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QUANTITATIVE DETERMINATION OF BISPHENOL A FROM HUMAN SALIVA USING BULK DERIVATIZATION AND TRAP-AND-ELUTE LIQUID CHROMATOGRAPHY COUPLED TO ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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Abstract—Endocrine disruptors cause adverse health effects as a result of their ability to shift the hormonal balance that is essential to the body. Bisphenol A (BPA) is an endocrine disruptor that has garnered much attention because of its presence in many consumer materials, which generates a significant risk for exposure. A method is presented for rapid detection of oral exposure to BPA directly from human saliva. Saliva was chosen because it serves as a noninvasive sampling route to detect BPA exposure; however, it is one of many complex biological matrices that have traditionally posed problems in quantitative analysis. Such analyses usually require extensive sample preparation to reduce interferences contributed by the sample matrix. Three validated methods are presented here that feature a streamlined sample-preparation strategy (bulk derivatization) prior to accurate and sensitive analysis by trap-and-elute liquid chromatography coupled to electrospray ionization mass spectrometry. Validated methods include standard addition calibration with variable injection volumes and multiple injection loading, as well as with incorporation of an internal standard. Reported limits of detection reached as low as 49.0 pg/ml (2.9 pg loaded on-column; equivalent to parts per trillion in saliva) among the presented methods with good accuracy and precision throughout. A proof-of-concept study is demonstrated to show that the final validated method has potential application to specific studies for trace-level BPA detection from real samples. Environ. Toxicol. Chem. 2011;30:1243–1251.

Keywords—Bisphenol A Bulk derivatization Human saliva Restricted-access media

INTRODUCTION

Endocrine disruptors are a particular class of compounds that has garnered much attention from the scientific community. The cause for such alarm is their ability to cause adverse health effects in the human body, such as feminization of the male [1–3], abnormal sex organ growth [4–6], and cancer [7–9]. Compounding the problem is their nearly ubiquitous presence in all aspects of the environment, which has become increasingly evident with advancements in analytical instrument sensitivity. Numerous reports have cited the presence of endocrine disruptors in a variety of different sources ranging from natural drinking water to food products to materials used in the manufacture of consumer goods [10–12]. Endocrine disruptors specifically target the hormone balance that is maintained by the endocrine system, which comprises an intricate web of hormone receptors and ligands that regulates metabolism and bodily function. The ability of endocrine disruptors to affect this system lies in their structural similarities to endogenous hormones. These similarities confer the ability to bind key interaction sites on target membrane receptors triggering unintended signal transduction through the cell [13–15]. Even more distressing are reports indicating that endocrine disruptors impart epigenetic effects, in which negative effects are proliferated from the affected parent through to offspring generations [16].

Given the numerous different types of hormones, the most prevalent and well-studied types of endocrine disruptors are those that mimic the biological function of estrogen. Estrogen is a steroid hormone that plays a critical role in the development and maturation of male and female sexes. Studies have been reported implicating these estrogen mimics as the culprit responsible for causing many disastrous medical anomalies over many decades [17,18]. Since the early 1970s, large numbers of compounds have been identified as having estrogenic properties and, as a result, are now tightly regulated. A good example is dichlorodiphenyltrichloroethane (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane; DDT), which was a commonly used pesticide from the 1930s [17]. Another is diethylstilbestrol (4,4′-(3*E*)-hex-3-ene-3,4-diyldiphenol; DES), which was used as a drug to prevent miscarriages and as a constituent in daily household products such as cosmetics and shampoos [17]. Ultimately, DES was found to be the cause of multiple reproductive abnormalities in both male and female infants and was banned from use [16,19,20].

Recently, the use of bisphenol A (BPA) in consumer products has sparked debate and deliberation, especially over the actual repercussions of exposure to BPA and whether the establishment of regulations is justified. BPA is present in the epoxy resins used in the sealants of canned foods [21–24]. It is used as the monomer unit in the synthesis of polycarbonate polymers [25], a material that is used in many manufactured products. In addition, it is also used as a plasticizer in certain polyvinyl chloride and structural polymers [26]. Because of these ubiquitous uses, a significant chance exists of oral

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exposure to BPA by organisms [10,27–29]. Despite a wide body of research that reports the deleterious bioactivity of the compound, the actual effect of BPA on the human body is still an area of significant debate. Binding studies show that BPA has a thousandfold lower binding affinity to the estrogen receptor than endogenous estrogen [30,31]. Many argue that, even though BPA is prevalent in the environment, its effects are negligible in the trace quantities that are generally detected in the environment. On the other hand, biological studies in test animal subjects have already shown adverse side effects of BPA exposure, even at very low levels [32]. Another aspect of the debate deals with the effects on the human body of long-term exposure. Regardless of the outcome of these debates, a growing need for fast, accurate methods to detect and quantify trace amounts of this endocrine disruptor is apparent. Only then can data be collected that will allow a fair assessment of BPA exposure and its associated effects for potential establishment of regulations of this compound.

To probe for oral exposure of BPA, a suitable and representative biological fluid from the body must be analyzed. Human saliva fits many of the criteria for this quantitative analysis for a number of reasons. Oral exposure of BPA passes through the mouth before being transferred into the rest of the body; therefore, direct sampling of the saliva can indicate oral exposure [33,34]. In addition, sampling of human saliva is one of the simplest and least invasive routes for biomonitoring compared with collection of fluids such as blood and urine, among others. However, saliva still provides similar problems as a matrix for quantitative analysis compared with other biological fluids. Traditional methods of preparation involving biological fluids suffer a wide variety of potential interferences and complexities stemming from these matrices. Consequently, time-consuming sample preparation steps (e.g., liquid–liquid extraction, solid-phase extraction, and protein precipitation) are often needed prior to use of these analytical methods to reduce interferences contributed by the sample matrix [34–36]. In addition, these methods can require the use of costly consumables, materials, and chemicals.

Here we report a series of methods for quantitative analysis that incorporate a bulk derivatization strategy to reduce sample preparation time, to improve recovery of analytes in biological fluids, and to enhance sensitivity. Emphasis is placed on the reduction of sample preparation time for the quantitative analysis of BPA from biological fluids and the overall increase in efficiency of analysis given by bulk derivatization compared with previous methods [27,37–39]. The approach is further aided by the use of a trap-and-elute liquid chromatography–mass spectrometry (LC-MS) instrumental configuration, which features restricted-access separation media [40]. The bulk derivatization strategy involves crude saliva being directly subjected to a derivatization reaction with dansyl chloride, as shown in Figure 1. Dansylated BPA is easily protonated under acidic conditions, which, in addition to the added hydrophobicity conferred on the product, enhances sensitivity in positive ionization mode electrospray ionization–mass spectrometry (ESI-MS). Three separate analytical methods (standard addition, standard addition with multiple injection loading [MIL], and standard addition with internal standard and MIL) were developed for the quantitative analysis of BPA from human saliva using these proposed strategies. Each was subjected to rigorous method validation. Applications were performed to extend these validated methods to practical case studies in a real-world scenario. In the present study, canned food products suspected of BPA contamination were ingested, after which saliva samples were immediately collected for analysis by the methods developed.

MATERIALS AND METHODS

Chemicals and materials

Bisphenol A was purchased from Sigma-Aldrich, and the internal standard, d_{16}-bisphenol A, was obtained from Supelco. Dansyl chloride was obtained from Fluka; sodium hydroxide was obtained from EMD Chemicals. Concentrated hydrochloric acid was obtained from Pharmaco–AAPER, and concentrated formic acid was obtained from J.T. Baker. High-performance liquid chromatography-grade acetone was also purchased from J.T. Baker. Liquid chromatography–mass spectrometry-grade water and acetonitrile were supplied by Burdick and Jackson; LC-MS–grade Chromasolv 2-propanol was purchased from Fluka. Blank saliva and application saliva samples were obtained from the authors of this paper. All saliva samples were collected in glass vials, and contact with plastic products was minimized during every step of the procedure for all samples and chemicals.

Optimized sample preparation method

The optimized method was finalized and is outlined in Figure 2. The total sample preparation was devised to achieve a final volume of 1 ml after completion of the procedure. Initial volumes of the saliva samples (720 μl) were adjusted to compensate for additional BPA spike and internal standard spike volumes, which were both 50 μl each, dissolved in acetonitrile. After the standards were added, 40 μl NaOH (1 M) was added to the vial, followed by 150 μl dansyl chloride (8 mg/ml in acetone). Sufficient reaction time was allowed, which can be qualitatively monitored by observing the color changes of the reaction solution (approximately 3 min). After the reaction reached a transparent, pale yellow color, the solution was incubated in an oven at 60°C for 15 min to ensure complete reaction. The sample was then removed from heat, briefly

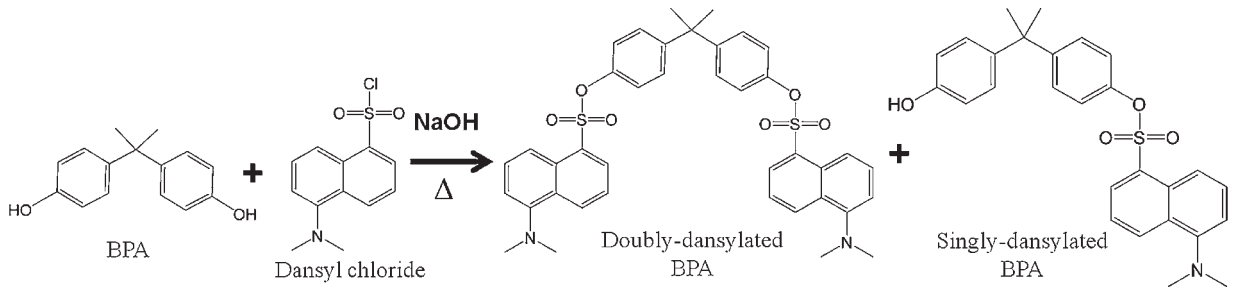

Fig. 1. Reaction diagram of derivatization reaction of bisphenol A with dansyl chloride. NaOH = sodium hydroxide.

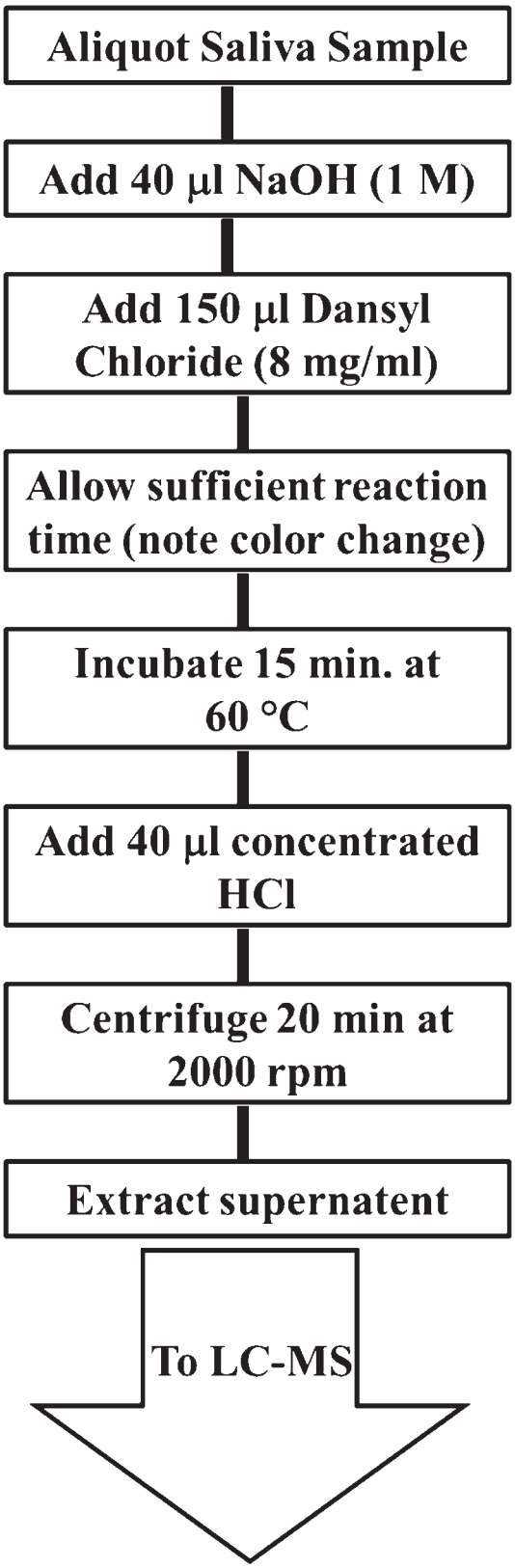

Fig. 2. Flow diagram of bulk derivatization sample-preparation protocol for human saliva. NaOH = sodium hydroxide; HCl = hydrogen chloride; LC-MS = liquid chromatography–mass spectrometry.

vortexed, treated by addition of 40 µl concentrated hydrogen chloride (HCl), and centrifuged for 20 min at 2,000 rpm. The supernatant was transferred into a conical-bottomed autosampler vial for injection onto the HPLC system. Completion of the entire sample preparation protocol is achievable in less than 1 h, and multiple samples can be processed in parallel.

Chromatographic method

High-performance liquid chromatography analysis was performed with a Shimadzu Prominence LC system comprising four LC-20AD pumps, a SIL-20A HT autosampler with a 40-µl sample loop, and a CTO-20AC column oven with a six-port valve-switching system. An instrumental diagram of the LC system is shown in Figure 3. The trap column used in the analyses was the Shimadzu Shimpack MAYI-ODS (4.6 mm i.d. × 10 mm L, 3 µm d_p); a Varian Pursuit XRs C18 column (2.0 mm i.d. × 100 mm L, 3 µm d_p) was used as the analytical column. With reference to Figure 3, the mobile-phase compositions used in the binary pump consisted of water/acetonitrile (95:5) + 0.1% formic acid for mobile phase A and acetonitrile/isopropyl alcohol (90:10) + 0.1% formic acid for mobile phase B. At the start of the chromatographic method, the binary pump mixes the mobile phase at 20% B to equilibrate the column. The loading mobile phase used for injection of the saliva sample through the autosampler was an isocratic composition of water/acetonitrile (80:20). The binary pump was operated at a constant flow rate of 0.1 ml/min. The secondary pump system used for sample loading was operated at 0.6 ml/min to inject the saliva sample into the trap column and wash out unretained biomolecules from the trap. The flow rate was then reduced to 0.05 ml/min after the system configuration was switched from configuration A (trap loading phase) to configuration B (analysis phase). In the multiple injection–loading scheme, the samples were successively injected at intervals of 1 min each. After 5 min of washing, the six-port valve was switched to configuration B, and the retained analytes were eluted from the trap column onto the analytical column for separation and into the ESI-MS instrument for analysis. For each method, to separate components on the analytical column, a gradient program of 20 to 95% organic content in 12 min was carried out by the binary pump. The pump was then held at isocratic flow with 95% organic content for 20 min. To wash the trap column, the configuration was switched back from the analysis phase (B) to the loading phase (A) to wash the trap column with a mixture of water/isopropyl alcohol (10:90) for 10 min before reverting back to the loading mobile-phase composition of water and acetonitrile (80:20) for equilibration. Sufficient pre-equilibration of the entire chromatographic system (35 min) is allowed between each analysis to ensure precision.

Mass spectrometry

A Shimadzu ion trap—time of flight electrospray ionization—mass spectrometry (IT-TOF ESI-MS) system was used for the mass analysis in this method. Electrospray ionization was performed in the positive-ionization mode with a spray capillary voltage of 4.5 kV, and the detector voltage was 1.6 kV. Mass analysis was operated in the automatic-mode setting with a scan range of 150 to 600 (*m/z*) and an ion accumulation time of 56 m/sec. The curved desolvation line (CDL) and heat block were both heated to 200°C. The time-of-flight mass analyzer was maintained at 40°C. Nebulizing gas was introduced at 1.5 L/min, and the drying gas was set to 75 kPa. Data analysis was performed using LCMSolutions (version 3.5) software (Shimadzu). The instrument was regularly calibrated to <5 ppm error in mass accuracy with an external standard of sodium trifluoroacetic acid solution (sodium hydrate 0.1 g/L, trifluoroacetic acid 0.25 ml/L).

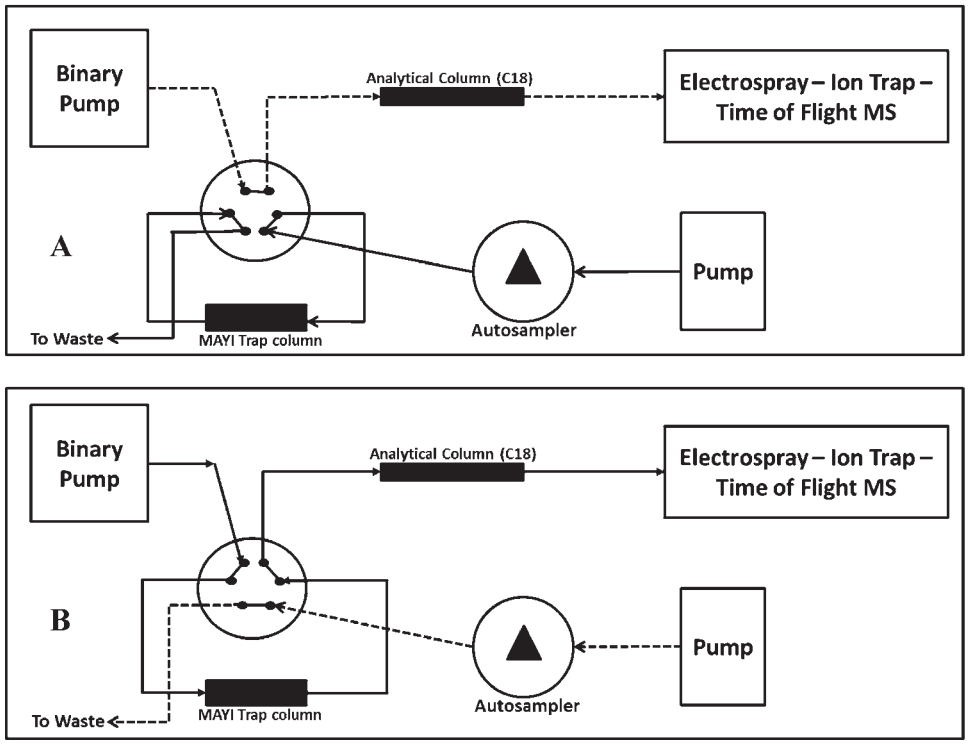

Fig. 3. Schematic diagram of trap-and-elute liquid chromatography system in trap column–loading phase (**A**) and analysis phase (**B**).

RESULTS AND DISCUSSION

Optimization of bulk derivatization protocol

Initial studies yielded promising results and indicated the viability of the proposed method for biomonitoring applications using a 5-μl injection volume. In these experiments, the free, unmetabolized form of BPA was monitored, as opposed to any possible enzymatically modified forms. We assume that the time frame between ingestion of BPA and saliva sampling was short enough that the BPA had not been subjected to enzymatic conversion to the glucuronidated form to a significant degree. In addition, the BPA should be in the free form for derivatization by dansyl chloride. The chromatographic peak for dansylated BPA was assigned using the extracted ion chromatogram from its exact mass. One major problem that was encountered was the division of the derivatized BPA between singly dansylated and doubly dansylated forms, as shown Figure 4A. Splitting of analyte signal between two forms generally leads to a loss of sensitivity. The cause for the two different forms of BPA could be attributed to an insufficient amount of dansyl chloride available for bulk derivatization. However, adding a large excess of dansyl chloride leads to excessive dilution and loss of sensitivity, because a large aliquot of dansyl chloride must be added into the sample mixture. The chosen concentration of 8 mg/ml dissolved in acetone is a concentration that is close to its solubility limit, so increasing the concentration of the dansyl chloride was not a viable option. Theoretically, a certain threshold of dansyl chloride, upon being reached, would provide for complete derivatization to convert all BPA to the doubly derivatized form, even in the presence of other matrix components. Optimization of the bulk derivatization reaction was performed in order to determine a set of reaction conditions in which the derivatization was most efficient. The efficiency of the reaction was evaluated by monitoring the signal response of both derivatized forms of BPA against varying mass quantities of dansyl chloride added. Figure 5 shows the findings from an investigation of the effects of the dansyl chloride (8 mg/ml in acetone, a concentration close to its solubility limit) mass quantity on the BPA signal response (a sum of both singly and doubly derivatized forms). Twenty-microliter injections of a spiked sample of 10 μM BPA in blank saliva were used in this experiment, which is a concentration that is well above the expected levels of BPA exposure in saliva. Saliva was collected and combined from multiple sources into a single pool to accommodate the variability of different factors (pH, amylase content) in saliva from each source [41]. From the plot, the optimal volume of dansyl chloride was determined to be 1.2 mg (150 μl of 8 mg/ml in acetone) for bulk derivatization.

To enhance recovery, the effects of postderivatization additives were also investigated. It was found that the addition of concentrated HCl to the preparation dramatically enhanced the analyte signal. It is speculated that the HCl accentuates signal response by both protonating the dansylated BPA and cleaving the solid salivary protein contained in the biological matrix, thus disrupting hydrophobic–hydrophobic interactions between the dansylated BPA and the solid proteins in the saliva. By releasing the BPA from the solid matter, the BPA is not removed from the supernatant during pellet formation via centrifugation. Recovery studies using a sample size of 1 ml showed that 91.9% (±0.2% RSD) of the BPA was recovered from the sample with this optimized procedure. Figure 4C shows a representative chromatogram from the final optimized method, in which the BPA has been completely converted into the doubly derivatized form and with the addition of the HCl to the sample preparation. A 10-fold increase in signal intensity was observed from the optimized sample preparation (compare Fig. 4C and B).

The method was subjected to rigorous method validation in accordance with the U.S. Food and Drug Administration (FDA) bioanalytical method validation guidelines [42]. The method

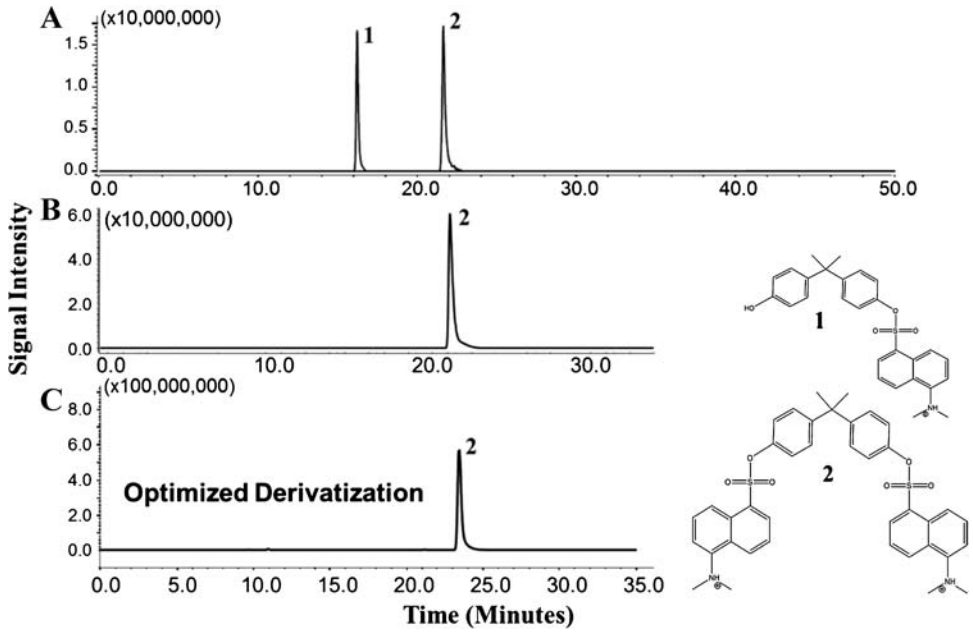

Fig. 4. Representative chromatograms demonstrating division of different forms of 10 μM bisphenol A (BPA) with an addition of 20 μl dansyl chloride (8 mg/ml in acetone; **A**) and 150 μl chloride (8 mg/ml in acetone; **B**) with both singly (1) and doubly (2) derivatized forms. (**C**) Effects of the incorporation of a 40-μl HCl additive is also shown in comparison with the division of BPA signal.

limit of detection (LOD) was determined by taking seven replicates at a concentration point near the limit of quantification along with generation of a full calibration curve containing seven points. The LOD was then calculated from the equation, $\mathrm{LOD} = t(s)/\mathrm{m}$, where t is the $t-$table value for 7 degrees of freedom (3.1), s is the standard deviation of the seven replicates, and m is the slope of the calibration curve generated from method validation. The LOD is reported as both a concentration (e.g., picograms per milliliter) and a mass loaded on column (e.g., picograms). The accuracy and precision of the method were determined by sampling three different concentration points (0.01, 0.05, and 0.08 μM) in replicates of five. These particular concentrations were chosen to represent a low, medium, and high region of the calibration curve. Linearity was evaluated based on the correlation coefficient of the best-fit line of the empirical data points. The standards and samples used in method validation were spiked into blank pooled saliva, performed on a single day by a single researcher. Water blanks were injected between all runs; the use of blank-corrected signals for calibration and LOD determination was not necessary, because of the negligible signal for BPA in the blank samples. The results of the method validation are summarized in

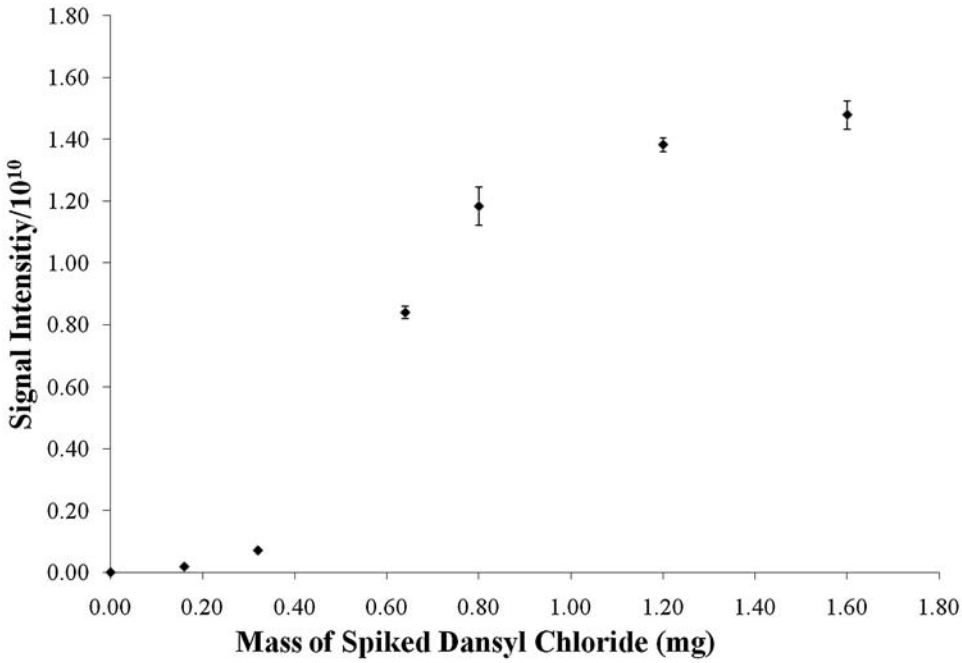

Fig. 5. Optimization of aliquot volume for bulk derivatization using an 8 mg/ml dansyl chloride solution, a concentration that was near to the solubility limit of dansyl chloride in acetone. The intensity of the total bisphenol A (BPA) signal (both singly and doubly derivatized) was plotted versus mass quantity of dansyl chloride used in the derivatization reaction.

Table 1. Method validation parameters for single-injection method (5 μl)

Limit of detection (ng/ml)	3.25 (16 pg loaded on column)	
Reported concentration (μM)	0.500	8.00
Calculated concentration (μM)	0.60 ± 0.04	7.9 ± 0.1
Accuracy error (%)	20.2	0.24
Coefficient of variance (%)	6.3	1.7

Table 1. The correlation coefficient (R^2) of the calibration curve was 0.996. The LOD was determined to be 3.25 ng/ml (16 pg on column). Accuracy and precision evaluated for quality control standards were judged to be satisfactory.

Multiple injection–loading strategy

Extension of the validated method to application studies revealed a significant problem. The signals generated from several application studies were below the reported LOD (data not shown). New strategies were thus explored to enhance the sensitivity or detectability of BPA. The use of a multiple injection–loading (MIL) scheme was explored, in which the saliva sample was injected onto the MAYI trap column multiple times, essentially converting the MAYI column into a preconcentration device. The negative aspect to using MIL is the band broadening associated with repeated injections of a large sample slug. An investigation to find the optimal loading scheme was conducted, the results of which are shown in Figure 6, which shows the plots of different injection schemes (10, 20, and 40 μl) along with their peak asymmetry factors. The optimal MIL scheme was determined to be three injections of 20 μl. This setting offered a good compromise between increased signal response (sample loading) and reduced peak asymmetry. A second method validation was performed on the multiple-injections method, the data for which are summarized in Table 2. The correlation coefficient (R^2) of the calibration curve was 0.996. It is important to note that, although the LOD expressed in terms of picograms per milliliter decreased, because of the higher signal intensities seen when using the MIL scheme, the LOD of the method when considered in terms of mass loaded on column was not significantly affected by the MIL scheme. The MIL scheme was simply a means by which more saliva sample could be loaded onto the system and preconcentrated by the trap column. It does not alter the lowest amount of analyte that the analytical system can see. Nevertheless, acceptable accuracy and precision were again demonstrated from method validation.

A proof-of-principle application is presented to demonstrate the utility of this validated method in the detection of BPA exposure from human saliva. It is well known that a primary source of widespread BPA exposure is canned foods [21–23]. A source of BPA in these canned products comes from the epoxy resin lining and sealants used in the preservation and packaging process. A series of different canned goods imported from different countries, purchased from local supermarkets, was investigated. Each food product was first ingested, and a saliva sample was collected immediately afterward. To ensure that BPA contamination was solely from the canned product, a blank saliva sample was also collected prior to ingestion of any food substance. From these canned food analysis studies, the product with the highest BPA contamination was determined to be a can of green salsa chili from Mexico (data not shown). Concentrations were calculated by generation of a standard addition calibration curve and extrapolation of the zero point concentrations from the curve. Despite the larger BPA signal relative to the single 5 μl injection method, the calculated concentration was determined to still be below the reported limit of detection, even though a seemingly reasonably sized signal was recorded at the retention time of interest.

Incorporation of internal standard

A method that incorporates an internal standard, d_{16}-bisphenol A, was explored as a solution to the problem. Addition of an internal standard to the analysis allowed normalization of the analyte signal to the internal standard signal, which significantly enhanced the precision (lower standard deviation of each concentration point) of the method. According to the calculations of LOD set by the FDA bioanalytical method validation guidelines, precision is directly proportional to the LOD, so accentuated precision inherently lowers the LOD. The MIL method, now with internal standard, was once again subjected to method validation, the results of which are reported in Table 3. The correlation coefficient (R^2) of the calibration curve was 0.998. With this method, a significantly lower limit of detection of 49.0 pg/ml (2.9 pg on column) was calculated; very good accuracy and precision were also demonstrated.

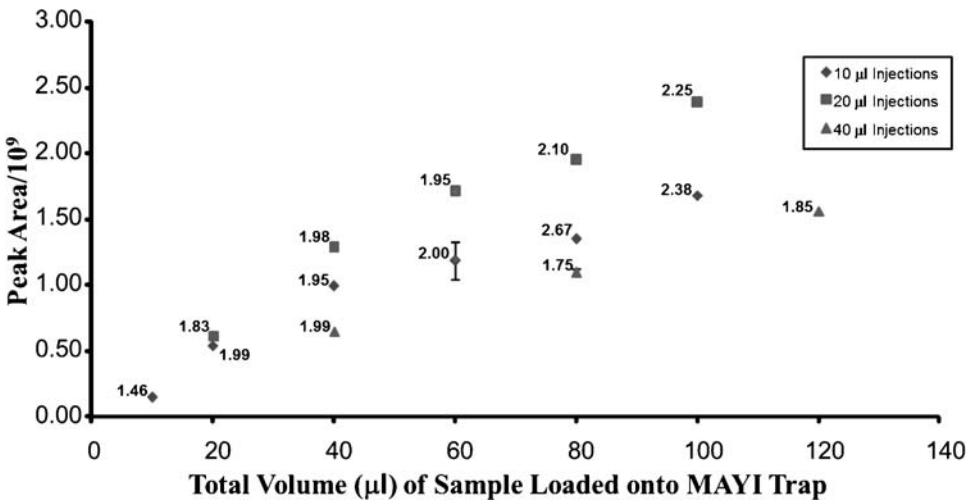

Fig. 6. Optimization of multiple loading injection with multiples of different injection volumes (10, 20, or 40 μl). Area of the doubly dansylated bisphenol A peak was plotted against total injection volume. Peak asymmetry factors are indicated above each injection point.

Table 2. Method validation of multiple loading injection method ($3 \times 20\,\mu l$)

Limit of detection (pg/ml)	199 (12 pg loaded on column)		
Reported concentration (μM)	0.05	0.10	0.50
Calculated concentration (μM)	0.052 ± 0.002	0.096 ± 0.004	0.497 ± 0.002
Accuracy error (%)	3.4	−3.8	−0.5
Coefficient of variance (%)	3.6	3.6	3.5

Table 3. Method validation of multiple loading injection method ($3 \times 20\,\mu l$) with incorporation of an internal standard

Limit of detection (pg/ml)	49.0 (2.9 pg loaded on column)		
Reported concentration (μM)	0.01	0.05	0.08
Calculated concentration (μM)	0.0091 ± 0.0001	0.051 ± 0.001	0.082 ± 0.001
Accuracy error (%)	−8.6	2.8	3.5
Coefficient of variance (%)	0.79	1.9	1.6

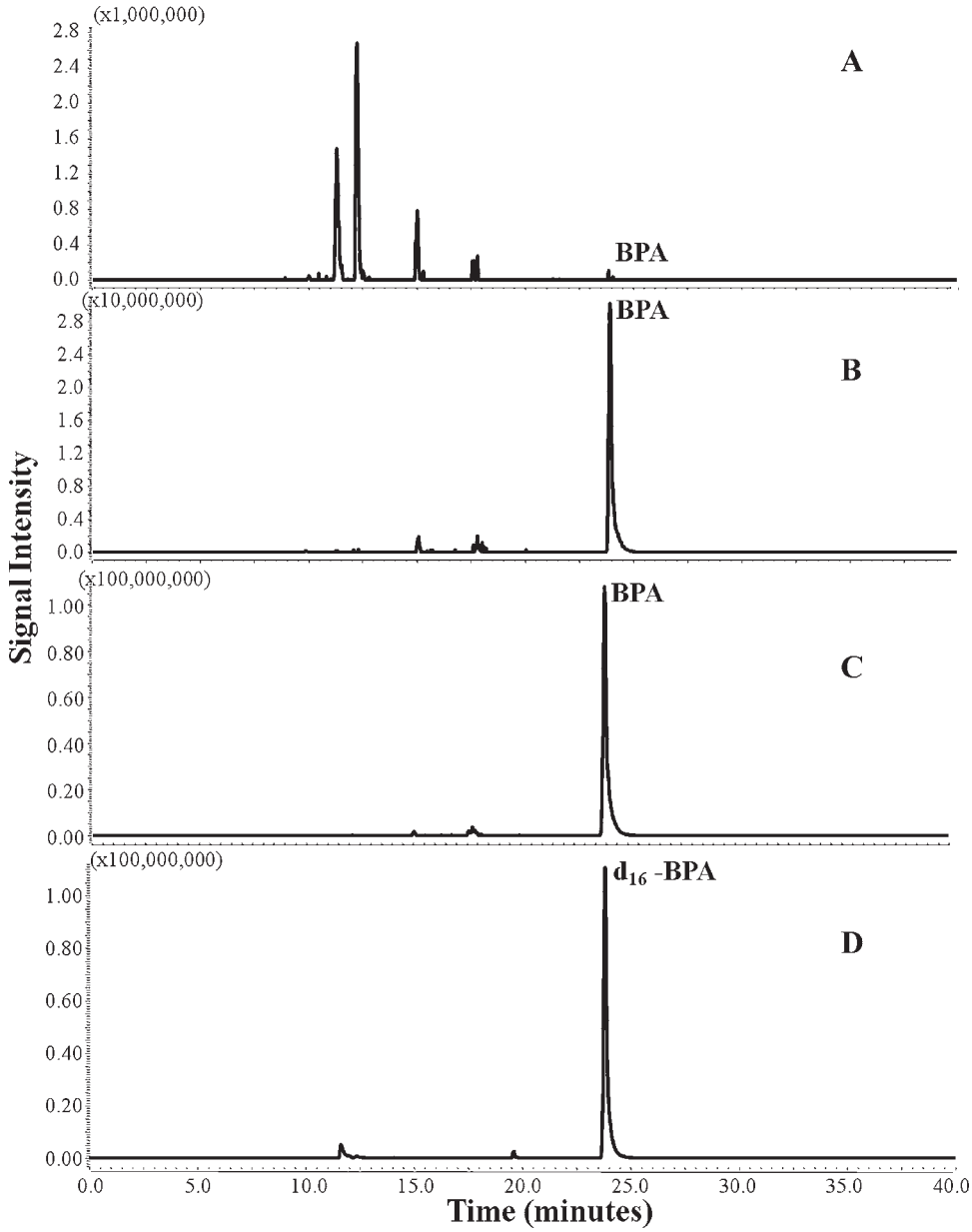

Fig. 7. Extracted ion chromatograms of doubly dansylated bisphenol A (BPA) (348.1107 *m/z*) in saliva sample obtained from exposure to canned hot and sour soup from the Republic of China (Taiwan) using multiple injection loading with incorporation of an internal standard. Figure shows the saliva blank sample before ingestion of food (**A**), the saliva zero point of the standard addition curve (**B**), the 0.04 μM BPA spike from the standard addition curve (**C**), and the representative internal standard spike of 0.05 μM d_{16}-BPA with an exact mass of 355.1557 *m/z* (**D**).

Another array of different canned food products was tested to screen for BPA contamination in human saliva after ingestion. Interestingly, one canned product, hot and sour soup from the Republic of China (Taiwan), revealed a high content of BPA contamination. The representative chromatograms are shown in Figure 7. The final calculation, obtained through standard addition, for the amount of BPA in saliva, presumably from oral contamination by ingestion of the hot and sour soup, was 2.39 ng/ml (or 137 pg on column). The background BPA levels in the pre-exposure samples were negligible. The entire analysis was replicated with a separate can of the same food product purchased from another local supermarket to ensure repeatability of these results. Instead of another standard addition experiment, a direct calculation of the BPA amount was determined directly by interpolation of the signal intensity of the canned food saliva from the equation obtained from a linear calibration curve of standards in blank saliva. The final BPA concentration was calculated to be 2.58 ng/ml (or 155 pg on column), which was in agreement with the first trial. This provides a proof-of-principle experiment to show that these methods are applicable for detection of BPA exposure sampling of human saliva.

CONCLUSIONS

Widespread BPA contamination from a variety of sources has been an issue of great concern, which increases the need for rapid, high-throughput, and sensitive biomonitoring methods. With such analytical methods, proper steps can be taken to evaluate toxicity and establish appropriate regulations on a compound that is currently receiving a great deal of attention. Three separate, viable methods are presented to serve as tools in the study to detect trace quantities of BPA from a biological fluid such as human saliva. Part-per-trillion levels of oral exposure can be detected directly from sampling of saliva after ingestion of BPA. The extensive work presented here represents a new approach to sample preparation of biological fluids for analysis in the form of bulk derivatization coupled to restricted access media as a means to expedite the entire preparation protocol compared with conventional methods. Such methods can be expanded to a wider range of analytes and metabolites in addition to a wider range of biological matrices, studies for which are currently being undertaken in our laboratory.

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