 12 peer-reviewed studies have been published reporting BPA conjugates in various human matrices such as, urine, plasma, serum, and umbilical cord blood. A summary of these studies with key details of the study populations, sample type, analytical method features and performance parameters, BPA and conjugates detection rates and concentrations in human matrices is presented in Table 2.

BPAG was more frequently detected than BPAS or BPADS. The detection rates for BPAG in the reported study matrices ranged between (i) 61% [87] and 100% in urine [84, 85], and (ii) 0% [28] and 100% in blood [90] (Table 2). BPAG concentrations were comparatively higher than aglycone BPA in the reported matrices. For example, BPAG was in the range of <0.05–65.2 ng/mL in urine and <0.05–11.9 ng/mL in serum compared to aglycone BPA in the

range of <0.01–18.7 ng/mL and <0.01–0.59 ng/mL in the respective matrices from the same study population [82]. BPAS was above detection limits in adult serum [82], umbilical cord serum [91], and urine [86, 94]. BPADS was less frequently included in the BPA conjugates analyses, and rarely above the limits of detection in studies where it was included (Table 2). Improvement in detection limits is required when the reported LODs are higher than the observed BPA levels in general population. For example, the detection limits of BPA and BPAG were 2 and 10 ng/mL in urine [88] and are not suitable for biomonitoring studies where the total BPA levels in urine from a general population are about 1 ng/mL. Even with a LOD of 1 ng/mL, BPAG went undetected in 39% of urine samples in one human study (n = 110) [87].

Urinary BPAG concentrations represented 90 – 95% of the total BPA while aglycone BPA constituted only 1 – 2% [86, 94]. In contrast, one study reported BPAG and aglycone BPA as 57% and 32% of the total BPA in urine [82]. The lower proportion of BPAG in this study was attributed to possible external contamination of samples with BPA and underestimation of BPAG from using an inappropriate internal standard (labeled BPA instead of BPAG). In the case of umbilical cord serum, BPAS was frequently detected and was the predominant fraction contributing to 45% of the total BPA followed by 36% aglycone BPA and 19% BPAG [91]. The authors concluded that the sulfonation pathway was preferred over glucuronidation for BPA detoxification during midgestation. A very interesting approach was taken to compare BPAG and BPAS levels with total BPA by multiplying BPAG level with 0.5614 (ratio of the molecular weight of BPA to that of BPAG) to obtain concentration of BPA in glucuronide form, and multiplying BPAS level with 0.7404 (ratio of the molecular weight of BPA to that of BPAS) to obtain concentration of BPA in sulfate form [91]. These conversion factors are important when BPAG and BPAS are measured directly before they can be compared with previous BPA exposure data.

Efficiency of BPA conjugation metabolism in neonates was assessed by measuring urinary aglycone BPA and BPAG [83, 84]. A difference in BPA conjugates composition and detection frequency in different bio-matrices collected at different life stages can further enhance our understanding on BPA metabolism and its effects in humans. Detectable aglycone BPA was higher in lipid-rich bio fluids such as breast milk [95, 96] versus urine [27, 28]. This observation could help to further explore and understand the partition preference of conjugated and aglycone BPA in systemic circulation [90]. Measuring conjugated BPA forms in infant and children matrices could help to investigate their potential health effects during the development of the detoxification mechanisms. Interestingly, urinary BPAG concentrations were tested for associations with postmenopausal breast cancer in a case-control study and found no statistical significant relationship [93]. Aglycone BPA was detected only in 37 out of 1150 samples and was not evaluated for statistical relationships with breast cancer prevalence in this study. The authors conclude that BPAG measurement in a spot urine sample may not represent the long-term exposure and/or critical window exposure in relation to breast cancer development.

A mismatch in BPA exposure assessment was observed between the two approaches, total BPA versus sum of the individual forms of BPA in urine [82].The sum of the geometric mean concentration of six forms of BPA (aglycone BPA, conjugates BPAG and BPADS, and

chlorinated derivatives BPAMC, BPADC and BPATrC) in urine (GM: 3.119 ng/mL) was approximately 0.2 fold lower than the total BPA in urine (GM: 5.40 ng/mL) [82], which indicates the presence of unidentified forms that are unaccounted for in the total BPA pool. Rapid metabolism of BPA should not be considered as risk-free [67] because BPA may undergo different metabolic pathways resulting in a different subset of metabolites ranging in estrogenic activity [61, 64, 65, 97–113].Underexplored metabolic pathways of BPA yielding higher estrogenic activity metabolites needs attention towards understanding the role of such understudied metabolites in endocrine disruption outcomes [114]. Effects of unconventional metabolites of BPA in in vitro assays were recently discussed [115].

6. Challenges and opportunities

The major limitation in using BPAG and BPAS as additional biomarkers of exposure to BPA is that conjugated forms could degrade within a day or two if the urine samples are stored at room temperature [78, 87, 116]. This results in underestimating the BPA exposure when the conjugates alone were analyzed and measured directly. BPAG was stable at −80°C for the entire study duration of 28 days and stable for at least 3 freeze and thaw cycles [87].Though the urine samples are stored appropriately, it is likely that they are exposed to ambient temperatures for certain periods during collection, handling and shipping that could potentially compromise the utility of these alternative biomarkers. A field blank and reagent blank are used as a quality control measure to assess and/or control background contamination of BPA when using total BPA as a biomarker, but no such measure is available to estimate the degradation of urine sample between post-specimen collection and pre-analysis in the laboratory. This remains a major caveat for BPA conjugates application in human exposure assessment until and unless the appropriate storage conditions are practiced for urine specimen integrity. Other potential limitations of this approach are (i) that it is unclear at the moment what fraction of the total BPA is precisely covered by measuring BPAG and BPAS conjugates, (ii) lack of documentation whether a statistically significant, positive, and linear correlation exists between BPAG and total BPA, and (iii) unknown and unidentified conjugates of BPA other than BPAG and BPAS go unmonitored and unmeasured. Except for the study by Kannan’s group [82], no other study calculated the percent contribution of concentration of each metabolite of BPA towards total BPA.

Glucuronide and sulfate conjugates are the most common metabolites of xenobiotics and exogenous chemicals that undergo phase II metabolism in humans as a detoxification measure [117–126]. This creates a critical analytical challenge of lack of MRM transitions selectivity and possible interferences from other conjugates. For example, one of the dietary isoflavones namely resveratrol (*m/z* 227→185) and its conjugated urinary metabolites such as resveratrol-4’-O-glucuronide (*m/z* 403→227) and resveratrol-4’-sulfate (*m/z* 307→227) share similar molecular weights and fragmentation patterns as that of BPA, BPAG and BPAS, respectively [28]. Utilization of labeled standards for BPAG and BPAS that became recently available commercially can help to overcome this situation by including additional mass transitions as well as retention times for better exposure biomarker validation.

Further studies are deemed necessary to accurately determine the difference between the conjugated fraction assessed by taking the difference between total and aglycone BPA as per

the conventional approach and the sum of aglycone BPA, BPAG and BPAS from the novel simultaneous detection approach. This effort will also shed light on the occurrence and approximate concentration of unknown metabolites of BPA in human matrices. Though BPAG is a major fraction of total BPA in urine specimens, there is not sufficient evidence that it is a good biomarker for BPA exposure because the ratios of individual BPA conjugates to total BPA vary depending on exposure sources. Associations with a disease or risk factor are not established at this time and hence additional studies are deemed necessary before considering BPAG as a good alternative biomarker. Future research directions include finding correlations between total BPA with individual conjugates to strengthen the usefulness of conjugates as biomarkers of exposure to BPA and predicting total BPA exposure based on the slopes of the exposure-response curves for the individual conjugates.

7. Conclusion

This review presents novel approaches and analytical methods for the simultaneous extraction and detection of BPA and conjugates in biological matrices. Recent advances in the commercial availability of standards and respective labelled internal standards, SPE phases, LC columns, and MS detectors have enabled highly specific and sensitive analysis of BPA conjugates. Detection limits at trace levels is possible and documented. Because BPA conjugates are formed only after the exposure to BPA, biomonitoring of BPAG and other minor conjugates can be considered as reliable biomarkers of exposure, and avoid misinterpretations while paving new directions in assessing human risks to BPA. The analytical approaches can also be useful for further understanding of BPA pharmacology and pharmacokinetics. Though BPA conjugate measurement is possible because of the recent advances in analytical methods, there are certain limitations and challenges to overcome before considering their use in human biomonitoring studies. If successful, then the analysis of BPA conjugates as additional biomarkers might contribute to a better understanding of life-stage differences in metabolism and distribution, inter-individual variability, and the potential health effects of bio-active forms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Bisphenol A conjugates require in vivo metabolism and not prone to external contamination.
2. Analytical trends in the measurement of BPA conjugates are reviewed in this work.
3. BPA conjugates can be potential additional biomarkers of human exposure to BPA.

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Table 1

Summary of the novel analytical approaches for the determination of BPA aglycone form and its glucuronide and sulfate conjugates in bio-matrices.

Study #	Sample (i) Subject (ii) Matrix (iii) Analysis volume	Sample preparation: key steps (i) Enzymatic deconjugation (Yes/No) (ii) Internal standards	Analytical method: key parameters (i) Injection volume (ii) LC column (iii) Mobile phase (iv) Ionization source (v) Mass transitions (*m/z*)	Analytical performance: key features (i) LOD, LOQ definition (ii) LOD (iii) LOQ	Reference (chronological)
1	(i) Humans (ii) Urine and Plasma (iii) Urine: 100 μL; plasma: 100 μL	(i) No enzymatic deconjugation for the analysis of aglycone BPA and BPAG. For total BPA, an enzymatic hydrolysis was performed with glucuronidase/ sulfatase from *Helix pomatia* (EC 3.2.1.31). (ii) BPA-d_{16}; BPAG-d_{16}	(i) Urine: 10 μL; plasma: 20 μL (ii) ReproSil-Pur ODS-3 column (150 mm × 4.6 mm × 5 μm) (Maisch, Ammerbuch, Germany) (iii) *Mobile phase:* Solvent A: water; and Solvent B: acetonitrile (iv) ESI, negative ion mode (TurbolonSpray was also operated in negative ion mode) (v) *BPAG:* 403.2 → 113.1, 227.0; *BPAG-d_{16}* 417.2 → 113.0, 241.1; *BPA:* 227.0 → 212.0, 133.0; *BPA-d_{16}* 241.1 → 223.3), 142.1.	(i) NA (ii) BPA (1.14 ng/mL); BPAG (10.1 ng/mL). (iii) BPA (3.42 ng/mL); BPAG (26.26 ng/mL).	[28]
2	(i) Rats (ii) Plasma (iii) 0.1 mL	(i) No enzymatic hydrolysis (ii) NA	(i) 10 μL (ii) Two columns were tested: Kinetex C18 column (100 mm × 4.6 mm × 2.6 μm, Phenomenex, CA, USA); and Discovery C8 column (50 mm × 4.6 mm × 5 μm, Supelco, MO, USA). Guard column: Krud Katcher Ultra In-Line Filter guard column (0.5 μm, Phenomenex, CA, USA). (iii) *Mobile phase:* Solvent A: ammonium acetate (2 mM, pH 9) and Solvent B: acetonitrile. (iv) ESI, negative ion mode (v) *BPAG:* 403 → 227; *BPA:* 227 → 212.	(i) NA (ii) BPA (0.1 ng); BPAG (0.25 ng). (iii) NA	[89]
3	(i) Sheep (ii) Urine and plasma (iii) Urine: 100 μL; plasma: 100 μL	(i) No enzymatic hydrolysis for simultaneous analysis of aglycone BPA and BPAG. However, β-hydrolysis was also performed for comparison between direct assay and after enzymatic deconjugation. (ii)BPA-d_{16}	(i) NA (ii) Acquity BEH C18 column (100 mm × 2.1 mm × 1.7 μm; Waters, MA, USA) (iii) *Mobile phase:* Solvent A: water in acetonitrile; and Solvent B: acetonitrile. (iv) ESI, negative ion mode (v) *BPAG:* 403 → 227; *BPA:* 227 → 212; *BPA-d_{16}* 241 → 142.	(i) LOD: concentration that has a signal to noise ratio greater than three times the mean noise of six blanks. LOQ: lowest concentration with a precision <20% and accuracy within 80–120%. (ii) *Urine:* BPA (2 ng/mL); BPAG (10 ng/mL). *Plasma:* BPA (0.6 ng/mL); BPAG (2 ng/mL). (iii) *Urine:* BPA (25 ng/mL); BPAG (1000 ng/mL). *Plasma:* BPA (1 ng/mL); BPAG (20 ng/mL).	[88]
4	(i) Humans (ii) Urine and serum (iii) Urine: 0.5 mL;	(i) (a) Yes for analyzing total BPA (aglycone + conjugated); and (b) No for the analysis of aglycone BPA, BPAG, and	(i) 10 μL (ii) Betasil C18 column (100 mm × 2.1 mm i.d. × 5 μm particle size; Thermo Electron Corp., MA, USA) and Javelin Betasil C18 guard column	(i) LOD: three times the standard deviation of five replicates of 0.01 ng/mL standard. LOQ: ten times the standard	[82]

Study #	Sample (i) Subject (ii) Matrix (iii) Analysis volume	Sample preparation: key steps (i) Enzymatic deconjugation (Yes/No) (ii) Internal standards	Analytical method: key parameters (i) Injection volume (ii) LC column (iii) Mobile phase (iv) Ionization source (v) Mass transitions (*m/z*)	Analytical performance: key features (i) LOD, LOQ definition (ii) LOD (iii) LOQ	Reference (chronological)
	serum: 0.5 mL	BPADS. (ii) $^{13}C_{12}$-BPA	(20 mm × 2.1 mm i.d. × 5 μm particle size; Thermo Electron Corp., MA, USA) (iii) *Mobile phase:* Solvent A: Methanol and 10 mM ammonium acetate; Solvent B: Methanol. (iv) ESI, negative ion mode (v) *BPA:* 227 → 212; *BPAG:* 403 → 113; *BPADS:* 387 →307.	deviation of five replicates of 0.01 ng/mL standard. (ii) BPA (aglycone): 0.003 ng/mL; BPAG: 0.02 ng/mL; and BPADS: 0.02 ng/mL (iii) BPA (aglycone): 0.01 ng/mL; BPAG: 0.05 ng/mL; and BPADS: 0.05 ng/mL	
5	(i) Humans (ii) Umbilical cord serum (iii) 0.25 mL	(i) No enzymatic hydrolysis for simultaneous analysis of aglycone BPA and BPAG. (ii)BPA-d_{16} (Note: internal standards for BPAG and BPAS were unavailable at the time of analysis).	(i) 25 μL (ii) Agilent Extend-C18 column (100 mm × 2.1 mm × 1.8 μm). Temperature at 50°C. (iii) *Mobile phase:* Solvent A: Water with 0.05% ammonium acetetate (pH 7.8) Solvent B: Methanol with 0.05% ammonium acetetate (pH 7.8). (iv) ESI, negative ion mode (v) *BPA:* 227.0 → 133.1, 212.1; *BPA-d_{16}* 241.0 → 142.2, 222.1; *BPAG:* 402.9 → 112.9, 226.9; *BPADS:* 307.0 → 227.0, 212.1.	(i) LOD: lowest concentration that has a signal to noise ratio ≥3. LOQ: lowest concentration that has a signal to noise ratio ≥10 and yield ≥ 0.95 regression coefficient for the calibration curve. (ii) BPA: 0.05 ng/mL; BPAG: 0.05 ng/mL; BPAS: 0.025 ng/mL (iii) BPA: 0.1 ng/mL; BPAG: 0.1 ng/mL; BPAS: 0.1 ng/mL	[91]
6	(i) Humans (ii) Urine (iii) 0.5 mL	(i) No enzymatic hydrolysis for simultaneous analysis of aglycone BPA and BPAG. (ii) $^{13}C_{12}$-BPA	(i) 20 μL (ii) Agilent Pursuit 3 penta floura-phenyl propyl (PFP) column (100 mm × 3.0 mm internal i.d. × 3 μm particle size; Agilent Technologies). Column temperature at 20°C (iii) *Mobile phase:* Solvent A: 2 mM ammonium acetate in water; and Solvent B: Methanol. (iv) ESI, negative ion mode (v) *BPA:* 227.0 → 132.8; *BPA:* 227.0 → 212.1; *BPA-$^{13}C_{12}$* 239.2 → 224.1; *BPAG:* 403.1 → 113.1; *BPAG:* 403.1 → 227.0.	(i) LOD: lowest concentration that gives signal to noise ratio ≥ 3. LOQ: lowest concentration that gives signal to noise ratio ≥ 10. (ii) BPA: 0.03 ng/mL; BPAG: 0.10 ng/mL (iii) BPA: 0.08 ng/mL; BPAG: 0.33 ng/mL	[85]
7	(i) Humans (ii) Urine (iii) 1.0 mL for aglycone BPA, and 1.5 mL for BPAG, BPAS, and BPADS analysis	(i) No enzymatic hydrolysis for the analysis of aglycone BPA, BPAG, BPAS, and BPADS. (ii) $^{13}C_{12}$-BPA, BPAS- d_6; BPADS-d_6; BPAG-d_6	(i) 10 μL (ii) Acquity UPLC HSS T3 column (50 mm × 2.1 mm × 1.8 μm). Column temperature @ 30°C (iii) *Aglycone BPA*:: *Mobile phase:* Solvent A: 0.1% aqueous formic acid solution; and Solvent B: acetonitrile. *BPAG, BPAS, and BPADS*:: *Mobile phase:* Solvent A: Solvent A: 2% NH_4OH in H_2O pH=11.0; and Solvent B: 0.1% NH_4OH in methanol. (iv) *Aglycone BPA* ESI, positive ion mode *BPAG, BPAS, and BPADS* ESI, negative ion mode (v) *BPA:* 695.5 → 171.2, 156.2; *BPA-$^{13}C_{12}$:* 707.5 → 171.2, 156.2; *BPAS:* 307.3 → 212.3, 227.2; *BPAS-d_6* 313.3 → 215.3, 233.3; *BPADS:* 387.2 → 227.3, 212.3, 307.3; *BPADS-*	(i) LOD: estimated concentration that gives a signal to noise ratio ≥3. A sample with 7 to 10 fold concentration of the estimated LOD is analyzed in ten replicates on the same day and by the same analyst. Final LOD is three times the standard deviation of these runs. LOQ: Ten times the standard deviation of the ten replicates. (ii) BPA (0.012 ng/mL); BPAS (0.011 ng/mL); BPADS (0.38 ng/mL); BPAG (0.097 ng/mL). (iii) BPA (0.039 ng/mL); BPAS (0.037 ng/mL); BPADS (1.3 ng/mL); BPAG (0.32 ng/mL).	[86]

Study #	Sample (i) Subject (ii) Matrix (iii) Analysis volume	Sample preparation: key steps (i) Enzymatic deconjugation (Yes/No) (ii) Internal standards	Analytical method: key parameters (i) Injection volume (ii) LC column (iii) Mobile phase (iv) Ionization source (v) Mass transitions (*m/z*)	Analytical performance: key features (i) LOD, LOQ definition (ii) LOD (iii) LOQ	Reference (chronological)
			d_6 393.2 → 233.4; *BPAG:* 403.4 → 212.3, 227.3; *BPAG-d_6* 409.5 → 215.3, 233.3.		
8	(i) Humans (ii) Serum (iii) Lab 1: 0.2 mL; Lab 2: 0.5 mL; Lab 3: 0.25 mL; Lab 4: 0.55–1 mL.	(i) No enzymatic hydrolysis for the analysis of aglycone BPA and BPAG. (ii) Lab 1: BPA-d_6 and BPAG-$^{13}C_{12}$; Lab 2: BPA-$^{13}C_{12}$; Lab 3: BPA-d_6 and BPAG-$^{13}C_{12}$; Lab 4: BPA-$^{13}C_{12}$ and BPAG-$^{13}C_{12}$.	(i) Lab 1: 5 μL; Lab 2: 10 μL; Lab 2: 25 μL; and Lab 2: 10 μL. (ii) Lab 1: Shimadzu Shimpack XR-ODS III (2.0 mm × 50 mm, 1.6 μm). Column temperature @ 50°C Lab 2: C18, Betasil(Thermo Electron Corporation), (100 × 2.1 mm, 5 μm). Column temperature @ room temperature. Lab 3: Agilent Extend C-18 (4.6 × 100 mm, 1.8 μm). Column temperature @ 50°C. Lab 4: C18 BDS, Hyperclone (Phenomenex, CA, USA), (100 × 4.6 mm, 3 μm). Column temperature @ not heated. (iii) *Mobile phase::* Lab 1: Acetonitrile (10 to 100%). Aqueous ammonium actetae (1 mM) as a co-solvent. Lab 2: Solvent A: methanol; Solvent B: ammonium acetate (10 mM) @ pH ~5.0. Lab 3: Solvent A: a mixture of water and ammonium acetate (0.05%) @ pH 7.80; and Solvent B: a mixture of methanol and ammonium acetate (0.05%) @ pH 7.80. Lab 4: Solvent A: a mixture of acetonitrile and water with 0.01% ammonia (pH 9) (10:90 v/v); Solvent B: acetonitrile. (iv) Lab 1, 2, 3, and 4: ESI, negative ion mode (v) Lab 1: *BPA:* 227.0 → 212.0, 133.0; *BPA-d_6* 233.0 → 215.0; *BPAG:* 403.0 → 227.0, 133.0; *BPAG-$^{13}C_{12}$* 415.0 → 239.0. Lab 2: *BPA:* 227.0 → 212.0; *BPA-$^{13}C_{12}$* 239.0 → 224.0; *BPAG:* 403.0 → 113.0. Lab 3: *BPA:* 227.0 → 133.1, 212.1; *BPA-d_6* 233.0 → 85.0, 122.8; *BPAG:* 402.9 → 112.9, 226.9; *BPAG-$^{13}C_{12}$* 415.0 → 112.8, 239.1. Lab 4: *BPA:* 227.0 → 212.0; *BPA-$^{13}C_{12}$* 239.0 → 224.0; *BPA-d_6* 233.0 → 138.0; *BPAG:* 403.0 → 113.0; *BPAG-$^{13}C_{12}$* 414.0 → 239.0.	(i) Lab 1:: Standard FDA guidelines Lab 2:: LOD: 3xSD, and LOQ: 10xSD (standard deviation) of five replicate analyses of a 0.01 ng/mL standard solution. Lab 3:: LOD: signal to noise ration > 10, and LOQ: lowest concentration on the calibration curve that yield ≥ 0.95 linear regression coefficient. Lab 4:: LOD: 3xSD, and LOQ: 10xSD (standard deviation) of three replicate analyses of the lowest calibration standard solution. (ii) Lab 1:: BPA: 0.02 ng/mL; BPAG: 0.002 ng/mL Lab 2:: BPA: 0.003 ng/mL; BPAG: 0.02 ng/mL Lab 3:: BPA: 0.05 ng/mL; BPAG: 0.05 ng/mL Lab 4:: BPA: 0.04 ng/mL; BPAG: 0.02 ng/mL (iii) LLOQ Lab 1:: BPA: 0.10 ng/mL; BPAG: 0.01 ng/mL Lab 2:: BPA: 0.01 ng/mL; BPAG: 0.05 ng/mL Lab 3:: BPA: 0.10 ng/mL; BPAG: 0.10 ng/mL Lab 4:: BPA: 0.13 ng/mL; BPAG: 0.06 ng/mL	[90]
9	(i) Humans (ii) Urine (iii)[a] 0.2 mL	(i)[a] Noenzymatic hydrolysis for simultaneous analysis of aglycone BPA and BPAG. (ii) BPA- d_6; BPAG-d_6	(i)[a] 6 μL (ii)[a] Thermo-Scientific BetaBasic C8 column (50 mm × 1.0 mm × 5 μm). Note: Changed to a C18 column with 2.0mm internal diameter in [83]. (iii)[a] *Mobile phase:* Solvent A: 0.1% formic acid in water;	(i) LOD: NA LOQ: five times LOD (ii) BPA: 0.02 ng/mL; BPAG: 0.02 ng/mL (iii) BPA: 0.1 ng/mL; BPAG: 0.1 ng/mL	[83]; [84] [a][56]

Study #	Sample (i) Subject (ii) Matrix (iii) Analysis volume	Sample preparation: key steps (i) Enzymatic deconjugation (Yes/No) (ii) Internal standards	Analytical method: key parameters (i) Injection volume (ii) LC column (iii) Mobile phase (iv) Ionization source (v) Mass transitions (*m/z*)	Analytical performance: key features (i) LOD, LOQ definition (ii) LOD (iii) LOQ	Reference (chronological)
			and Solvent B: 0.1% formic acid in acetonitrile. (iv)[a] ESI, positive ion mode (v)[a] $BPA(dansyl)_2$ 695 → 170.		
10	(i) Humans (ii) Urine (iii) 1.0 mL	(i) No enzymatic hydrolysis for simultaneous analysis of aglycone BPA and BPAG. (ii) BPAG-$^{13}C_{12}$	(i) 2 μL (ii) Acquity UPLC BEH amide column (100 mm × 2.1 mm × 1.7 μm; Waters, MA, USA) (iii) *Mobile phase:* acetonitrile with 1 mM ammonium acetate in water. (iv) ESI, negative ion mode (v) *BPAG:* 403 → 113, 175; BPAG-$^{13}C_{12}$ 415 → 113, 175.	(i) NA (ii) BPAG: 1 ng/mL (iii) BPAG: 5 ng/mL	[87]

[a] Nachman et al. (2013 and 2015) *analyzed dansyl chloride-derivatized BPAG in addition to free BPA as per their primary method reported by* Fox et al. (2011).

Table 2

Use of BPA conjugates in humans' exposure assessment and biomonitoring studies.

Study #	**Study** **(i) Size** **(ii) Location** **(iii) Year**	**Analysis** **(i) Matrix** **(ii) Extraction** **(iii) Instrumentation** **(iv) References of sample analysis relevance cited in the study**	**BPA-aglycone** **(i) LOD** **(ii) LOQ** **(iii) Detection frequency** **(iv) Concentration**	**BPAG** **(i) LOD** **(ii) LOQ** **(iii) Detection frequency** **(iv) Concentration**	**BPAS** **(i) LOD** **(ii) LOQ** **(iii) Detection frequency** **(iv) Concentration**	**Study Reference (chronological)**
1	(i) n = 19 (7 males and 12 females) (ii) Germany (iii) NA	(i) Urine and plasma (ii) No enzymatic de-conjugation. LLE. (iii) HPLC-MS/MS (iv) NA	(i) 1.14 ng/mL (ii) 3.42 ng/mL (iii) Percent above LOD (no intentional exposure to BPA):: Urine: 0%; Plasma: 0%. (iv) Range (no intentional exposure to BPA):: Urine: <LOD; Plasma: <LOD.	(i) 10.1 ng/mL (ii) 26.26 ng/mL (iii) Percent above LOD (no intentional exposure to BPA):: Urine: Most samples; Plasma: 0%. (iv) Range (no intentional exposure to BPA):: Urine: <LOQ; Plasma: <LOD.	NA	[28]
2	(i) n = 31 (urine) and 14 (serum) (ii) Albany, United States of America (iii) August-September 2011	(i) Urine and Serum (ii) No enzymatic de-conjugation. SPE. (iii) HPLC-MS/MS (iv) [62, 89, 127]	(i) 0.003 ng/mL (ii) 0.01 ng/mL (iii) Percent above LOD:: Urine: 96.8%; Serum: 57.1%. (iv) Range:: Urine : <LOQ-18.7 ng/mL; Serum : <LOQ-0.588 ng/mL.	(i) 0.02 ng/mL (ii) 0.05 ng/mL (iii) Percent above LOD:: Urine: 87.1%; Serum: 50.0%. (iv) Range:: Urine : <LOQ-65.2 ng/mL; Serum : <LOQ-11.9 ng/mL.	(i) 0.02 ng/mL (ii) 0.05 ng/mL (iii) Percent above LOD:: Urine: 35.5%; Serum: 50.0%. (iv) Range:: Urine : <LOQ-6.16 ng/mL; Serum : <LOQ-1.77 ng/mL. Note : BPADS conjugate was monitored.	[82]
3	(i) n = 85 (ii) Northern and Central California, United States of America (iii) 2010–21012	(i) Umbilical cord serum (ii) No enzymatic de-conjugation. SPE. (iii) HPLC-MS/MS (iv) [88, 128, 129]	(i) 0.05 ng/mL (ii) 0.10 ng/mL (iii) Percent above LOD: 47% (iv) Range: <LOD-52.26 ng/mL	(i) 0.05 ng/mL (ii) 0.10 ng/mL (iii) Percent above LOD: 76% (iv) Range: <LOD-5.41 ng/mL	(i) 0.025 ng/mL (ii) 0.10 ng/mL (iii) Percent above LOD: 96% (iv) Range: <LOD-12.65 ng/mL Note : BPAS conjugate was monitored.	[91]
4	(i) n = 12 (ii) Baltimore, United States of America (iii) January-February 2012	(i) Urine (ii) No enzymatic de-conjugation. No solvent extraction. Dansyl chloride derivatization. (iii) HPLC-MS/MS (iv) [56]	(i) 0.02 ng/mL (ii) 0.1 ng/mL (iii) Percent above LOQ:: 7–44 days age: 0% (iv) Median: <LOQ	(i) 0.02 ng/mL (ii) 0.1 ng/mL (iii) Percent above LOQ:: 7–44 days age: 100% (iv) Median: 0.66 ng/mL	NA	[84]
5	(i) n = 15 (ii) Mersin, Turkey	(i) Urine (ii) Protein precipitation.	(i) 0.03 ng/mL (ii) 0.08 ng/mL	(i) 0.10 ng/mL (ii) 0.33 ng/mL	NA	[85]

Study #	**Study** **(i) Size** **(ii) Location** **(iii) Year**	**Analysis** **(i) Matrix** **(ii) Extraction** **(iii) Instrumentation** **(iv) References of sample analysis relevance cited in the study**	**BPA-aglycone** **(i) LOD** **(ii) LOQ** **(iii) Detection frequency** **(iv) Concentration**	**BPAG** **(i) LOD** **(ii) LOQ** **(iii) Detection frequency** **(iv) Concentration**	**BPAS** **(i) LOD** **(ii) LOQ** **(iii) Detection frequency** **(iv) Concentration**	**Study Reference (chronological)**
	(iii) NA	Dilution and LLE. (iii) HPLC-MS/MS (iv) [85]	(iii) Percent above LOD: 93.33% (iv) Range: 0.06–1.39 μg/g	(iii) Percent above LOD: 100% (iv) Range: 2.22–105.97 μg/g		
6	(i) n = 200 (adult male = 74, adult female = 69, and children = 57) (ii) Mersin, Turkey (iii) March-September 2012	(i) Urine (ii) Protein precipitation. Dilution and LLE. (iii) HPLC-MS/MS (iv) [85]	(i) 0.03 ng/mL (ii) 0.08 ng/mL (iii) Percent above LOD:: Adult male: 9%; Adult female: 12%; Children: 4%. (iv) Mean:: Adult male: 0.54 μg/g; Adult female: 0.31 μg/g; Children: 0.60 μg/g.	(i) 0.10 ng/mL (ii) 0.33 ng/mL (iii) Percent above LOD:: Adult male: 41%; Adult female: 39%; Children: 39%. (iv) Mean:: Adult male: 31.37 μg/g; Adult female: 57.57 μg/g; Children: 56.68 μg/g.	NA	[92]
7	(i) n = 46 (males = 26, females = 20) (ii) Quebec, Canada (iii) March 2013	(i) Urine (ii) No enzymatic de-conjugation. Dansyl chloride derivatization and LLE for aglycone BPA. SPE for BPAG, BPAS and BPADS. (iii) HPLC-MS/MS (iv) NA	(i) 0.012 ng/mL (ii) 0.039 ng/mL (iii) Percent above LOD: 41.3% (iv) Range: <LOD (0.012 μg BPA eq/L)-0.215 μg BPA eq/L	(i) 0.097 ng/mL (ii) 0.32 ng/mL (iii) Percent above LOD: 95.7% (iv) Range: <LOD(0.11 μg BPA eq/L)-20.6 μg BPA eq/L	(i) BPAS: 0.011 ng/mL; BPADS: 0.38 ng/mL (ii) BPAS: 0.037 ng/mL; BPADS: 1.30 ng/mL (iii) Percent above LOD: BPAS: 28.3%; BPADS: 0%. 95.7% (iv) Range: BPAS: <LOD(0.03 μg BPA eq/L)-0.192 μg BPA eq/L; BPADS: NA	[86]
8	(i) n = 1150 (cases with post-menopausal breast cancer = 575, controls = 575) (ii) Warsaw and Lodz, Poland (iii) January 2000-January 2003	(i) Urine (ii) No enzymatic de-conjugation. No solvent extraction. Dansyl chloride derivatization. (iii) HPLC-MS/MS (iv) [56, 84]	(i) NA (ii) NA (iii) Percent above LOD: 3% (iv) Geometric Mean:: NA	(i) NA (ii) NA (iii) Percent above LOD: 97.2% (iv) Geometric Mean:: Cases with post-menopausal breast cancer: 4.11 ng/mg; Controls: 3.92 ng/mg.	NA	[93]
9	(i) n = 5 (unspiked samples from healthy donors) (ii) Four laboratories in the United States participated in a Round Robin experiment. The labs were located in San Francisco (CA), Chicago (IL), Albany (NY), and Columbia (CO). (iii) NA	(i) Serum (ii) No enzymatic de-conjugation. Lab 1: protein precipitation and LLE; and Lab 2–4: SPE. (iii) HPLC-MS/MS (iv) [82, 91, 130]	(i) Lab 1: 0.02 ng/mL, Lab 2: 0.003 ng/mL, Lab 3: 0.05 ng/mL, and Lab 4: 0.04 ng/mL. (ii) LLOQ:: Lab 1: 0.10 ng/mL, Lab 2: 0.01 ng/mL, Lab 3: 0.10 ng/mL, and Lab 4: 0.13 ng/mL. (iii) Percent above LOD: 100% in all the four labs.	(i) Lab 1: 0.002 ng/mL, Lab 2: 0.02 ng/mL, Lab 3: 0.05 ng/mL, and Lab 4: 0.02 ng/mL. (ii) LLOQ:: Lab 1: 0.01 ng/mL, Lab 2: 0.05 ng/mL, Lab 3: 0.10 ng/mL, and Lab 4: 0.06 ng/mL. (iii) Percent above LOD: 100% in all the four labs.	NA	[90]

Study #	**Study** **(i) Size** **(ii) Location** **(iii) Year**	**Analysis** **(i) Matrix** **(ii) Extraction** **(iii) Instrumentation** **(iv) References of sample analysis relevance cited in the study**	**BPA-aglycone** **(i) LOD** **(ii) LOQ** **(iii) Detection frequency** **(iv) Concentration**	**BPAG** **(i) LOD** **(ii) LOQ** **(iii) Detection frequency** **(iv) Concentration**	**BPAS** **(i) LOD** **(ii) LOQ** **(iii) Detection frequency** **(iv) Concentration**	**Study Reference (chronological)**
			(iv) Range: Mostly below 0.5 ng/mL and few between 0.5 and 1.0 ng/mL.	(iv) Range: Mostly below 0.5 ng/mL and few between 0.5 and 1.0 ng/mL. Except for one sample with a mean 18.9 ng/mL for the data from four labs.		
10	(i) n = 1890 (ii) 10 cities in Canada (iii) 2008–2011	(i) Urine (ii) No enzymatic de-conjugation. LLE for aglycone BPA and SPE for BPAS, BPASD, and BPAG. (iii) HPLC-MS/MS (iv) [86]	(i) 0.012 μg equivalents/L (ii) NA (iii) Percent above LOD: 43.2% (iv) Range: <LOD-2.82 μg equivalents/L	(i) 0.11 μg equivalents/L (ii) NA (iii) Percent above LOD: 94.8% (iv) Range: <LOD-136.15 μg equivalents/L	(i) BPAS: 0.03 μg equivalents/L; BPADS: 0.47 μg equivalents/L (ii) BPAS: NA; BPADS: NA (iii) Percent above LOD: BPAS: 23.4%; BPADS: 0%. (iv) Range: BPAS: <LOD-1.79 μg equivalents/L; BPADS: <LOD-0.36 μg equivalents/L	[94]
11	(i) n = 78 (ii) Baltimore, United States of America (iii) December 2012-August 2013	(i) Urine (ii) No enzymatic de-conjugation. No solvent extraction. Dansyl chloride derivatization. (iii) HPLC-MS/MS (iv) [56]	(i) NA (ii) 0.1 ng/mL (iii) Percent above LOQ:: 3–6 days: 0%; 7–27 days: 0%. (iv) Median:: 3–6 days: <LOQ; 7–27 days: <LOQ.	(i) NA (ii) 0.1 ng/mL (iii) Percent above LOQ:: 3–6 days: 77%; 7–27 days: 64%. (iv) Median:: 3–6 days age: 0.49 ng/mL; 7–27 days age: 0.16. ng/mL.	NA	[83]
12	(i) n = 110 (ii) Chicago, United States of America (iii) NA	(i) Urine (ii) No enzymatic de-conjugation. SPE with mixed-mode reversed phase/ strong-anion exchange sorbent (iii) UHPLC-MS/MS (iv) NA	NA	(i) 1.0 ng/mL (ii) 5.0 ng/mL (iii) Percent above LLOD: 61% (iv) Mean: 58 ng/mL; Range: 6–236 ng/mL	NA	[87]