Rafidain Journal of Science
https://rsci.uomosul.edu.iq
Vol. 35, No. 2, pp. 158-169, 2026 (June)

Azo-Dye Estimation of Bisphenol A Release from Plastic Bottles to Baby's Milk Via Diazotization reaction

Ahlam A. Shihab
Department of Biology/ College of Education for Pure Sciences/ University of Mosul

Hana Sh. Mahmood **Ayad Ch. Khorsheed**
Department of Chemistry/ College of Science/ University of Mosul

p-ISSN: 1608-9391
e -ISSN: 2664-2786

Article information

Received: 3/ 9/ 2025
Revised: 20/ 10/ 2025
Accepted: 4/ 11/ 2025

DOI: 10.33899/rsci.v35i2.63630

Corresponding author:
Ahlam Ahmad Shihab
abdelrahmanbasil@uomosul.edu.iq
Hana Shuker Mahmood
hanashukermahmood@uomosul.equ.iq
Ayad Chachan Khorsheed
ayad_chachan@uomosul.edu.iq

ABSTRACT

Bisphenol A (BPA) is an organic compound commonly used in modern industries, particularly in the production of polycarbonate plastics and epoxy resins. It is classified as an endocrine-interfering compound due to its chemical structure, which is similar to that of estrogen. In this study, BPA is forced to release from plastic baby bottles to milk by heating; then it is extracted from milk and determined spectrophotometrically using an azo coupling reaction with the diazotized p-nitroaniline (PNA)reagent to yield the azo dye which shows maximum absorbance at 478 nm. The method demonstration range is from 0.1 to 5.5 µg/ml, with a limit of detection (LOD) of 0.02861 µg/ml, limit of quantitation (LOQ) 0.09538 The calculated molar absorptivity confirmed 3.2× 104 l. mol -1.cm -1. The proposed method has high sensitivity, reliability, and it is applied for determination of BPA in milk after accelerating release of BPA under the effect of heat. The results show that the plastic used in the bottles released BPA into the milk at similar levels, but the Chinese AVEHINI plastic bottle released the highest levels. The validity of the method was confirmed using standard addition and t-test.

Keywords: Bisphenol A, Milk, azo-coupling, p-nitroaniline, plastic baby bottles

INTRODUCTION

Bisphenol A (BPA) is an organic compound from the phenol family, with the molecular formula $C_{15}H_{16}O_2$. It is composed of two phenol units linked by an isopropylidene bridge, a structural feature that imparts distinctive physical and chemical properties. BPA appears as a white crystalline solid (Juan *et al*., 2021) with a melting point of approximately 158–159 °C (PubChem, 2024).

It has low solubility in water (120–300 mg/L at 25 °C) but dissolves readily in organic solvents such as acetone and ethanol (EFSA, 2023). Its density is about 1.2 g/cm³ (PubChem, 2024) and its molecular weight is 228.29 g/mol (Yuksel *et al*., 2013; Wang *et al*., 2023). The molecule contains two aromatic rings connected by a central bridge and bears two hydroxyl (–OH) groups (Catron *et al*., 2019; Pelch *et al*., 2019), which conferring phenolic reactivity and the ability to interact with other compounds, including oxidizing agents (Chang *et al*., 2024). BPA maintains the thermal stability of epoxy-resin (Manzoor *et al*., 2022).

BPA is widely recognized for its endocrine-disrupting effects due to its ability to bind to estrogen receptors in the body (Zamri et al., 2021; Nahar et al., 2024). Bisphenol A(BPA) is a chemical compound widely used in manufacturing various plastics It is found in products such as polycarbonate plastics epoxy resins and vinyl acetate resins ,as well as in other polymers like polyethylene chloride, polyurethane and thermal paper used for receipts and invoices (Tsai, 2013; Ighalo *et al*., 2024), these materials are utilized in producing infant bottles, eyeglass lenses (Kimand and Lee, 2019), automotive parts , and spots equipment, indicating BPA's expensive presence in daily life (Vandenberg , 2007; Sutiakova *et al*., 2012; Kim *et al*., 2019.

Besides BPA, derivatives such as Bisphenol S (BPS), Bisphenol F (BPF) and Bisphenol B (BPB) (Wu LH *et al*., 2018), have emerged as alternatives, purportedly safer, However, recent research suggests that some of these substitutes exhibit similar or even greater toxic and endocrine-disrupting effects compared to BPA (Sharma, 2025). BPA migrates form manufactured materials into food through several mechanisms primary migration occurs directly from plastic surfaces or inner can coatings into food substance, additionally, heat exposure (Khalili *et al*., 2023). Such as during autoclaving or microwaving, accelerates BPA release, especially in fatty foods like milk due to BPA's lipophilic properties (Krishnan, 1993; Li *et al*., 2007; Hagera *et al*., 2025), factors influencing BPA migration include temperature, fat content of food, and contact time between food (Ondrej *et al*., 2024).

Regrading health effects, BPA acts as an endocrine, disruptor mimicking estrogen, posing significant during pregnancy. Studies have linked maternal BPA exposure to fetal developmental issues and subsequent neurobehavioral disorders in offspring (Sutiakova *et al*., 2012; Manzoor *et al*., 2022). Infants and children are particularly vulnerable due to immature metabolic Systems, BPA has been detected in urine samples from many children, with associations found between exposure and increased risks of hyperactivity, obesity and cognitive development alterations (Kim *et al*., 2019; Nahar *et al*., 2024), in adults, BPA exposure correlates with reproductive problems in women, including menstrual irregularities and reduced fertility, and adversely affects sperm quality and hormone levels in men (Ma, *et al*., 2019; Escrivá *et al*., 2021; Sutiakova *et al*., 2012; Manzoor *et al*., 2022).

Systemic health issues linked to BPA include cardiovascular diseases, obesity, diabetes and endocrine gland dysfunctions, alongside neuroimmune disturbances driven by BPA s interference with hormonal and inflammatory signaling pathways (Caporossi & Papaleo, 2017; Kim *et al*., 2019; Manzoor *et al*., 2022). Permissible exposure limits for BPA vary internationally. The European Food Safety Authority (EFSA) updated its tolerable daily intake (TDI) to 0.2 ng/kg body weight /day-one of the strictest limits globally-due to evidence of immune system effects (EFSA, 2023).

The U.S. Food and Drug Administration (FDA) currently consider typical dietary exposure to BPA use has been restricted or banned in infant products like baby bottles in many countries alongside enhanced regulation for food contact materials (FDA, 2024; Ondrej *et al*., 2024).

BPA is determined in carbonated beverages using MSPE-MS concerning in the impact of packaging type on contamination PET bottles showed the highest BPA concentrations indicating potential health risk for children (Kooti *et al*., 2025).

A diazotization –coupling reaction with sulfamethoxazole was developed for the determination of BPA (BPA). The method showed high accuracy with recoveries between 99.0% and 102.3% and a detection limit of 0.05 µg/ml. The optimal absorbance was measured at 445 nm, making the method suitable for routine analysis in beverages milk samples (Xu, *et al*., 2017). This study presents a green solvent-free analytical method for determination bisphenol A(BPA) in commercial milk using stir-bar sorptive extraction (SBSE) conjugated with thermal-desorption gas chromatography-mass spectrometry (TD-GC//MS). The method achieved ultra-trace detection limits of approximately 45ng/L, with recoveries improved by over 50% compared to conventional extraction approaches (Encerrado *et al*., 2025).

A multi-commuted fluorometric sensor was developed to measure the native fluorescence of BPA separated by C_{18} from milk samples. The method demonstrated high sensitivity with a detection limit (LOD) of 0.06 ng/ml and a quantification limit (LOQ) of 0.20 ng/ml, with a relative standard deviation (RSD) below 6% It was successfully applied to liquid milk, powdered milk, and infant formula samples, yielding results below the maximum residue limit set by European Union regulation (Cunha *et al*., 2012). In this research; BPA is forced to release from plastic baby bottles to milk by heating; then it is extracted from milk and determined spectrophotometrically using an azo coupling reaction with the diazotized p-nitroaniline (PNA)reagent to yield the azo dye

MATERIAL AND METHODS

Instruments used:

All absorption measurements for the proposed method were performed on a double-beam spectrophotometer of the type (UV-Vis Spectrophotometer, UV-1900, Shimadzu, Japan), use 1 cm glass cuvettes, and the weight was measured using a sensitive balance of type Sensitive balance model Kern & Sohn analytical balance ABS 120-4N-(Germany). A water bath of the type Elektro-mag Turkey was used for heating the solutions and the milk extract. Additionally, an Edmund Buhler B1 Mixers shaker, a Wincom China Vortex Mixer for laboratory Test Tubes, and a centrifuge were used Centrifuge OHAUS, American.

Chemicals Used

All chemicals used were of high purity.

BPA Solution (4×10^{-4} M):

Was prepared by dissolving 0.0091 g of pure BPA, supplied by SRL (Srlchem), in 80 mL of 99% ethanol (Cristalco France Alcohols). The solution was then brought up to a final volume of 100 ml with distilled water using a volumetric flask.

p-nitroaniline diazotized reagent solution (3×10^{-4} M):

This solution was prepared by dissolving 0.0552 g of the para-nitroaniline reagent (Fluka) in 50 ml of distilled water in a 100 ml beaker. The solution was heated for about 25 minutes at 70°C, then cooled to 0-5°C in the ice-water bath. While stirring, 20 ml of 2 M hydrochloric acid (Fisher Scientific) was added. The solution was then transferred to a 100 ml volumetric flask in an ice bath.

While shaking vigorously, 0.0276 g of sodium nitrite (supplied by BDH) was added. The color of the yellow reagent changed to colorless as it underwent diazotization. The volume was brought up to 100 ml with cold distilled water (Al-Saffar, 2017). The solution was stored in a dark bottle and prepared every 5 days (stable).

Sodium Hydroxide solution (1M):

Was prepared by diluting a concentrated plastic ampoule 10 M in 100 ml (BDH, Poole England) to in 1000 ml distilled water.

Release of BPA

30 ml of KDD Kuwaiti artificial milk was placed into four different types of baby bottles: Mini bab/China, Bebedor Birlik company /Thailand, Aillin Kala Koodak Toos company /Iran, AVEHINI/

China. The bottles were then placed in a water bath for one hour at a temperature of 45°C. Following this, they were shaken with a mechanical shaker for 20 minutes.

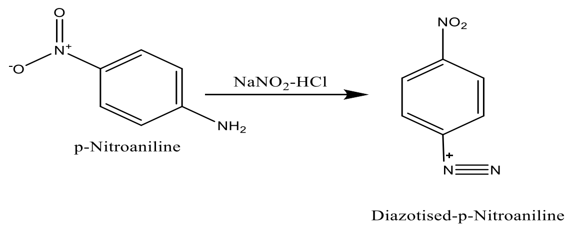
Extraction of BPA from milk

5 ml of the milk from each of the four plastic bottles was transferred to centrifuge tubes, 5 ml of acetonitrile was added. The tubes were then homogenized using a vortex mixer for 5 minutes. Finally, the samples were centrifuged for 21 minutes at 5000 rp/min, the filtrate is then taken to perform the method for estimating BPA (Kamal *et al*., 2025).

The principle of the proposed method:

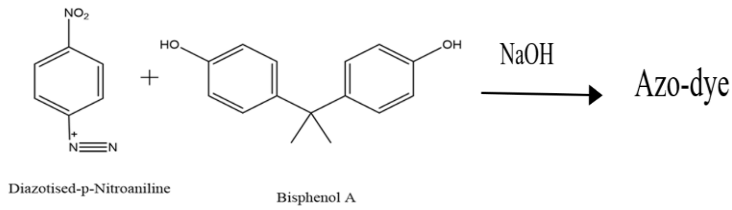
The reaction involves two steps as shown below:

1- Formation of the diazotized p-Nitroaniline (DPNA) as shown in scheme (1):



Scheme 1: The reaction steps (1)

2- Coupling of the nitrogenous agent with BP A as shown in scheme (2):



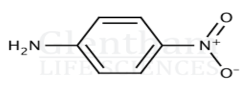
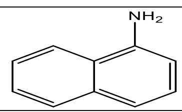
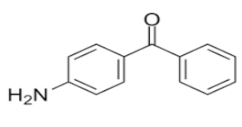
Scheme 2: The reaction steps (2)

Optimization of reaction conditions

Selecting the diazotized agent

In this study, 2 ml of 1×10^{-3} M of three organic reagents were tested as diazotized reagents in acidic medium with 2 ml of BPA 1×10^{-3} M as coupling agent in a basic medium, the results are shown in (Table 1).

Table (1): Selection of the azo-coupling reagent

0.5 ml of diazotized reagent (0.004 M)	Structure	Absorbance
p-Nitroaniline		1.614
Naphthyl amine		1.2
p-Amino benzophenone		0.2

In (Table 1), it is noted that the diazotized p-nitroaniline (PNA) reagent gave the highest absorbance for the colored product, therefore, it was chosen as the diazotizing reagent for subsequent experiments.

Select the Base

The effect of different types of available bases was studied to determine their influence on the absorption of the formed azo dye. This was done by adding 1 ml of 1M of the selected bases to the reaction medium (0.5 ml of 1×10^{-3} M of diazotized reagent with 0.5 ml of 1×10^{-4} M BPA; i.e 4.5 µg /ml as coupling agent). The results in (Table 2) shows that NaOH is the best choice.

Table (2): Selection of the Base

1ml of base (1M)	**Absorbance**
NaOH	0.429
Na_2CO_3	0.002
NH_4OH	No color contrast
KOH	0.385

Amount of the coupling agent

The effect of different amounts of the diazotized reagent on the absorption of the resulting dye was studied using from 0.25ml to 0.75 ml of 4×10^{-3} M with 1-4.5 µg of BPA/ml, as (Table 3) shown, 0.75 ml of the diazotized reagent yielded the highest absorbance.

Table (3) Select the amount of the Diazonium Reagent

Volume of (PNA) soln. (ml)	**Absorbance/µg of BPA/ml**								
	1	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**R^2**
0.25	0.063	0.113	0.118	0.164	0.190	0.197	0.133	0.137	0.95
0.5	0.083	0.137	0.197	0.255	0.275	0.293	0.365	0.355	0.97
0.75	0.105	0.143	0.188	0.275	0.338	0.369	0.412	**0.508**	**0.98**

Selection of base amount:

Different amounts of the base, ranging from 1.0-2.5 ml of 1 M, were used to follow from 2.0 to 5.5 µg/10 ml of BPA. The results shown in (Table 4) exhibit that 1.5 ml of the base gave the highest absorbance and the highest determination coefficient.

Table (4): Effect of the Base Amount

Volume of 1M NaOH (ml)	**Absorbance, µg/ml of BPA**								
	2.0	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**R^2**
1	0.233	0.325	0.383	0.469	0.491	0.590	0.665	0.616	0.966
1.5	0.230	0.323	0.428	0.469	0.534	0.633	0.656	**0.776**	**0.986**
2	0.251	0.339	0.427	0.474	0.664	0.625	0.527	0.572	0.972
2.5	0.246	0.349	0.377	0.496	0.575	0.552	0.639	0.647	0.938

Study the coupling time

To study the time required for complete the coupling step, the reaction mixture was left for times 2, 2.5,3,4, and 5 minutes before dilution, the results in (Table 5) shows time of 2.5 minutes is sufficient for complete coupling, more time may cause in partial dissociation of the formed dye.

Table (5): Study the effect of coupling time

Absorbance	Coupling time, minutes				
	2	2.5	3	4	5
	0.517	0.630	0.614	0.564	0.424

Effect of surfactants

To determine the effect of surfactants on the absorbance and color contrast of the formed colored product, 1 ml of different types of surfactants (cationic, anionic, and neutral) was added to the reaction medium. From the results recorded in (Table 6), it was observed that there was no improvement in absorbance, so their use was excluded in subsequent experiments.

Table (6): The effect of surfactants on the absorption of the formed product.

Volume of ml	Absorbance / 1 ml of surfactant solution			
	SDS* (1x10^{-3}M)	CPC*(1x10^{-3}M)	CTAB* (1x10^{-3}M)	TritonX-100 (1%)
1	0.104	0.531	0.613	0.607

*Cetyltrimethylammonium bromide (CTAB), Sodium dodecyl sulphate (SDS), Iso-octylphenoxypolyethoxyethanol (Triton X-100), Cetylpyridinium chloride (CPC), absorbance = 0.630 without surfactant

Stability of the dye

The stability of the formed dye was studied by measuring the absorbance against a blank solution at time periods. The results in (Table 7) show that the dye forms immediately and remains stable for a maximum of 60 minutes at room temperature.

Table (7): The stability of the dye

Temperature	**Absorbance /Time (min)**								
	0	**5**	**10**	**15**	**20**	**30**	**40**	**60**	**(overnight)**
7-10 C^{o}	0.634	0.645	0.642	0.647	0.653	0.640	0.639	0.600	0.565
20-25 C^{o} (R.T)	0.634	0.629	0.622	0.625	0.625	0.626	0.628	0.627	0.601

The final absorption spectrum

A water-soluble orange azo dye is formed between the diazotized p-nitroaniline reagent and BPA in NaOH medium, the former stable azo dye shows maximum absorption at 478 nm, compared to the blank solution which shows very little absorption at the same maximum wavelength. Fig. (1) shows the final absorption spectrum.

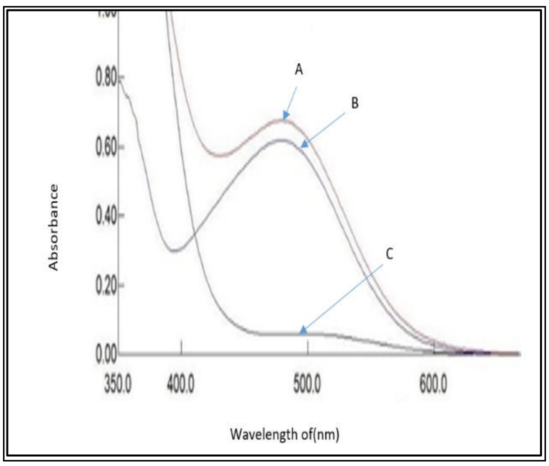


Fig. 1: Absorption spectrum of 4.5 µg/ ml of BPA measured: (A) against distilled water (B) against the blank solution (C) blank solution against distilled water.

Approved working method and standard curve

Increasing volumes of a BPA solution (4×10^{-4} M) were added to a series of 10 ml volumetric flasks containing 0.75 mL of a diazotized para-nitroaniline reagent solution at a concentration of (4×10^{-3} M), 1.5 mL of 1 M sodium hydroxide was added, left for 2.5-minute as a coupling period, and diluted to 10ml with distilled water. The absorbance was measured immediately after diluting; the absorbance was measured at a wavelength of 478 nm. Fig. (2), it was found that Beer's Law applies in the range of 0.1-5.5 µg.ml^{-1}, There's a positive deviation after the upper Beer's law limit.

The calculated value of the limit of detection (LOD) for the standard curve was found to be 0.02861 µg/ml, and the limit of quantification (LOQ) was 0.09539 µg/ml. The determination coefficient is 0.9992, statistically, this indicates excellent linearity specifications. The molar absorptivity was determined to be 3.230305×10^{-4} l·mol^{-1}·cm^{-1}, which suggests the method has high sensitivity.

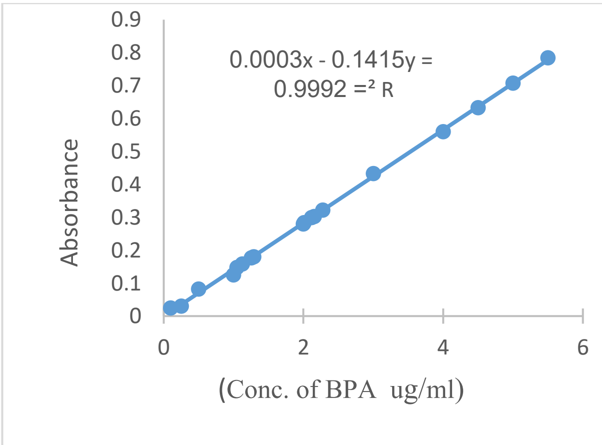


Fig. 2: Standard curve for the determination of BPA by the azo coupling method using the diazotized p-nitroaniline reagent

Accuracy and precision

The accuracy and agreement of the method were examined by calculating the relative error (RE)and relative standard deviation (RSD) of three different concentrations (5, 20, 45 µg/10 ml) of the standard solution under study. The results, shown in (Table 7), indicate that the method has good accuracy and precision.

Table (7): Accuracy and selectivity of the method

BPA µg / ml	Recovery%*	RE %*	RSD%*
0.5	99.2	-0.8	1.428
2	99.5	-0.5	0.540
4.5	99.444	-0.556	0.315

*Average of three determinations

The nature of the resulting dye.

The ratio of the reaction between BPA with the diazotized PNA, two methods were used: the continuous variation method and the mole-ratio method (Job's method; Delevie, 1997).

The method of continuous variations (Job's method) and mole ratio method:

A number of solutions containing different volumes were prepared (0.2–0.8 mL) of BPA and decreasing quantities (0.2–0.8 mL) of the diazotized PNA of the same concentration (4×10^{-4}M).

Fig. (3) illustrates the Job's method and Fig. (4) illustrate molar ratio, in which 0.4 ml of a BPA (4×10^{-4} M) was added to different volumes (0.2-0.4 ml of the diazotizing PNA of the same concentration. It was observed from both methods, that the molar ratio of BPA to the diazotized reagent is 1:1.

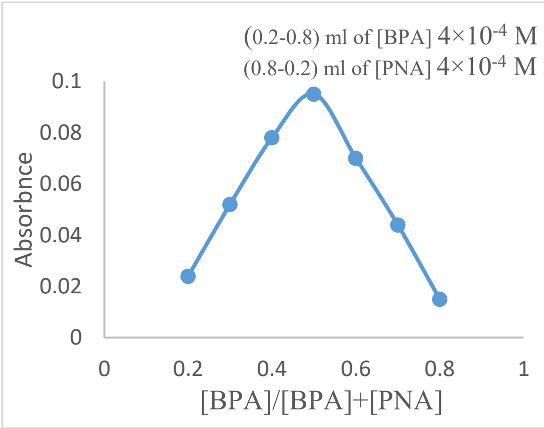


Fig. 3: Job's method curve for the azo dye

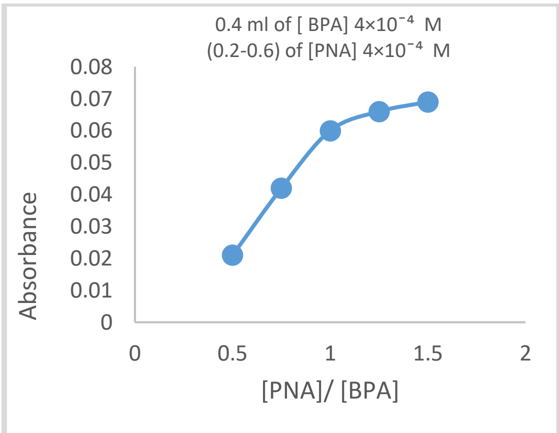
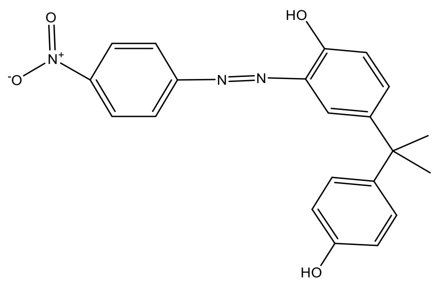


Fig. 4: Mole ratio curve for the azo dye

According to the ratio of the formed azo dye, the suggested structure between BPA and the diazotized PNA is as follows:



Azo dye

Application of the method

The proposed method was applied to estimate BPA in different samples of artificial milk extracts for four types of baby bottles. From the prepared solutions (see 'Preparation' section), 0.5 and 1 ml were taken from the Mini brand, as well as from the other types of baby bottles. The results are shown in (Table 8).

Table (8): Application of the method

Plastic baby bottles contain the milk	Standard		Extract sample			Recovery %	RSD%	t-test
	Amount taken µg/ml	Average* Absorbance	Amount Found µg/mL	Average *absorbance	Amount Found µg/xml			
Mini baby Made in China	1.253	0.177	1.224	0.173	0.0612	97.685	0.578	2.158
	2.009	0.284	2.018	0.2853	0. 2018	100.947	0.537	1.129
Bebedor Birlik company /Thailand	1.123	0.158	1.132	0.160	0.0566	100.801	0.625	3.4
	2.118	0.299	2.122	0.3003	0.2122	100.188	0.357	1.700
Aillin Kala Koodak Toos company /Iran	1.288	0.180	1.297	0.1833	0.06485	100.698	0.315	6.855
	2.155	0.302	2.171	0.307	0.2171	100.742	0.564	4.907
AVEHINI/ China	1.050	0.148	1.062	0.150	0.0531	101.142	1.333	1.7
	2.277	0.322	2.274	0.3216	0.274	99.868	0.181	0.582

The results presented in (Table 8) confirm the success of the proposed method in estimating the organic compound BPA milk extracts from different types of baby bottles. The results show that the plastic used in the bottles released BPA into the milk at similar levels, but the Chinese AVEHINI plastic bottle released the highest levels. The reliability of the method was confirmed by t-test.

Standard addition method

The standard addition method (Harvey, 2000) was used to demonstrate the efficiency and accuracy of the proposed method in estimating BPA in milk extract. The results are shown in Fig. (5) and Fig. (6), which represent the standard addition curves for estimating BPA in milk extract 1.123 and 2.118 µg/ ml of Be Bedor Birlik company /Thailand and 1.288 and 2.155 µg/ mL^{-1} for the Iranian-made baby bottle. The results of standard addition method were listed in (Table 10), in which the recovery ranged from 96.055 to 99.922%.

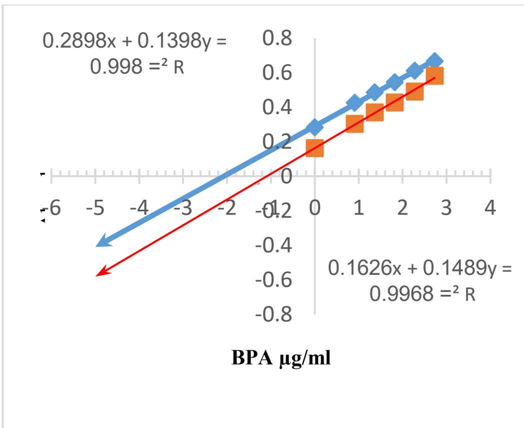


Fig. (5): standard addition curve for the determination of BPA in milk extract from Bebedor Birlik company /Thailand

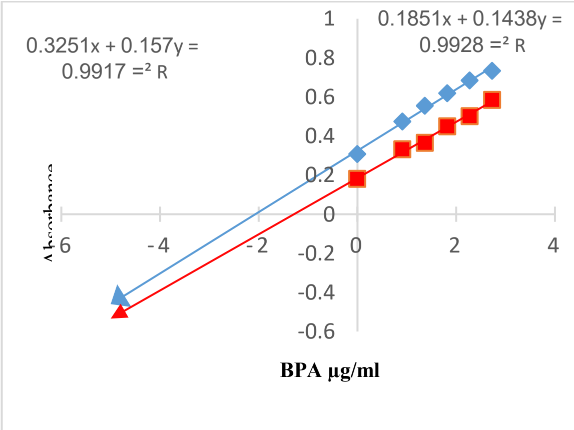


Fig. (6): Standard addition curve for the estimation of BPA in Iranian baby bottle milk extract.

Table (10): The results of standard addition method

Milk extract	Amount taken µg/10ml	Amount found µg/10ml	Recovery%
Bebedor Birlik company /Thailand	1.123	1.092	97.239
	2.118	2.072	97.828
AVEHINI Made in Iran	1.288	1.287	99.922
	2.155	2.070	96.055

CONCLUSIONS

The proposed method is simple, sensitive, and fast. It relies on the azo coupling reaction between bisphenol A and the diazotized reagent p-nitroaniline, in a basic medium of sodium hydroxide to form a stable, water-soluble orange azo dye. This dye shows maximum absorbance at a wavelength of 478 nm. The method demonstrates good accuracy and precision, and it is highly sensitive. It was successfully applied to the determination of BPA in milk extract from various types of baby bottles.

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الصبغة الازوية لتقدير البيسفينول أ المنطلق من الزجاجات البلاستيكية الى حليب الاطفال الرضع

احلام احمد شهاب
قسم علوم الحياة/ كلية التربية للعلوم الصرفة/ جامعة الموصل

هناء شكر محمود **اياد جاجان خورشيد**
قسم الكيمياء/ كلية العلوم/ جامعة الموصل

الملخص

يعتبر مركب البيسفينول A (BPA) من المركبات العضوية واسعة الاستخدام في الصناعات الحديثة، خصوصاً في تصنيع لدائن البولي كربونات وراتنجات الايبوكسي. يعمل البيسفينول *A* على اضطراب الغدد الصماء بسبب بنيتهُ الكيميائية المشابهة لهرمون الاستروجين. في هذه الدراسة تم إجبار مادة BPA على الانطلاق من زجاجات الأطفال البلاستيكية إلى الحليب عن طريق التسخين؛ ثم يتم استخلاصها من الحليب وتقدير BPA طيفيا باستخدام تفاعل الاقتران الازوي. مع بارا–نترو أنيلين المؤزوت اذ تتكون الصبغة الازوية التي تظهر أعلى امتصاص عند الطول الموجي 478 نانومتر. وتميزت الطريقة بخطية جيدة ضمن مدى التراكيز 0.1–5.5 ميكروغرام/مل، مع حد كشف (LOD) بلغ 0.02861 ميكروغرام/مل وحد التقدير الكمي (LOQ) مقداره 0.09538 ميكروغرام/مل. كما أكدت قيمة الامتصاصية المولارية المحسوبة 3.230305×10^{4} لتر. مول$^{-1}$. سم$^{-1}$ الحساسية العالية والموثوقية المميزة للطريقة اذ تم تطبيق الطريقة لتقدير BPA المنطلق من بلاستيك أربع انواع من رضاعات الاطفال اذ اظهرت الطريقة ان البلاستيك المصنوع منه الرضاعات قد أطلق المركب BPA الى الحليب بنسب متقاربة ولكن بلاستيك الرضاعة الصينية AVEHINI أطلق النسبة الاعلى من المركب كما تم التأكد من موثوقية الطريقة باستخدام الاضافة القياسية واختبار t.

الكلمات المفتاحية: بيسفينول أ، حليب، اقتران الآزو، بارا–نيتروأنيلين، زجاجات الأطفال البلاستيكية