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Determination of BPA in commercial baby foods in India, using QuEChERS extraction, one pot derivatization and GC-MS analysis

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ABSTRACT

The growing popularity of marketed baby foods in India has raised concern about exposure to bisphenol A (BPA) which is a well known endocrine disruptor and a food contaminant. In absence of BPA related strict legislations, investigation of risk associated with dietary levels of BPA in sensitive population of India has gained vital importance. In present study, BPA was effectively extracted from 12 different types of baby food products using QuEChERS salts and dispersive-solid phase extraction (d-SPE). The rapid, rugged and reliable derivatization method was developed for analysis of BPA using Gas Chromatography-mass spectrometry. The method was validated and verified with high reproducibility (<10% RSD), sensitivity (2.967 ng g^{-1}LOD, 9.891 ng g^{-1}LOQ) and recovery (>80-98%). The concentration of BPA found in various powdered, fruit based and milk based baby foods ranged from < 1.04 to 26.19 ng g^{-1}. The baby foods under study were found to be safe for consumption; however, periodical determination of BPA in food products on a larger statistical scale is recommended.

Key words *- baby foods, BPA, derivatization, GC-MS, QuEChERS.*

I. INTRODUCTION

Baby food, major source of food and nutrients for younger children, is soft and easily consumed food which is introduced to babies roughly between the ages of four to six months up to two years. The packaged baby foods and infant formulae are available in multiple varieties, tastes and various forms like liquids, solids, ready to serve and easy to cook dry powdered formulae. The industrial revolution, fortification of baby foods, changing lifestyle and modernization are the major causes behind increasing demand of commercial baby foods. Although homemade food has not lost its relevance but it is losing popularity among working mothers. The increasing numbers of nuclear families which are mostly being supplemented with income from both parents, working women are ready for the convenience of ready-to-serve baby foods and the market is waiting to explode. The Indian baby food market is one of the fastest growing segments and is estimated at Rs.1500 crores. In the period 2007-12, sales of baby foods increased (in real terms) by nearly 80 percent **[1]**.

The growing popularity of baby foods raises concern about the safety issues of their consumption. The organic pollutant BPA [2,2-bis(4-hydroxyphenyl)propane] is a monomer and raw material in epoxy resins which is widely used as food contact coating materials. The presence of BPA into food may be sourced from leachable

plastic food packages, water and environment **[2-5].** BPA contamination through environmental may occur during production, processing, use or via physical and chemical degradation of end products during disposal of BPA containing substances (products or articles) or recycling operations **[6-7]**. BPA has recently received considerable attention worldwide from both scientists and regulatory authorities due its recognized endocrine-disrupting properties [8]. Recent studies have shown that BPA is associated with increased incidence of reproductive cancers, oxidative toxicity, neurotoxic effects, genotoxic effects, liver enzyme abnormalities [9-10]. There is also evidence that low level exposure to BPA, particularly at sensitive life cycle stages (fetuses, infants and children), may lead to permanent alterations in hormonal, developmental or reproductive capacity as neurological and endocrine systems are developing and hepatic system is immature [11]. The effects of BPA are agonistic to action of estrogens that alter developmental processes even at a very low dose [12]. Tolerable Daily Intakes (TDI-5 to 50µg/kg bw/day) of BPA have been fixed with strict limit by most of the countries. However, no such standard limit is set by Indian Food Safety Authority which raises concern about the BPA exposure from commercial baby foods **[13]**.

The extensive literature survey showed that BPA is detected at ng g^{-1} levels in food samples **[2, 14-17]**. Therefore, the methods of extraction and detection have to be highly sensitive and efficient. Solvent extraction is one of the most common and effective techniques for extraction of BPA from food; acetonitrile as a solvent plays an important role in precipitation of proteins in food samples **[18]**. Further cleanup of the extracts using d-SPE is necessary to remove co-extracted interferences. The various sensitive techniques like LC combined with various detectors including UV, fluorescence, ECD, MS and tandem mass spectrometry (MS/MS) and GC-MS have been used for detection of BPA **[19]**. GC-MS is one of the methods frequently used for determination of BPA in food analysis because of its higher resolution and lower LOD compared with LCMS methods, despite the tedious derivatization step required. Extracts were rarely analyzed directly by GC-MS without derivatization **[20]**. Derivatization is an essential step for accurate and sensitive quantitative analysis of BPA using GC-MS due to presence of two hydroxyl groups in BPA.

In present study, the popular QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method was selected for effective extraction of BPA from fatty and non-fatty baby foods. The one pot derivatization method was developed to simplify the extraction after acetylating BPA and to avoid contamination from environment. The representative 12 samples belonging to seven commercial brands available in Indian local market were tested with validated method, for presence of BPA. The study is an attempt to provide a baseline regarding safety issues concerned with branded and non-branded commercial baby foods available in India. It will help to decide if any safety and control measures are required in India with respect to BPA as a potential contaminant.

II. MATERIALS AND METHODS

2.1. Materials and Reagents

Analytical standard of BPA (purity grade > 99%) and a surrogate standard Deuterated bisphenol A (BPA d-16) were purchased from Sigma Aldrich. HPLC grade reagents, including water (CAS No. 7732-18-5) and acetonitrile (ACN) were purchased from Merck, India.

The buffering salt pouches of QuEChERS extraction kit (4g MgSO4, 1g NaCL, 1g Na citrate, 0.5g disodium citrate sesquihydrate) and QuEChERS dispersive kit (Kit contents part no. 5982-5156) contained 15 ml d-SPE tubes (150 mg PSA, 150 mg C18 EC, 900 mg MgSO4) were purchased from Agilent, India.

The derivatizing agent Acetic Anhydride (purity > 99%, CAS No. 540-84-1), pyridine (99.5%, CAS No.-108-24-7) and non-polar solvent iso-octane ((99.5%) were used.

2.2 GC-MS Instrumental Parameters

Baby food samples extracts were analyzed using Thermo Fisher Scientific gas chromatograph with single quadropole mass detector. MS Capillary column: DB-5 (30 m, 0.25mm, 0.25 µm) with helium as a carrier gas (flow rate: 1ml/ min). Sample injection volume: Two µl; injector port temperature: 250˚C; Split less mode (split less time: 1 min.); Constant septum purge; Temperature program: Initial temp. 120˚C (held for 1 min.), ramped at 8˚C/min. to 270˚C (held for 3 min.); Ion source temp. 200˚C; The mass spectrometer was operated in positive ion mode.

2.3 Preparation of Standard Reference Materials

Stock (200 mg L^{-1}), intermediate (10 mg L^{-1}), and spiking solutions (0.1 and 1 mg L^{-1}) of BPA and BPA-d16 in acetonitrile were prepared in 25 mL volumetric flasks and stored at 4 °C. These standard solutions were stable for at least 6 months at 4°C.

Derivatized BPA calibration standard solutions were prepared in a dilution series as (1, 10, 25, 50, 100, 200) ng g^{-1}. BPA-d16 was added to each dilution level of BPA standard to obtain final concentration of 125ng $g^{-1}$of internal standard. Although these derivatized standards were stable more than 2 months when stored at 4°C, they were freshly prepared for every other batch.

2.4. Sampling

Twelve various types of baby foods belonging to nine different imported and local brands were broadly categorized into three groups i.e. powders based infant formulae (PIF), fruit based baby foods (FBBF) and milk based foods (MBF). Sample details are as shown in TABLE 1.

Table 1: Details of baby foods marketed in India and their dietary levels of BPA.

Types of baby foods	Sample code	Sample information	Branded/ Non-branded	Conc. Of BPA in baby foods (Mean ± STD in ug/kg)	Daily intakes of BPA for 6 kg wt of baby (in ug)

Powdered Infant Formulae (PIF)	PIF-1	Milk Powder Infant Formula	Branded	1.15 ± 1.63	0.19
	PIF-2	Powdered formula Wheat Stage 1	Branded	ND	0.00
	PIF-4	Powdered formula Organic food ragi& mixed fruits	Non-branded	ND	0.00
	PIF-5	Ragi Malt (NachaniSatva) Type 1	Non-branded	6.80 ± 9.62	1.12
Fruit Based Baby Foods (FBBF)	FBBF-1	Apple & mango puree	Branded	8.31 ± 0.21	1.41
	FBBF-2	Apple, pear & banana puree	Branded	26.19 ± 22.78	4.46
	FBBF-3	Apple Prunes	Branded	ND	0.00
	FBBF-4	Apple, red paper and sweet potato puree	Branded	10.76 ± 1.83	1.83
Milk Based Foods (MBF)	MBF-1	Milk & Egg Custard	Branded	20.99 ± 2.62	3.57
	MBF-2	Vanila flavoured milk Custard	Branded	1.65 ± 0.60	0.28
	MBF-3	Probiotic Milk (for 1 yr above)	Non-branded	0.01 ± 0.01	0.00
	MBF-4	Cheese	Non-branded	1.04 ± 0.25	0.02

The samples were stored at 4˚C and were analyzed in duplicates. All baby foods were purchased from local markets of Mumbai, India.

2.5. Sample Preparation

The packaged baby foods samples were homogenized and weighed 15g of liquid (for dry powders 5g of sample mixed with 10 ml of distilled water) samples in duplicate. The samples were spiked with 375µl of 1 mg L^{-1} of internal standard BPA-d16. The extraction was initiated by adding 15ml of acetonitrile. The sample was vortexed for one minute. The buffering salts were added using readymade pouch of QuEChERS extraction kit and then mixture was vortexed for a minute (European Method EN 15662). It was then centrifuged at 4000 rpm for 5 minutes. Eight ml of supernatant was taken out and placed into d-SPE tube. It was vortexed and centrifuged at 4000 rpm for one min. Five ml of supernatant was evaporated till dryness under nitrogen stream in low volume evaporator (LVE) system at 50˚C. The derivatizing reagents mixture (Acetic anhydride and pyridine, 300µl) was added to LVE tube which was kept at 60°C for 1 hr in oven. HPLC grade water (700 µl) was added to derivatized sample. Finally, the sample was extracted with 1 ml of iso-octane. The immiscible layers of sample were shaken and vortexed for one minute. The upper organic layer was carried in a vial for GC-MS analysis. The scheme for sample preparation method is described in Fig. 1.

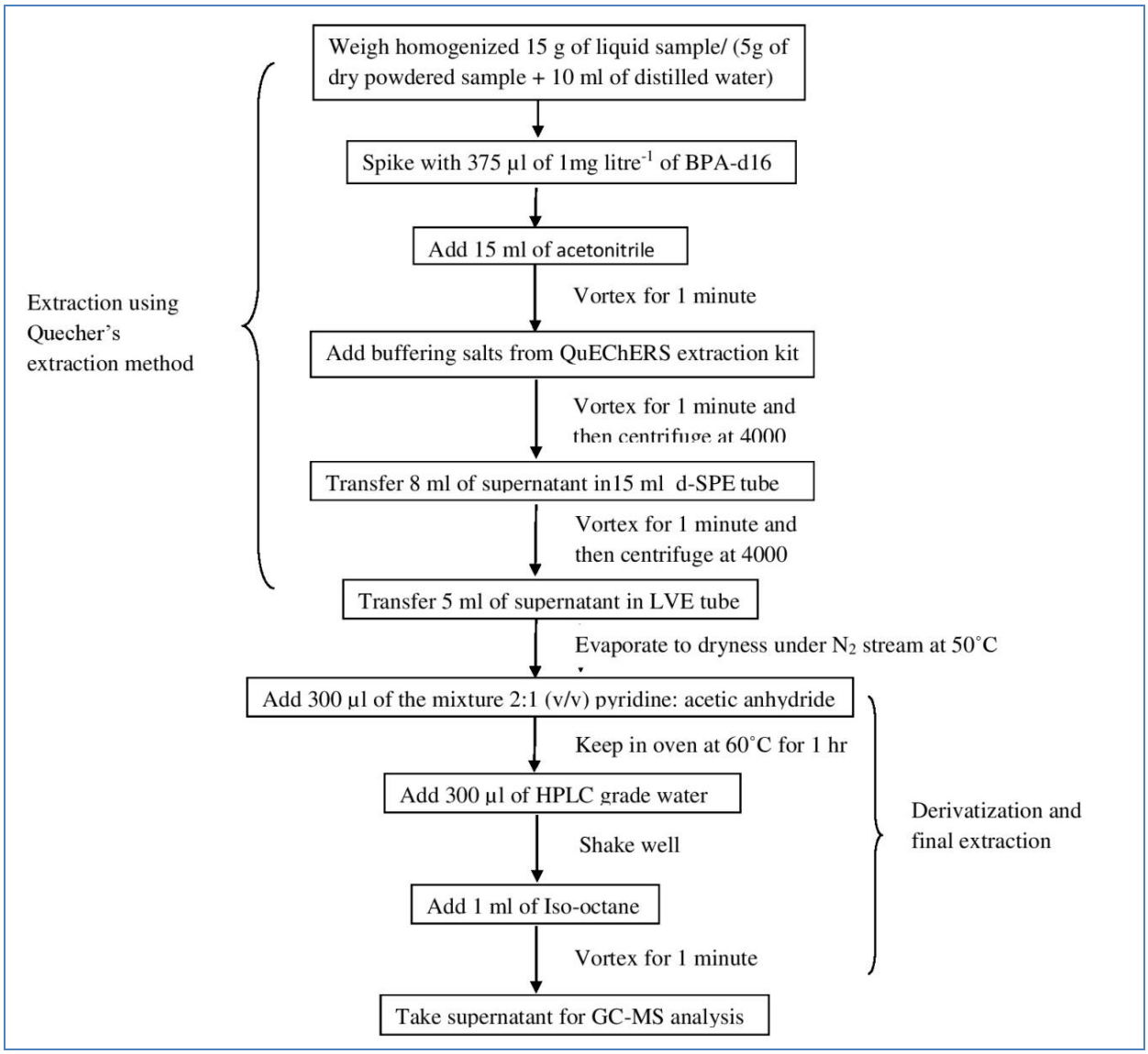


Fig.1 Scheme of sample preparation for BPA analysis

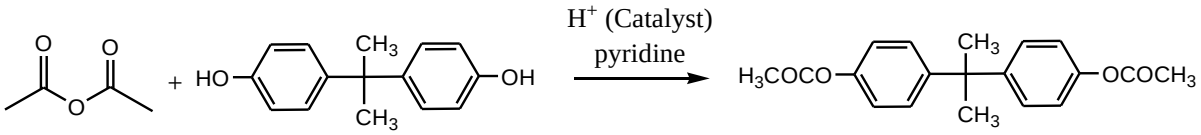
2.6. Quality Control Measures

All the glassware required for sample and standard solutions preparation were washed thoroughly and dried at 260˚C to get rid of BPA contamination from environment. Confirmation of BPA identity was based on the retention time and the ion ratios. The calculation of BPA concentrations in samples was based on the calibration curves of peak area ratios of BPA (ion m/z 213) over the internal standard peak area plotted (ion m/z 224) with the ratios of native BPA concentration over the internal standard concentration. The extraction batch contained the following control samples: (a) Two method blanks (15 mL of water); (b) One unknown sample for recovery study; and (c) the unknown sample spiked with three levels (10, 25 and 50 ng g^{-1}) of BPA.

Linearity, sensitivity, LOD, LOQ and recovery were tested for the validation of employed method.

III. RESULTS AND DISCUSSIONS

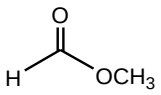
Since baby food is such a complex matrix, significant and often cumbersome cleanup and enrichment steps were employed following the initial extraction. Hence, a QuEChERS-based methodology was used for the effective extraction at ng g^{-1} levels, by providing a suitable partition co-efficient, when partitioned between acetonitrile and salt saturated water. The resulting extract is further cleaned up by removal of interfering compounds particularly carotenoid and lycopenic compounds, using dispersive SPE (dSPE). This method provides a faster analysis with less toxic reagents and allows extraction of BPA in various complex food matrices. The extracted BPA was then converted into acetate ester derivative by acylation using mixture of acetic anhydride as derivatizing agent and pyridine as a base catalyst. The derivatization reaction is as given below:



BPA (2,2-Bis(4-hydroxyphenyl)propane)

Derivetised BPA

+



The one pot derivatization method followed by polarity based simple liquid-liquid extraction provided good yield, prevented loss of analyte and improved detection of BPA using Single Ion Monitoring (SIM) mode of single quadropole GC-MS technique. The method was subjected to method validation in terms of sensitivity, linearity using internal standard calibration, limits of detection (LOD) and quantification (LOQ), accuracy and spiking study.

3.1. Method Optimization and Performance

The present study demonstrated effective QuEChERS extraction of BPA followed by d-SPE and one pot derivatization, with method detection limit below 2.97 ng g^{-1}. Thus, using pre-weighed ready to use QuEChERS buffering salt pouches has been proved to be quick, efficient, easy and cost-effective method. One pot derivatization was performed by acylation resulting in good yield of BPA derivative in diester form. The underivatized BPA standard (BPA dissolved in ACN) was analyzed for confirmation as shown in Fig.2 (a) and comparison with the peaks of BPA extracted by two different methods after derivatization. In method 1, after derivatization, derivatizing reagents were completely evaporated using LVE under nitrogen stream at elevated temperature 50ºC. It was then reconstituted using methanol (method 1). Though pyridine is used for faster and effective derivatization, complete evaporation of it during sample preparation was found to be tedious and time consuming process. The traces of pyridine caused interferences during analysis by GC-MS. The chromatogram is shown in Fig.2 (b). Hence, the method was optimized by shifting the polarity of the derivatized sample with

addition of water. The derivative of BPA was then extracted using the non-polar solvent iso-octane (Method 2). The desired sharp and well isolated peak of BPA was obtained after optimized liquid-liquid extraction using iso-octane, based on differences in polarity of the solvents and favorable partition co-efficient as seen in Fig.2 (c). Hence, method 2 was applied for derivatization of BPA in all standards and samples.

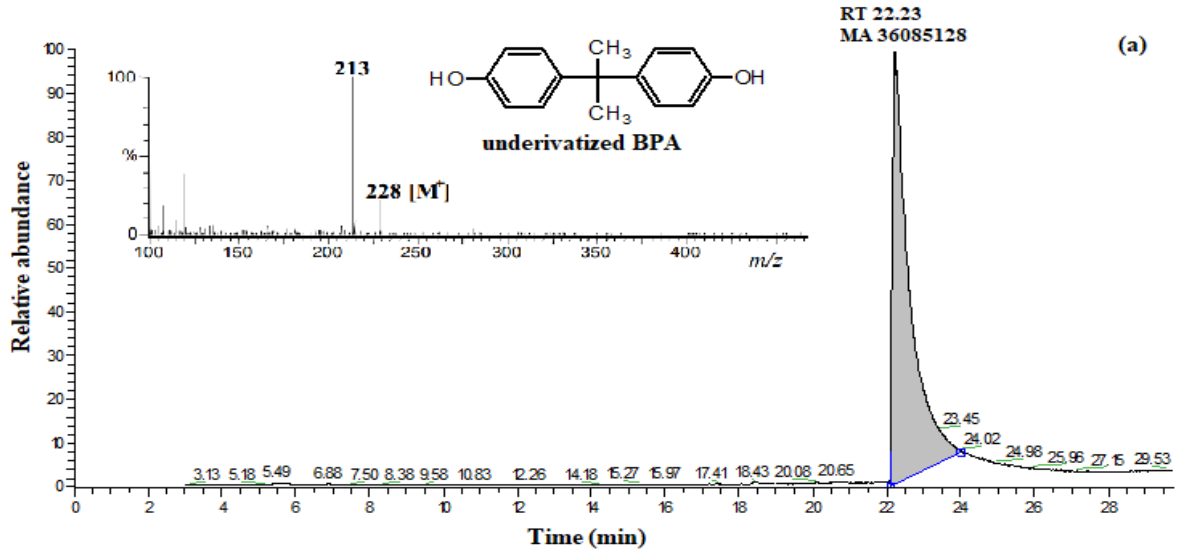


Fig.2 (a) Total Ion Chromatogram (TIC) of underivatized BPA 100 ppm std solution (along with full mass spectra of derivatized BPA at R 22.23)

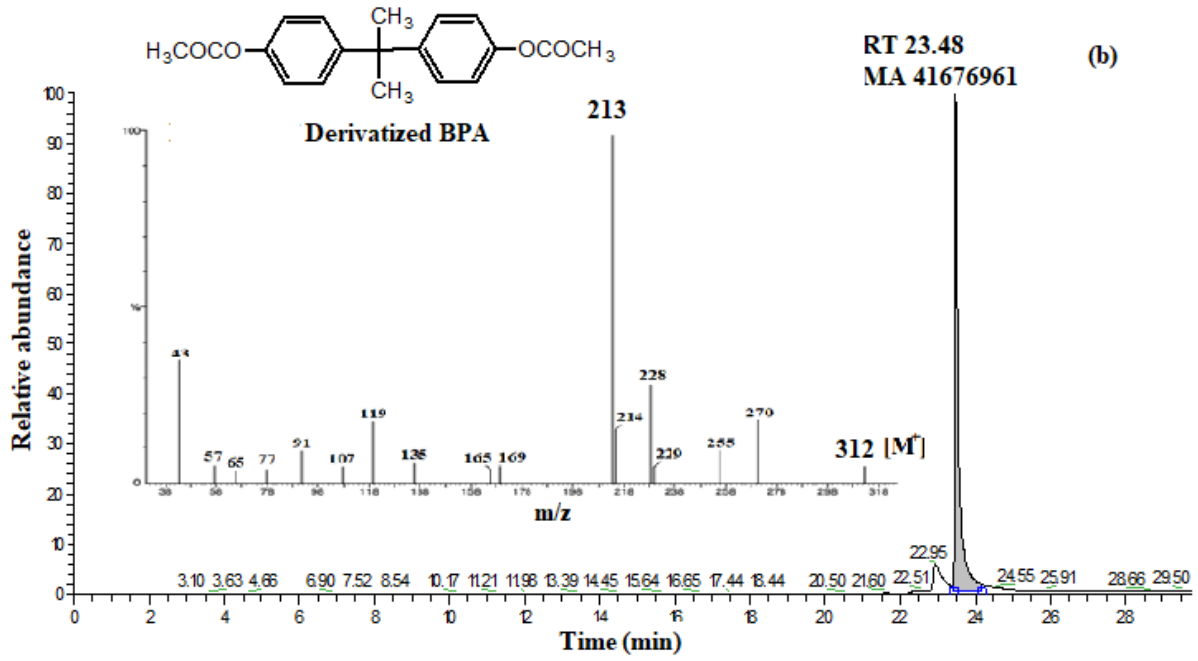


Fig.2 (b) TIC of BPA derivatized 100 ppm std solution after extraction by method 1 (along with full mass spectra of derivatized BPA at RT 23.48)

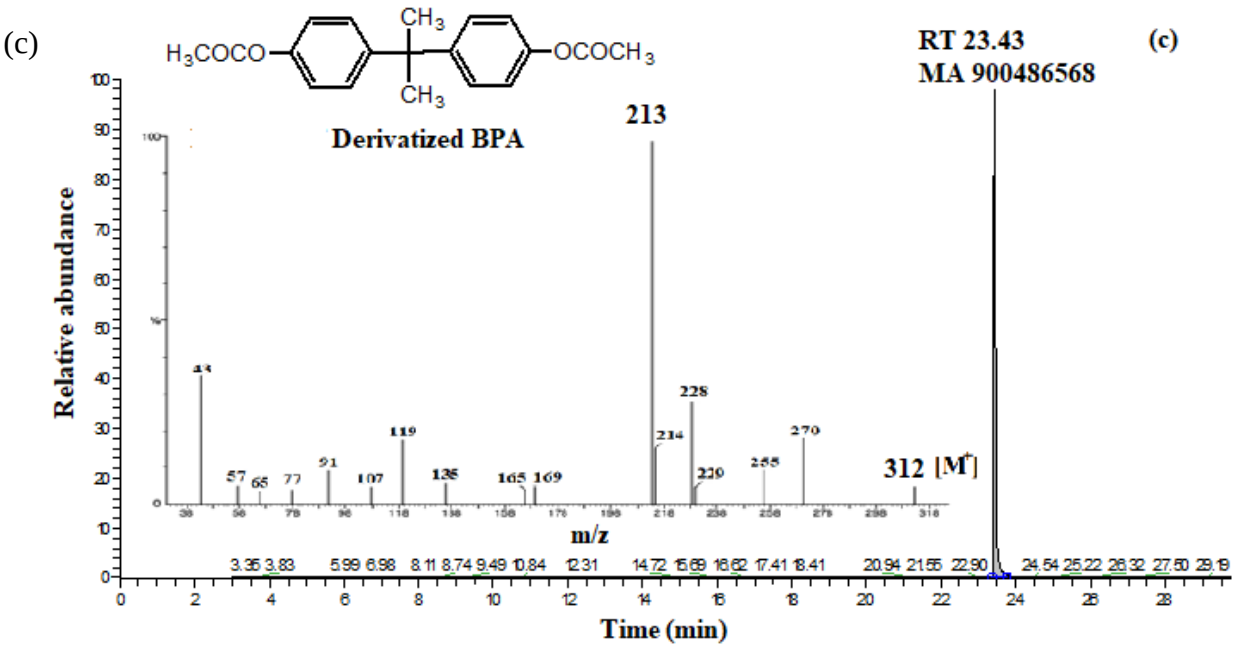


Fig.2 (c) TIC of BPA derivatized 100 ppm std solution after extraction by method 2 (along with full mass spectra of derivatized BPA at RT 23.43)

The derivatization using acetic anhydride in presence of pyridine enhanced the sensitivity of detection of BPA by approximate 25 times compared to the underivatized BPA considering the significant difference in their mass abundances (MA) as illustrated in Fig. 2 (a) and (c). Derivatization of BPA with acetic anhydride (m/z 102) resulted in the formation of its ester with m/z (312). The molecular ion peak at m/z(312) can be clearly seen in Fig. 2 (b) and (c), which is due to replacement of two hydrogen atoms of phenolic hydroxyl with two CH_3OCO- (m/z 60) from acetic anhydride. Thus, the derivatization of BPA was confirmed as shown in Fig. 2 (b) and (c) with the change of its molecular weight which indicated the peak at (m/z 312).

The Total Ion Chromatogram for the mixture of BPA and BPA-d16 is demonstrated in Fig.3 (a) using GC-MS with 5 channels i.e. 213, 224, 228, 270 and 312 ions (m/z). BPA was qualitatively and quantitatively determined using selective ion monitoring mode (SIM Mode); Quantitative data was obtained using 213 ion (m/z) for quantification of BPA (retention time 18.49) and 224 ion (m/z) was used for quantification of internal standard BPA-d16; retention time, 18.40). The performance of optimized method was tested in terms of method validation.

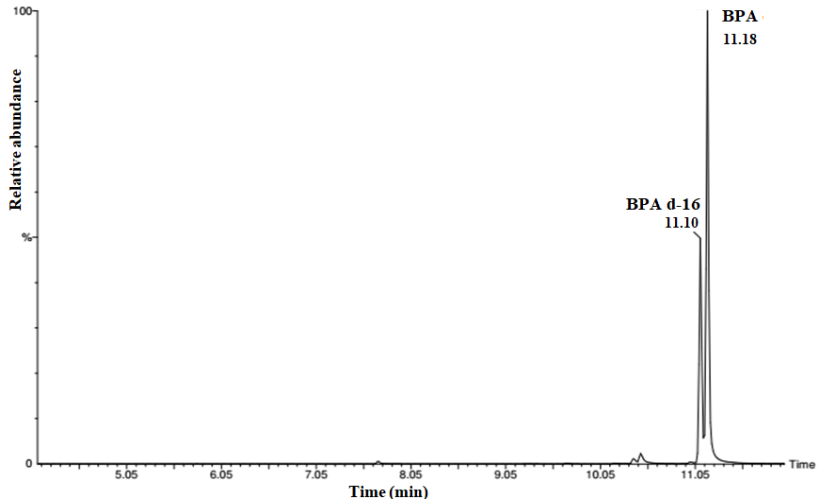


Fig.3 (a) TIC of std solution of 25 ng g^{-1} of BPA-d16 & 25 ng g^{-1} using 5 channels (213, 224, 228, 270 and 312) m/z

Linearity of the instrument and the method was demonstrated using five standard solutions with concentrations from 1 to 100 ng ml^{-1}. Linearity with R^2 value of better than 0.997 was observed for BPA's calibration plot with peak areas normalized to internal standard versus concentrations as shown in Fig.3 (b). The results were found to be reproducible.

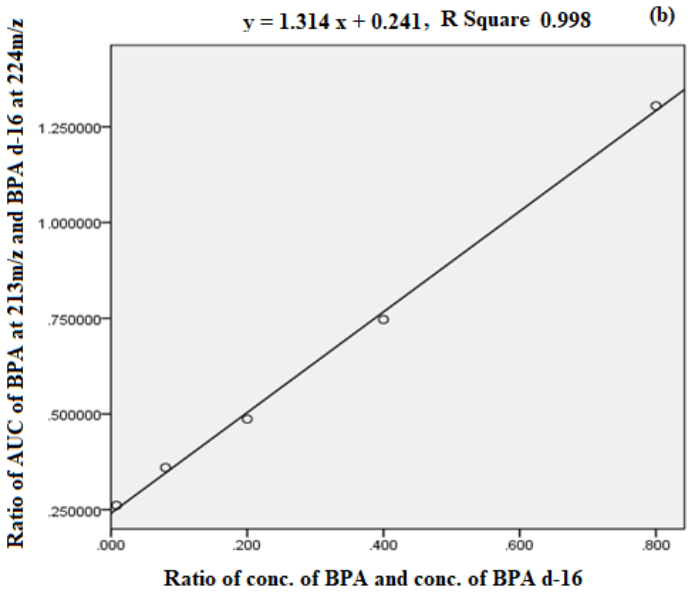


Fig.3 (b) Linear standard calibration curve of BPA by using BPA d-16 as an internal standard

Blank determination was applied to establish LOD and LOQ as the blank analysis gave results with nonzero standard deviation. Six replicates of blank were analyzed for presence of BPA. LOD was expressed as the analyte concentration corresponding to the sample blank value plus three standard deviations and LOQ was assessed as the analyte concentration corresponding to the sample blank value plus ten standard deviations as shown below **[21]**:

(1) $LOD=X_{b1} + 3S_{b1}$,

(2) $LOQ=X_{b1}+10S_{b1}$,

Where X_{b1} is the mean concentration of the blank and S_{b1} is the standard deviation of the blank.

The method detection limit was found to be 2.967 ng g^{-1} and method quantification limit was established at 9.891 ng g^{-1}. The extraction recoveries of the method were obtained from the analyses of three replicates of liquid formula samples spiked with BPA standard solutions at 10, 25, and 50.0 ng g^{-1}; recoveries were 80%, 98% and 92% respectively, with relative standard deviations (RSD) below 10%. The recovery was found to be maximum (98%) at 25 ng g^{-1} spike level.

3.2 Concentrations of BPA in Baby Foods:

BPA was detected in very low concentration i.e. 0.01 to 26.19ng g^{-1} in the analyzed samples. It was found to be present in 8 of 12 types of baby foods. The Apple pear banana puree contained highest content of BPA with concentration of 26.19ng g^{-1}. The findings were consistent with results of previous study which showed that BPA content in infant formulae ranged from 2.27 to 10.2 ng g^{-1} and 0.1 to 13.2 ng g^{-1} respectively **[2, 22]**. Another study indicated presence of high levels of BPA with concentrations of 45 to 113 ng g^{-1} in powdered formulae, may be due to contamination during processing **[23]**; however, the present study revealed low concentrations of BPA in dry powdered based infant formulae (< 6.80 ng g^{-1}).

It was reported that a 6-12 months old baby would require approximately 5 kg of powdered infant formula every month and in a year around 60 kg **[24]**. Thus, on the basis of the above study, the daily consumable weight of infant powdered formula was calculated to be 164.4g of infant formula powders. Similarly, as per the available guidelines, fruit juice intake should be limited to 4 to 6 oz i.e. 170 g a day in infants **[25]**. Also, the recommended daily intake for dairy products is 100 ml of milk and 15 g of cheese **[26]**. On the basis of dietary intakes of baby foods, the daily intake of BPA for PIF, FBBF and MBBF types of baby foods was evaluated. As per tolerable dietary intake of BPA, 6 months old baby with average body weight 6-7 kg can tolerate 50µg/kg i.e. 300-350µg of BPA per day. Table 1 indicated that the highest estimated dietary intake was found to be 4.46µg/day which was significantly below the TDI. The BPA content of contaminated samples was probably due to migration of residual monomer in can coating of due to the contamination from environment during processing baby foods.

IV. CONCLUSION

In present study, combination of QuEChERS extraction, d-SPE, one pot derivatization and liquid-liquid extraction was proposed for quantification of BPA at ppb levels in various fatty and non-fatty baby food matrices. The validated one pot derivatization method proved to be easy and reliable in preventing contamination of BPA and removal of interferences with minimum number of steps involved during sample preparation. The results confirmed that liquid-liquid extraction of BPA, after derivatization is more efficient over the method of evaporation of pyridine for GC-MS analysis resulting in enhanced resolution, avoiding irregular interfering peaks. The present study suggests that the dietary intakes of BPA from baby foods available in Indian market are unlikely to be concern to health of babies. However periodical evaluation of such contaminants in baby foods and other marketed canned food products is recommended using a larger sample

population. Thus, this study can be used as a baseline regarding safety issues with respect to BPA as a potential contaminant in baby foods. This assessment is needed as the information is limited on such matters.

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