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Development and validation of an ultra performance liquid chromatography-tandem mass spectrometry method for twelve bisphenol compounds in animal feed

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ABSTRACT

Bisphenol compounds (BPs) are a group of environmental contaminants with endocrine-disrupting effects both for humans and animals. The present work developed a sensitive analytical method for the detection of multiple BPs in the animal feed based on ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with post-column ammonium hydroxide (NH_4OH) infusion. A modified QuEChERS method was incorporated into the extraction and purification processes. The limit of detection (LODs) and quantification (LOQs) for the target BPs were in the ranges of 0.02–0.75 μg kg^{-1} and 0.04–0.95 μg kg^{-1}, respectively. Average recoveries were ranged between 82.6% and 112%. The proposed method was successfully applied to determine the concentrations of BPs in 20 actual feed samples, and the preliminary profiles of BPs in products from local feed factories were obtained. Each sample was simultaneously contaminated with at least 2 to 4 BPs, and bisphenol A (BPA) was the dominant analog of BPs found in animal feed.

Keywords: bisphenol; feed; determiation; post-column infusion; UPLC-MS/MS

1. Introduction

Bisphenol A (BPA), one of the most common endocrine-disrupting chemicals (EDCs), is used in large quantities as a monomer in the manufacture of epoxy resins and plastics, such as packaging materials, plastic bags, bottles, food containers, can linings, kitchenware, medical equipment, toys and sealants [1, 2]. BPA has been found to readily leach from plastic products into the surrounding medium, and many studies have confirmed that BPA is ubiquitous in the environment and food chain at present [3, 4]. Serious concerns about the adverse effects of BPA on human health and the ecosystem have led to strict regulations regarding the production and usage of this chemical in food contact materials in the European Union, United States of American, Canada and China [1, 5, 6]. Many BPA analogs are similar in molecular structure and exhibit the same or improved plasticizer properties, and so some of these, such as bisphenol E (BPE), bisphenol F (BPF), bisphenol S (BPS) and bisphenol AF (BPAF), have been used as substitutes for BPA and have been identified in environmental, food and biological samples as well as in consumer goods [7-9]. Unfortunately, recent studies have suggested that these analogs show comparable biological activities to BPA, including the potential for hormonal disruption, toxicity and genotoxicity [10, 11]. Furthermore, some compounds, such as BPS and BPAF, may have higher estrogenic activity than BPA [12, 13]. Even so, for most of the BPA analogs, the understanding of the environmental, biological, toxicological and health impacts remains minimal.

Over the past several decades, there have been numerous studies of human dietary exposure to BPA and its analogs based on migration from food packaging or storage containers, and various potential health risks have been reported [14-18]. Although BPA has only moderate potential for bioaccumulation as a result of its rapid metabolism into glucuronides and sulfate conjugates [19], several more highly hydrophobic analogs, such as bisphenol C (BPC), bisphenol Z (BPZ) and BPAF, have been shown to bioaccumulate in the aquatic food chain [20]. Besides, one human study determined that the frequency of detection and concentrations of BPA in adipose tissues was higher than in other organs (such as the liver and brain), indicating that BPA could accumulate in such tissues [21]. One survey conducted in China showed that non-packaged fresh meats (including pork, chicken, beef and mutton) were also contaminated by BPA and BPS [16], suggested that the exposure of animals to these compounds in the general environment and/or in feed could lead to accumulation in animal products and contribute to human dietary exposure. However, the contamination of animal feed by BPA and its analogs (hereafter BPs) and the bio-accumulation of these chemicals have rarely been investigated, partly due to the lack of suitable methods for the analysis of BPs in complex feed samples. Therefore, it would be beneficial to develop a sensitive, high throughput method for the analysis of BPs in animal feed. Unfortunately, the simultaneous identification and quantification of multiple BPs are complicated because of the significant variation in the lipophilic and acid-base properties (see Table 1), as well as the likely presence of low concentrations of these compounds in test specimens as background contamination. To date, the primary method for the analysis of trace levels of BPs in food and biological samples has been liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) because this technique is selective, reliable and sensitive [5]. Other studies have used gas chromatography (GC) in conjunction with MS or MS/MS [12, 22]. Even so, it is still very challenging to analyze trace level BPs using LC-MS/MS in complex matrixes such as animal feed. Besides, BPs are almost always analyzed while operating in the negative ionization mode (ESI^-), which tends to produce relatively weak responses and so greatly reduces sensitivity [23]. Incorporating alkaline additives such as ammonium hydroxide (NH_4OH) in the mobile phase can enhance ionization efficiency, and thus improve the detection responses for most BPs [5], but can also interfere with the peak shape and retention behavior of certain analog BPS [24]. The chemical derivatization of BPs followed by LC-MS/MS operating in the positive mode (ESI^+) is another approach that can be used to increase sensitivity, but this technique is more complex and leads to various side-effects. Samples with complex matrices can not only interact with the BPs to inhibit extraction, but can also generate numerous interferences that reduce analytical performance. Thus, the analysis of trace level BPs in complex matrices requires suitable sample preparation. However, the traditional sample preparation methods for BPs, including liquid-liquid extraction (LLE) [5] and solid-phase extraction (SPE) [24, 25], are often time-consuming and laborious. Molecular imprinting (MI) is sensitive, but is only applicable to a few BPs (such as BPA) and multiple BPs cannot be tested simultaneously [26, 27]. In recent years, a liquid-liquid micro-extraction (LLME) method has been developed that is both simple to operate and provides high extraction efficiency, although this process is most suitable for use with simple matrices such as water [28] and urine samples [29]. Because of its simplicity and wide range of applicability, the QuEChERS ("quick, effective, cheap, rugged and safe") approach coupled with LC-MS/MS or GC-MS/MS has also been used to analyze BPs in milk [30], urine [22] and baby food [12] samples. Even so, in these prior studies, only a maximum of seven BPs was detected simultaneously. Moreover, to the best of our knowledge, the analysis of multiple BPs in animal feed has not yet been reported.

The objective of the present work was to develop a sensitive and accurate analytical method for the simultaneous determination of BPA and 11 bisphenol analogs (BPB, BPC, BPE, BPF, bisphenol M (BPM), bisphenol P (BPP), BPS, BPZ, BPAF, bisphenol AP (BPAP) and bisphenol FL (BPFL)) in feed samples intended for different species. In the method developed, samples were prepared using the modified QuEChERS method and analyzed using the ultra performance UPLC-MS/MS in conjunction with isotopically labeled standards. Besides, a novel post-column NH_4OH infusion technique was employed to achieve both high detection sensitivities and good peak shapes for the target BPs. Finally, the proposed method was validated and applied to investigate the BPs occurrence in animal feed products from local feed factories in Beijing, China.

2. Material and methods

2.1. Chemical and reagents

Analytical BPA, BPB, BPC, BPE, BPF, BPM, BPP, BPS, BPZ, BPAF, BPAP and BPFL standards were sourced from Sigma-Aldrich (St. Louis, MO, USA). Isotopically labeled internal standards (ISs; BPA-d_8, BPB-d_8, BPF-d_{10}, BPS-d_8 and BPAF-$^{13}C_{12}$) were obtained from TRC Inc. (Toronto, Canada). The physicochemical properties, chemical structures and purities of the target analytes are summarized in Table 1. Stock solutions of individual analyte were prepared in methanol (MeOH) (100 µg mL^{-1}) after which a mixed working standard solution containing all 12 BPs was prepared with each at a concentration of 5 µg mL^{-1} by combining suitable aliquots of the various stock solutions and diluting the mixture with the appropriate volume of MeOH. An IS mixture containing all five isotopically labeled BPs at 2 µg mL^{-1} each was also prepared in MeOH and stored in a brown glass bottle at -20 °C.

An LC-MS grade NH_4OH solution was acquired from CNW Technologies GmbH (Dusseldorf, Germany), while LC-MS grade MeOH, acetonitrile (MeCN), and formic acid (FA) were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water (resistivity 18.2 MΩ cm) used throughout the experiment was obtained from a Milli-Q water purification system equipped with an EDS-Pak cartridge to remove EDCs (Millipore RiOs-DITM, Bedford, MA, USA). Analytical grade sodium chloride (NaCl) and anhydrous magnesium sulfate ($MgSO_4$) were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Octadecyl bonded silica (C_{18}, 50 µm) and primary secondary amine (PSA, 40-60 µm) were purchased from the Bonna-Agela Technologies Company (Tianjin, China).

2.2. *Instruments*

Analysis of the 12 BPs was performed using a UPLC system (Acquity) coupled with an MS/MS instrument equipped with an infusion pump (XEVO TQ-S, Waters, USA). The Waters Masslynx software package (version 4.1) was used to control the instruments and to acquire and process data. A BEH Shield RP18 column (100 mm × 2.1 mm i.d. and 1.7 µm particle size, Waters, USA) with a guard column (0.5 cm × 2.1 mm, 2.7 µm, Waters, USA) was used. Other laboratory equipment included an RVC 20-18 desktop vacuum centrifugal concentrator (CHRIST, Germany), a 3K15 high-speed refrigerated centrifuge (Sigma, USA), a D37520 high-speed centrifuge (Kendro, USA) and a VX-III multi-tube vortexer (Targin Technology, China).

2.3. Sample preparation

In each trial, a portion of a ground feed sample (2.00 g) was weighed out and transferred into a 30 mL round bottom glass tube with a cap, after which 20 µL of the IS solution (2 µg mL^{-1}) was added such that the final concentration of each IS component in the sample was 20 ng g^{-1}. Following this, 4 mL of water was added and the feed was allowed to soak for 10 min, after which the sample was extracted using 10 mL of a 0.5% (v/v) solution of FA in MeCN in conjunction with agitation at 2000 rpm with a multi-tube vortexer for 10 min. After this step, 1 g of NaCl and 1 g of $MgSO_4$ were added consecutively to the mixture and the tube was manually agitated to prevent the formation of agglomerates, followed by another 1 min of high-speed vortexing and centrifugation for 5 min at 5000 rpm. A 6 mL aliquot of the organic layer was removed and transferred to a 10 mL round bottom glass tube containing 500 mg $MgSO_4$ and 400 mg PSA as clean-up sorbents. The mixture was vigorously shaken for 1 min and centrifuged for 5 min at 5000 rpm, after which 5 mL of the supernatant was transferred into a 10 mL conical-bottom glass tube and evaporated to dryness in a vacuum centrifugal concentrator at 1500 rpm and 60 °C. Finally, the dry residue was dissolved in 1 mL MeOH:water (80:20, v/v) and then passed through a 0.22 µm polyvinylidene fluoride (PVDF) syringe filter (Bonna-Agela, Tianjin, China) before the UPLC-MS/MS analysis.

2.4. UPLC-MS/MS conditions

Each chromatographic separation was carried out using a BEH Shield RP18 column with MeOH:water as the mobile phase. A gradient elution was employed, starting with 60% (by volume) MeOH for 2 min then increasing to 90% MeOH over 4 min and finally increasing to 95% MeOH over a further 2 min, before returning to 60% MeOH over 1 min. This composition was held constant for 1 min before the next injection (for a total run time of 10 min). The column was kept at 40 °C, the mobile phase flow rate was 0.2 mL min^{-1} and the injection volume was 2 μL. A post-column NH_4OH infusion was employed to enhance the targeted compounds' ionization efficiencies and increase detection sensitivities. This was performed by setting the flow-state of the instrument to the Combine mode to add a 0.4% (v/v) NH_4OH solution at an infusion rate of 5 μL min^{-1}.

The mass spectrometer was operated in the ESI$^-$ mode with a capillary voltage of 2.0 kV, source offset of 60 V, desolvation temperature of 500 °C, source temperature of 150 °C, desolvation gas flow of 900 L h^{-1} and cone gas flow of 150 L h^{-1}. Nitrogen (99.999%) was employed as the nebulizer, desolvation and cone gas. Analyte detection was performed in the multiple reaction monitoring (MRM) mode using argon (99.999%) as the collision gas at a flow rate of 0.14 mL min^{-1}. To optimize the tandem mass parameters, a 1 μg mL^{-1} solution of each compound in MeOH was infused into the MS system through a peristaltic pump at a flow rate of 10 μL min^{-1}. The optimized instrument parameters for the target BPs and the IS compounds were generated automatically using the IntelliStart mode of the Masslynx software, and are provided in Table S2. To reduce possible contamination of the ion source, signals were collected only from 1.3 min to 7.2 min in each injection, and all materials eluted outside this time range were discharged to waste.

2.5. *Box-Behnken experimental design*

Both the sample extraction parameters and clean-up sorbents were optimized using a Box–Behnken response surface design process followed by mapping of the mathematical models corresponding to each response. The experimental design and the modeling analysis were performed using the Design Expert statistical software package (version 8.0.6.1; Stat-Ease Inc., Minneapolis, USA).

2.6. Method validation

Thorough method validation was performed for three selected model matrices that were typical of energy, protein and mixed feeds, respectively. Five IS compounds were used as surrogates during these trials to compensate for the loss of analytes during sample preparation and for matrix effects.

Three calibration curves having six concentration levels within the range of 0.2–200 ng mL^{-1} for the analytes and fixed concentrations of the IS compounds (20 ng mL^{-1}) were generated. The calibration standards were in 80% (v/v) solutions of MeOH in water. These curves were constructed based on the ratios of the peak areas of the analytes to those of the IS compounds plotted against the analyte concentration.

Accuracy and precision were expressed by determining values for recoveries and relative standard deviations (RSDs), using data obtained from six replicate analyses of blank feed samples fortified with the mixed standard solution at three concentrations (2, 10 and 100 μg kg^{-1}). The concentrations of the analytes were calculated using matrix-matched calibration curves with corrections based on the IS compounds (present at 20 ng mL^{-1}). The inter-day precision was evaluated over 5 consecutive days using a spiked level of 10 μg kg^{-1}.

The sensitivity of the proposed method was evaluated by calculating the limit of detection (LOD) and limit of quantification (LOQ) values. Because both BPA and BPS were inevitably present in the blank specimens, ten blank samples were used to determine the LODs and LOQs for these two chemicals, based on defining the LOD and LOQ as 3 × SD and 10 × SD added to the mean concentration determined for the blank samples, respectively [31]. In the case of the other BPs, a sample of the most complex matrix (the pig formula feed) spiked at the lowest concentration level

(2 μg kg^{-1}) was employed to evaluate sensitivities. LODs were calculated as the average concentrations of compound producing a signal-to-noise ratio (S/N) of 3 using the less sensitive MS/MS transition (MRM2). This LOD was therefore the level that allowed the unambiguous identification of the analytes [24]. LOQs were estimated as the concentrations giving an S/N of 10 when using the most sensitive MS/MS transition (MRM1), provided that the S/N for the MRM2 transition was at least 3 [24].

3. Results and discussion

3.1. Separation column

Chromatographic conditions can significantly affect sensitivity and accuracy during the analysis of BPs. In the present work, an Acquity UPLC BEH Shield RP_{18} column in which polar groups shielded residual silanol surfaces was chosen, because this column provided high separation efficiency and relatively short analysis times. In contrast to the previous report [11] that the configurational isomers BPM and BPP could not be separated using a standard C_{18} column, this work showed the effective separation of BPM and BPP (Fig. S1 and Fig. 1).

3.2. Mobile phase and post-column NH_4OH infusion

The mobile phase and additive will not only affect the peak shapes but also the sensitivities of the analytes. Prior papers have reported that MeOH:water is a suitable choice for the mobile phase [30, 32], and that an acidic mobile phase greatly suppresses the ionization of BPs and thus reduces the signal [11, 24]. However, the BPs in this study gave relatively weak responses when using a pure MeOH:water mobile phase. Incorporating alkaline additive such as NH_4OH in the mobile phase has been proposed as a simple yet effective approach to enhancing ionization efficiency and improving sensitivity for BPs [23]. In this work, various amounts of NH_4OH solution (0%–0.01%, v/v) were added to the MeOH portion of the mobile phase as a test. The results confirmed that the addition of NH_4OH to the mobile phase significantly increased the peak areas for most of the target compounds, in agreement with the previous reports [5, 23, 33, 34], although some studies have suggested that pure MeOH:water provides the highest responses [24, 30]. Unfortunately, good peak shapes for all BPs were obtained in the current study only when using a pure MeOH:water mobile phase. Even at very low concentrations of NH_4OH, the most acidic compound BPS (with a p*Ka* in the range of 7.64−8.23) exhibited peak broadening, front-stretching, asymmetric tailing and shifts in RT (Fig. S2). For this reason, some authors have resorted to separately analyzing for BPS using a different method involving a pure MeOH:water mobile phase [11, 25], although this is obviously inconvenient. Those studies that simultaneously analyzed both BPS and the other BPs using a pure MeOH:water mobile phase showed low sensitivities [7, 35]. More recently, chemical derivatization followed by LC-MS/MS operating in the positive mode has been proposed as a means of increasing sensitivity while reducing the reliance on mobile phase modifications [24, 31, 36]. Even so, the associated derivatization procedures are time-consuming.

To resolve this problem, the present work employed a post-column NH_4OH infusion method. In this system, the mobile phase (MeOH:water) carries the analyte solution through the column, after which an NH_4OH solution transported via an infusion pump equipped with a micro-syringe is mixed with the effluent from the column before the ion source. This approach integrates the advantages of using a MeOH:water mobile phase to obtain good peak shapes and incorporating NH_4OH as an additive to improve the ionization efficiency and achieve high sensitivity. To permit a convenient evaluation of the effects of varying the NH_4OH level, the relative peak area of each BPs was monitored. The results (Fig. S3) showed that the optimal concentration of the NH_4OH solution for post-column infusion was 0.4% (v/v), which was equivalent to 0.01% (v/v) NH_4OH solution in the mobile phase at the ion source. Fig. 2 presented the peak areas of each compound acquired using the optimized NH_4OH concentration and without NH_4OH. As demonstrated in Fig. 2, other than BPS and BPAF, which showed high responses regardless of the presence of NH_4OH, the peak areas of the PBs were significantly increased by factors of 3.0 for BPM to 8.2 for BPF upon adding the NH_4OH.

3.3. *Optimization of the extraction procedure*

A pig formula feed with a complex matrix (12.5% moisture, 18.7% protein, 3.8% fat, along with other components as shown in Table S3) was used as a model sample. In initial trials, the effects of the pH of the extraction solution were assessed using water to simulate a feed sample, with 0.1%–2% FA in MeCN (that is, an acidic solution), pure MeCN (neutral) and 5% NH_4OH in MeCN (alkaline). The data in Fig. S4 demonstrate that the recovery of the most acidic compound (BPS) was decreased to zero when using the alkaline MeCN extraction, while the recoveries of the other BPs were almost unaffected by the pH value. Overall, for most of the target BPs, the highest recoveries were obtained with the 0.5% FA in MeCN extraction. Hence, this was selected as the extraction solution.

The Box-Behnken design process was used to investigate the critical extraction factors, including the volume of water, the soaking time and the extraction time, to assess the complex interactions between these parameters. A design matrix was generated with these three factors each at three levels that resulted in 17 experiments. The design matrix is shown in Table S4. The ANOVA results for the response surface quadratic model in Table S5 indicate that the data were well fitted by the model. In order to evaluate the different combinations of the parameters investigated conveniently, the average recovery rate of 12 analytes was used as the marker. Fig. S5 showed the corresponding different response plots. The curvature of the response for the water volume was found to be more significant than for the other two variables, meaning that the former variable had the greatest effect on the extraction efficacy. This effect can likely be attributed to the ability of a greater amount of water to promote the release of analytes bound to the matrix [37]. According to the response surfaces results, a 4 mL water volume, 10 min soak time and 10 min extraction time were the optimum parameters for these analytes. Using these optimized conditions, the recoveries of all analytes were close to 100%.

3.4. Optimization of salt amounts

The salting-out effect can be used to induce liquid-liquid phase separation and to improve the transport of analytes from the aqueous phase into an organic solvent [11]. In the QuEChERS method, NaCl and $MgSO_4$ are the most used salts and are typically employed in the ratio of 1:4 [30, 38]. $MgSO_4$ is also a drying agent as it can bind large amounts of water and readily form agglomerates. However, the solid dry feed samples also had strong affinities for water absorption, such that there was only a small volume of extra free water in each extraction solution. Thus, smaller amounts of $MgSO_4$ were required in this work. According to investigations of the various feed matrices, the addition of 1 g NaCl and 1 g $MgSO_4$ was chosen to partition the MeCN phase from the water phase in this work.

3.5. Optimization of the clean-up sorbents amounts

To remove any co-extracted interferences from the extract solutions, the effects of three sorbents ($MgSO_4$, C_{18} and PSA) were examined, using a simplified procedure to optimize the amount of these sorbents. In these trials, a standard matrix solution (Sm) was obtained at the concentration level of 20 ng mL^{-1} for each BP using the mixed standard solution spiked into extract solution of the blank sample. Subsequently, a 6 mL portion of each S_m sample was purified using different amounts of the sorbents for a 1 min time span. After centrifugation, 0.5 mL of the extraction solution was mixed with 0.5 mL water and subsequently analyzed by UPLC-MS/MS. In addition, a solvent standard solution (S_s) was prepared by adding a standard solution to a portion of a MeCN:water mixture (50:50, v/v) such that the final concentration of each BP was 10 ng mL^{-1}. The purification recovery values were used to find the optimal parameters, based on taking the ratio of the S_m peak area to the S_s peak area. The optimal amount of each sorbent was also estimated using a Box-Behnken design. The experimental design matrix and the ANOVA results are provided in Tables S6 and S7, respectively. To evaluate the different combinations of the parameters, the average recovery rates of all 12 analytes were used as indices, and Fig. S6 shows the various response plots. It is evident that the maximum response was obtained with 400 mg of PSA. Regardless of the amounts of $MgSO_4$ or C_{18} included, a lower amount of PSA was associated with lower recovery rates of the analytes, which ranged from over 90% (at 400 mg PSA) to under 55% (at 0 mg PSA). These results confirm that the PSA effectively removed some interferences and reduced the matrix suppression effects. With increases in the amount of C_{18}, the recovery rates initially decreased and then increased slightly, in agreement with prior reports in the literature [24, 26, 33]. This trend likely occurred because the C_{18} adsorbed both interferences and target analytes to various degrees. Concerning the $MgSO_4$ amount, 500 mg was sufficient to achieve good clean-up effects. Therefore, the optimal clean-up conditions were 400 mg of PSA and 500 mg of $MgSO_4$.

3.6. *Quality control*

During this work, the background level of BPA contamination in the LC system was controlled.

In our laboratory, the liquid lines used in the LC system were made of stainless steel to avoid the possible release of BPA from plastic lines. In addition, ultrapure water was filtered through EDS-Pak cartridges before use, to remove any trace amounts of BPs. The organic solvents used in the LC system were LC-MS grade and free from BPs. Finally, the peak areas of all target analytes were less than 30% of the LOD peak area according to the results of zero volume injections. An example of a solvent blank chromatogram is given in Fig. 3.

Contamination by BPs was also avoided throughout the entire analytical procedure, and all glassware and plasticware were washed with MeOH three times before use. The extent of background contamination was assessed by running procedural blanks spiked with the IS solution along with reagent blanks prepared for every batch in triplicate, in the same manner as employed during processing of the other samples. The background levels of BPA and BPS were found to be consistent in all method blanks and these concentrations were thus subtracted from the analysis results for samples.

3.7. Validation of the proposed method

The linearities of the calibration graphs were assessed using determination coefficients (R^2) and the lack of fit (LOF) test, with the results provided in Table 2. The R^2 value was greater than 0.99 for all compounds following linear regression analysis, while the F values were less than the F_{table} values and the p values were greater than 0.05 in all cases at a confidence level of 95%. Thus, the linear regression models adequately fit the data.

The calculated LODs ranged from 0.02 μg kg^{-1} for BPM to 0.75 μg kg^{-1} for BPFL while the LOQs varied from 0.04 μg kg^{-1} for BPS to 0.95 μg kg^{-1} for BPF. Compared to the reported work for the determination of BPs in mouse food [25] and baby food [12], the present method showed similar

LOQs for most analytes and 5- to 40-fold lower LOQs for BPS and BPP, indicating that good sensitivity was obtained. The results are summarized in Table 2.

Recoveries calculated using matrix-matched curves with corrections for the IS compounds were used to assess the accuracy of the method, due to a lack of certified reference materials (CRMs) for BPs in animal feed or similar samples. The resulting recoveries and RSD values are shown in Table S8. The average recoveries were between 82.6% for BPS and 112% for BPP while the RSD values for intra-day and inter-day precision trials were less than 12% and 18%, respectively. These data confirm that this method was both highly accurate and reproducible.

3.8. *Real samples analysis*

Eventually, the newly developed method has been employed to analyze a total of 20 animal feed products from local feed factories, including broiler, duckling, pig, beef, lamb, rabbit and fish formula feed. The results were shown in Table S9 and the profiles of detected BPs were summarized in Table 3. To better understand the occurrence characteristics of BPs in animal feed samples, the normalized proportions [39] were adopted and calculated using the equation (1).

$$\text{Normalized proportion} = \frac{C_{\text{BPx}}}{\Sigma\text{BPx}} \times 100\% \qquad (1)$$

Where the C_{BPx} is the concentration of the single BP analog in each sample and Σ BPx is the total concentration of all BPs in each sample. The Box diagram analysis result of normalized proportion was presented in Fig. 4. Among the 12 target analytes, 4 BPs, including BPA, BPF, BPS, and BPAF were found in these feed samples. Both BPA and BPS were quantified in all samples with concentrations ranging from 0.92 to 7.77 μg kg^{-1} and 0.32 to 7.51 μg kg^{-1}, respectively. A comparison to a previous report [25] indicates that the BPS concentration found in farm animal feed in the present work was close to that in mouse feed, while the level of BPA was lower. Interestingly, BPF and BPAF were also identified in these samples with lower detection ratios. BPF was detected in 6 of 20 samples, among which 3 samples could be quantified ranging from 1.51–54.12 μg kg^{-1}. BPAF, a halo-substituted BP analog, was found in only one sample at 0.45 μg kg^{-1}. According to Fig. 4, BPA was the dominant analog of BPs detected in animal feed samples. However, It should be noted that the multiple contaminations of BPs in animal feed products. For example, there were four BPs (BPA, BPF, BPS, and BPAF) were simultaneously present in a boiler feed sample. The MRM chromatograms of this sample (no. S02) are shown in Fig. S7. The possible cause of this phenomenon was the complexity of feed ingredient sources. Although the contamination levels were low, the long term health effects of chronic exposure to low concentrations of BPs are a concern. The source of BPs in animal feed and the cause of the numerous different contaminants require further study.

4. Conclusions

A UPLC-MS/MS method using QuEChERS for the sample preparation was developed for the simultaneous determination of 12 BPs in animal feed matrices, for the first time. Compared to methods published previously, the post-column NH_4OH infusion technique was employed in this study, providing higher detection sensitivities and excellent retention behaviors of the target BPs. The extraction and purification programs were systematically investigated and optimized. Good quality results, including recovery, precision, linearity, LOD and LOQ were achieved. Finally, the method was successfully applied to investigate the contamination of various animal feed products with BPs. The results indicated that BPA was the dominant BPs analog and that multiple BPs were present in some animal feeds. Further studies on occurrences and carry-over of BPs in animal feed products are needed, which will promote the risk evaluation of feed and food safety.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary materials for this paper is available in the online version of the paper.

Fig. S1. Typical chromatograms of BPs at concentrations of 0.5 ng mL^{-1} as reported in the literature.

Fig. S2. Chromatograms of BPs obtained using a mobile phase consisting of water (phase A):MeOH (phase B) containing (A) 0%, (B) 0.001%, (C) 0.002%, (D) 0.005% and (E) 0.01% of an NH_4OH solution.

Fig. S3. Relative peak areas of all BPs and isotopically labeled internal standards using different concentrations (0.1%-5%) of NH4OH in the mobile phase. The relative peak area of each BPs was calculated as a percentage value by dividing the peak area under the varying level of NH_4OH infusion by the area without any NH_4OH infusion.

Fig. S4. Effects of different extraction solvents on the recoveries of analytes from water at 20 μg kg^{-1} (n=3 in each case). Error bars represent the standard deviations.

Fig. S5. Response surface plots showing the average recovery of the target compounds based on varying the water volume, soak time and extraction time.

Fig. S6. Response surface plots showing the average recovery of the target compounds based on varying the amounts of $MgSO_4$, C_{18} and PSA.

Fig. S7. Typical MRM chromatograms of the target BPs extracted from an actual feed sample (no. S02).

Table S1. Analytical procedures previously reported for the determination of BPs in food and bio-samples.

Table S2. Characteristic ions and other mass spectrometer parameters used during the analysis of 12 target BPs and the internal standards.

Table S3. Ingredients and the calculated nutrient composition of the pig formula feed.

Table S4. Box-Behnken design matrix for the extraction parameters.

Table S5. ANOVA results for the response surface quadratic model of the extraction parameters.

Table S6. Box-Behnken design matrix for the sorbents amounts.

Table S7. ANOVA results for the response surface quadratic model of the sorbents amounts.

Table S8. Average recoveries along with intra-day and inter-day precisions.

Table S9. Results from the application of the method to actual animal feed samples.

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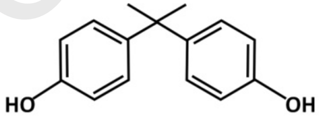
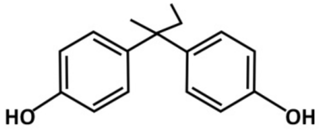
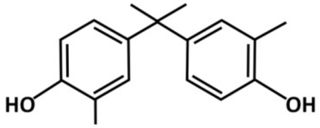
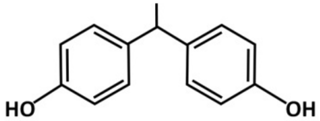
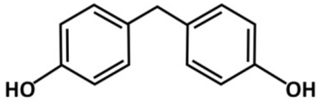
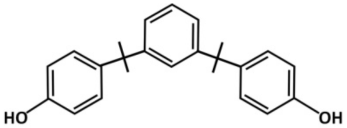
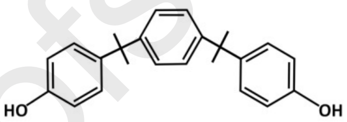
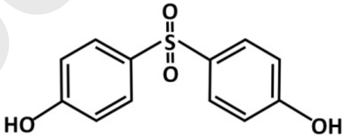
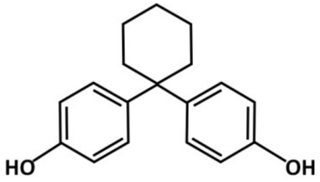
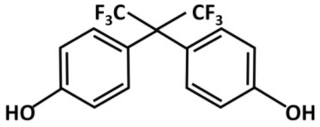
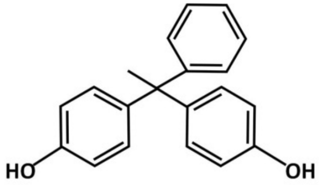
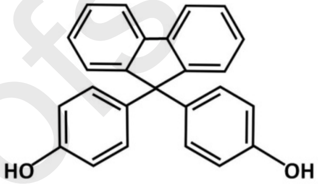
Table 1. Physicochemical properties, chemical structures and purities of 12 bisphenols.

Compound	Acronym	CAS number	Molecular formula	Mono isotopic mass	Log K_{ow}[a]	pKa[b]	Chemical structure	Purity (%)
Bisphenol A	BPA	80-05-7	$C_{15}H_{16}O_2$	228.12	3.64	10.29-10.93		⩾99.0
Bisphenol B	BPB	77-40-7	$C_{16}H_{18}O_2$	242.13	4.13	10.27-10.91		⩾98.0
Bisphenol C	BPC	79-97-0	$C_{17}H_{20}O_2$	256.15	4.74	10.45-11.09		⩾99.0
Bisphenol E	BPE	2081-08-5	$C_{14}H_{14}O_2$	214.10	3.19	9.91-10.64		⩾98.0
Bisphenol F	BPF	620-92-8	$C_{13}H_{12}O_2$	200.08	3.06	9.91-10.54		⩾98.0
Bisphenol M	BPM	13595-25-0	$C_{24}H_{26}O_2$	346.19	6.25	10.31-10.92		⩾99.0

Bisphenol P	BPP	2167-51-3	$C_{24}H_{26}O_2$	346.19	6.25	10.31-10.92		⩾99.0
Bisphenol S	BPS	80-09-1	$C_{12}H_{10}O_4S$	250.03	1.65	7.64-8.23		⩾98.0
Bisphenol Z	BPZ	843-55-0	$C_{18}H_{20}O_2$	268.15	5.00	9.91-10.51		⩾99.0
Bisphenol AF	BPAF	1478-61-1	$C_{15}H_{10}F_6O_2$	336.06	4.47	8.74-9.38		⩾99.0
Bisphenol AP	BPAP	1571-75-1	$C_{20}H_{18}O_2$	290.13	4.86	10.22-11.11		⩾99.0

Bisphenol FL	BPFL	3236-71-3	$C_{25}H_{18}O_2$	350.13	6.08	9.59-10.19		⩾99.0
Bisphenol A-d_8	BPA-d_8	92739-58-7	$C_{15}H_8D_8O_2$	236.16				⩾99.0
Bisphenol B-d_8	BPB-d_8	/	$C_{16}H_{10}D_8O_2$	250.18				⩾99.0
Bisphenol F-d_{10}	BPF-d_{10}	1794786-93-8	$C_{13}H_2 D_{10}O_2$	210.15				⩾99.0
Bisphenol S-d_8	BPS-d_8	/	$C_{12}H_2D_8O_4S$	258.08				⩾99.0
Bisphenol AF-$^{13}C_{12}$	BPAF-$^{13}C_{12}$	/	$C_3{}^{13}C_{12}H_{10}F_6O_2$	348.10				⩾99.0

[a] The octanol-water partition coefficient (LogK_{ow}) values were estimated using the KOWWIN v1.67 program (EPI Suite^{TM}package, USA).

[b] The p*Ka* values were calculated using the ACDLabs/p*Ka* dB software package, v. 11.0 (Toronto, Canada).

Table 2. Analytical and statistical parameters.

Compound	Internal standard	LOD (µg/kg)	LOQ (µg/kg)	Linear equation	R^2	ANOVA lack-of-fit	
						F-value[a]	p-value
BPA	BPA-d_8	0.51	0.52	0.051x+0.007	0.9916	0.041	1.00
BPB	BPB-d_8	0.60	2.00	0.107x-0.081	0.9962	1.271	0.33
BPC	BPAF-$^{13}C_{12}$	0.24	0.80	0.001x-0.001	0.9929	0.211	0.93
BPE	BPA-d_8	0.33	0.33	0.067x-0.026	0.9987	1.642	0.23
BPF	BPF-d_{10}	0.29	0.95	0.349x-0.292	0.9982	1.644	0.23
BPM	BPAF-$^{13}C_{12}$	0.02	0.06	0.003x+0.001	0.9867	0.020	1.00
BPP	BPAF-$^{13}C_{12}$	0.04	0.12	0.004x+0.005	0.9942	0.106	0.98
BPS	BPS-d_8	0.03	0.04	0.055x+0.014	0.9990	0.190	0.94
BPZ	BPAF-$^{13}C_{12}$	0.09	0.18	0.003x-0.003	0.9953	0.787	0.56
BPAF	BPAF-$^{13}C_{12}$	0.08	0.08	0.004x+0.003	0.9953	1.994	0.16
BPAP	BPAF-$^{13}C_{12}$	0.50	0.50	0.011x+0.009	0.9980	2.119	0.14
BPFL	BPAF-$^{13}C_{12}$	0.75	0.75	0.011x+0.009	0.9963	1.045	0.42

[a] $F_{table(4, 12)}=3.12$, α=0.05.

Table 3. Descriptive statistics of detected BPs (µg kg^{-1}) in the investigated feed samples.

	BPA	BPF	BPS	BPAF	Others
Mean	3.25	3.04	0.98	0.02	ND
Median	2.89	ND[a]	0.56	ND	ND
Range	0.92-7.77	ND-54.12	0.33-7.51	ND-0.45	ND
Detected ratio (%)	100	30	100	5	0
Quantified ratio (%)	100	15	100	5	0

[a] ND: not detected (< LOD).

Figure captions

Fig. 1. MRM chromatograms of the 12 target BPs and the IS compounds at concentrations of 20 ng mL^{-1} in MeOH:water (8:2, v/v) as obtained in this work.

Fig. 2. Peak areas for each compound acquired using the optimized NH_4OH infusion (0.4%, v/v) and without NH_4OH.

Fig. 3. A typical total ion chromatogram for a solvent blank.

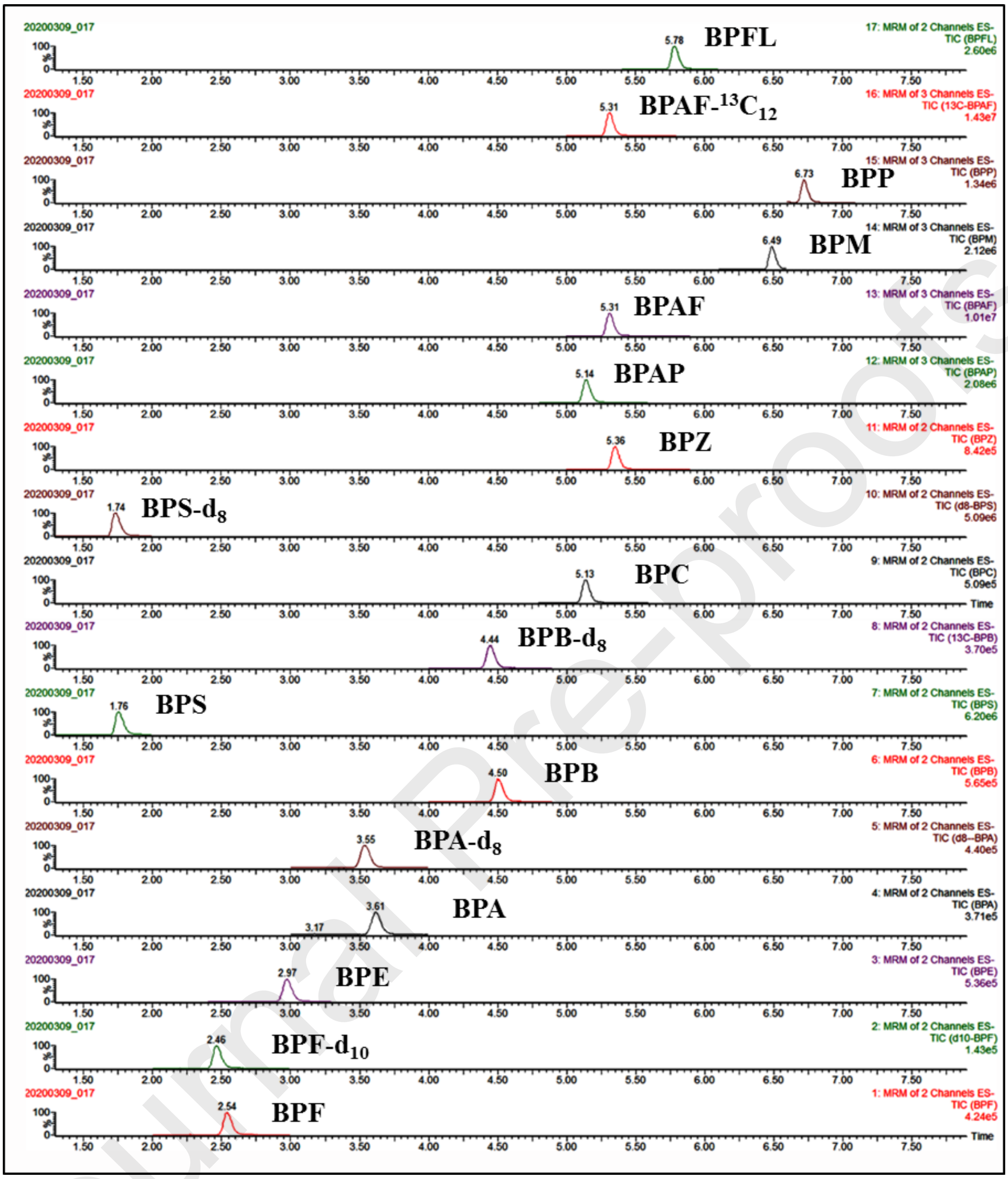
Fig. 4. The normalized proportions of target BPs in aminal feed samples.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT Author Statement

Ruiguo Wang: Conceptualization, Investigation, Writing-original draft, Writing-review & editing; **Shujun Dong:** Writing-review & editing; **Peilong Wang:** Supervision, Writing-review & editing; **Tong Li:** Validation; **Yuan Huang:** Investigation; **Lijuan Zhao:** Investigation; **Xiaoou Su:** Project administration.



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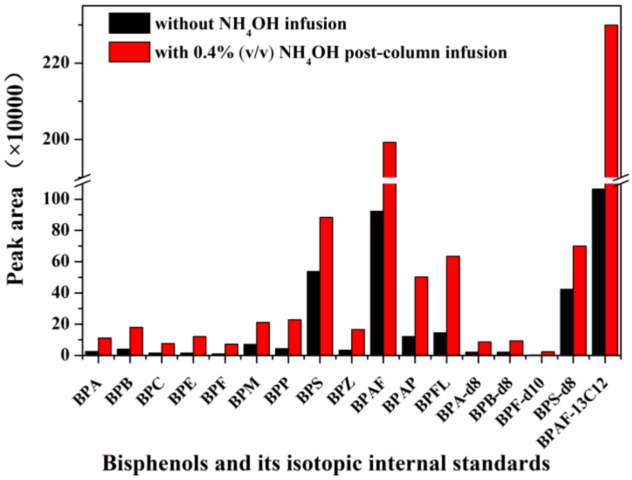

Fig. 2. Peak area for each compound acquired using the optimized NH_4OH infusion (0.4%, v/v) and without NH_4OH.

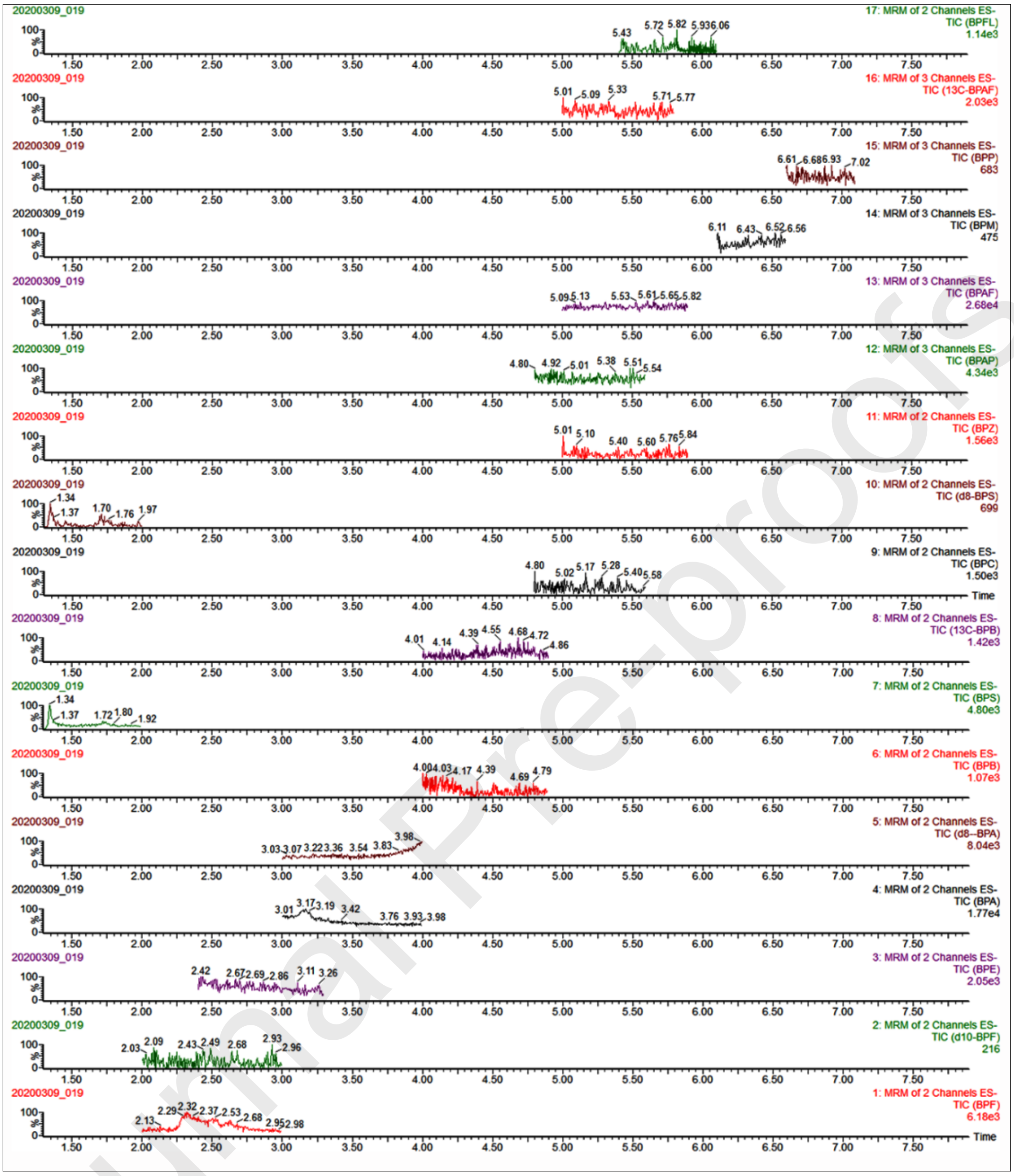

Fig. 3. A typical total ion chromatogram for a solvent blank.

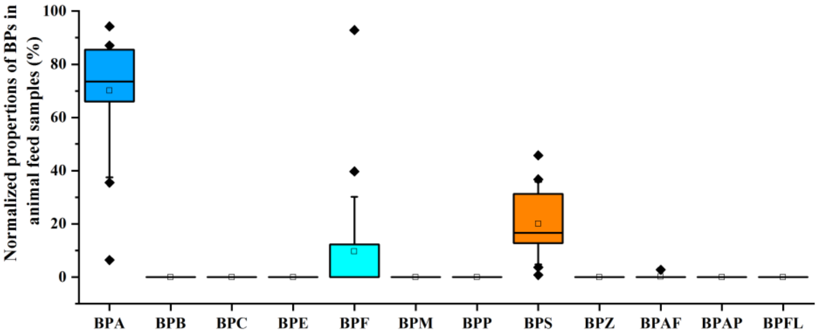

Fig. 4. The normalized proportions of target BPs in aminal feed samples. The lower and upper whiskers represent the 10th and 90th percentiles, and the bottom and top edges of the box show the 25th and 75th percentiles, respectively. The open square and the line within the box represent the mean and median concentrations, respectively. Diamonds are outliers.

Highlights

1. A UPLC-MS/MS method was developed for the determination of 12 bisphenols in animal feed.

2. The post-column ammonium hydroxide infusion was employed to improve sensitivities over 1.6-8.2 times.

3. Each investigated feed sample was simultaneously contaminated with at least 2 to 4 bisphenols.

4. BPA was the dominant analogue of BPs found in animal feed samples with concentrations ranging from 0.92 to 7.77 ng g^{-1}.