Developed and Rapid Extraction of Melamine in Infant Formulae by Combined Electromembrane with Nano Graphene Oxide Reinforced Hollow Fiber

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ABSTRACT: *Melamine is a high nitrogen compound used as an adulteration to high protein foods such as infant formulae. There are many different methods for extraction and analysis of melamine which are time-consuming, complex, and need large volumes of organic solvents. A validated method for extraction and cleanup of melamine (MEL) in infant formulae, water, and powdered coffee creamer was developed using a NanoGraphene Oxide (NGO) assisted with Electro Membrane Extraction (NGO/EME) followed by HPLC-UV detection. Supported Liquid Membrane (SLM) with NGO was used as the adsorbent interface in this study. Synthesized NGO was characterized by Fourier Transform InfraRed (FT-IR) spectroscopy and Scanning Electron Microscope (SEM). Effective parameters such as voltage magnitude, SLM solvent, pH of acceptor and donor phases, extraction time, and stirring rate were optimized. The method provided the LOD and LOQ 0.03, and 0.1µg/kg in infant formula, respectively. The accuracy was in the satisfaction recovery rate between 106-109% with RSD 4.83-5.31 for infant formulae as well as the other tested matrices. The developed method based on NGO/EME extraction presents a reliable and rapid analysis of melamine in infant formula.*

KEYWORDS: *Nanographene oxide; Melamine; Electromembrane; Infant formulae*.

INTRODUCTION

Melamine is a polar substance (see Fig. 1) that is widely used in industries. Because of the high nitrogen content (66% of MW) of MEL, it is added as adulteration to high-protein foods (see Fig.1) [1]. MEL is a white crystalline powder with pKa 5.11 with different applications in industries [2] such as papermaking, manufacturing laminates, and production of chemical fertilizers, and adhesives in the production of flame retardants and plastics, which are very common [3]. In 2007, MEL was detected in Chinese rice protein concentrate and wheat gluten that had been used in pet food in the United States. Consequently, some death in dogs and cats has happened due to kidney failure. MEL contamination was also reported from various brands of powdered infant formula, frozen yogurt desserts, and canned coffee drinks.

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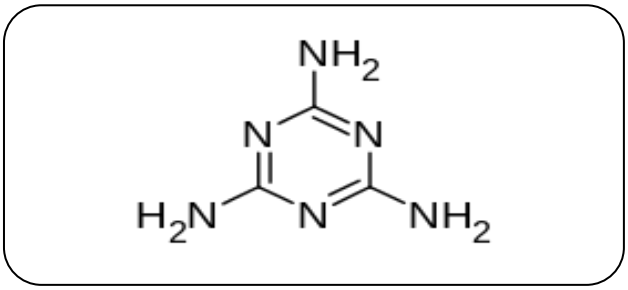

Fig. 1: The chemical structure of Melamine.

Adding MEL to milk to increase nitrogen content and pseudo increasing of protein index could be considered as an adulteration. The procedure leads to an error of about 16% in the measurement of milk product protein by the Kjeldahl method and Dumas tests. It is the second adulteration for covering the first adulteration (adding water to milk) [4]. In animal studies, melamine can cause bladder stones with the crystalline formation in combination with its metabolite Cyanuric acid, which also may be found in MEL powder [5]. Based on WHO regulations, the maximum residue limits (MRL) of melamine in infant formula and milk are 1 mg/L and 2.5 mg/L, respectively.

There are many different methods for the extraction and analysis of melamine in dairy products. Among the proposed methods, Liquid-Liquid Extraction (LLE) [6, 7], Solid-Phase Extraction (SPE, SPME), [8, 9] using a polymer molecular framework with a magnetic surface can be mentioned. These methods are time-consuming and complex and need a large number of organic solvents that are harmful to the environment and human health.

However, there are some challenges in MEL analysis such as the unsuitable sensitivity of conventional GC and HPLC detectors. Although derivation with suitable fluorophores or chromophores can enhance HPLC sensitivity and improve the chromatographic behavior of many compounds, such derivatization is associated with some disadvantages including lack of suitable deviating chemicals, the inefficiency of derivation, and the use of many different solvents.

Recently, Hollow Liber Liquid-Phase Microextraction (HF-LPME) is a relatively new technique proposed for pre-concentrate before appropriate analysis for complex matrixes. HF-LPME is a high degree of cleanup and an appropriate method for complex matrices. However, choosing a proper and suitable solvent and its time-consuming are major limitations of this method. The techniques were used to extract melamine from milk samples [10]. *Pedersen et al.* (2006), have published a new electro-membrane method based on liquid-phase microextraction for the first time [11].

The procedure is derived from three phases including the donor phase (sample matrix) that the analytes pass electro-kinetically migration through SLM (second phase) by applying an electric potential difference and finally enter the lumen of hollow fiber membranes (acceptor phase) [12].

Raman spectroscopy [13], an immunologic method like Enzyme-Linked ImmunoSorbent Assay (ELISA) [14], HPLC with UV detection and fluorescence spectrophotometry, capillary electrophoresis liquid and gas chromatography with mass spectrometry, and UPLC ultra-performance liquid chromatography-tandem mass are some of the instrumental methods which have been used to identify and determine MEL.

The application of Graphene (G) in modified electrochemical sensors has been increased because of its special properties such as large surface area as well as unique thermal, electrical, and mechanical properties [15-19]. There is a strong π stacking interaction with the benzene ring because of the presence of a large delocalized π-electron system [20-22]. Pre-concentration of the analyte and convenient separation occur due to the combination of the strong π stacking interaction of G. The presence of carboxyl, hydroxyl, and epoxy polar groups on the surface of the lamellar structure resulted in good dispersion of G in aqueous media [23]. Graphene Oxide (GO) can be used as a novel adsorbent in SPE and microextraction techniques because of the large surface area. The carboxyl groups in graphene oxide increase the electrical conductivity and migration of the analyte from the donor to the acceptor phase. With increasing electrical conductivity due to GO, the low voltage should be applied, and consequently, leads to better repeatability and migration of the analyte. It has been also found that GO increases detection sensitivity (low detection limit) and also increases recoveries of polar compounds such as MEL in a complex matrix. EME has less extraction time than other HF-LPME methods.

The hollow fibers play a significant role in filtering and increasing cleanup efficiency in complex matrices such as infant formula. Saturated fat and proteins cannot transfer through the wall pores of the HF, therefore the interested analyte is well purified and concentrated ready for the analytical process. [24].

The extraction of MEL by hollow fiber reinforced nanographene oxide coupled with HPLC- UV detector and

NH2 column in infant formula has been carried out. Additionally, two other matrices mean water and powdered coffee creamer were studied beside infant formulae to compare the ability of the method in different conditions. The parameters were considered to optimize for reducing the time of extraction and less consumption of solvents.

Several experimental parameters that influence the extraction of MEL were investigated. Finally, the table of merit of the proposed method was compared with previously published methods.

EXPERIMENTAL SECTION

Chemicals and reagents

MEL standard powder with 98% purity was purchased from Sigma-Aldrich (Germany). 1-Octanol, acetonitrile, hydrochloric acid, sodium hydroxide, and acetone were taken from Merck. Ultra-pure water was used from a Millipore system (Le monster-Lausanne, Switzerland). All reagents and solvents were analytical grades. The hollow fiber as a supported polymeric membrane was supplied from Membrana GmbH (Accurel Q3/2polypropylene hollow fiber, 600 μm i.d., 200).

Preparation of standard solutions and real samples

MEL stock solution (500 μgmL^{-1}) was prepared through the dissolving of melamine powder in distillation water Followed by sonication until the powder was completely dissolved. The stock solution was kept in a refrigerator. Working standard solutions were prepared from the stock solution by proper dilution. Dairy products including fresh bovine milk and infant formula were purchased from the market in Tehran Iran.

The infant formula sample was weighed and dissolved completely in hot water (50 °C) based on the manufacturer's instructions.

Synthesis of NGO Powder

NGO was produced through Hummer's method [25]. 1 g of graphite was added to 23 mL of H_2SO_4, 0.5 g of Sodium nitrate was added and the temperature was kept constant in the range 0–5 °C. 3 g of $KMnO_4$ was added in quota into the mixture, which was stirred for about 10 min. The beaker was heated and kept at 30-35°C for 12 h. Then, the deionized water and H_2O_2 were added. Then the reaction mixture was stirred at 60 0C for 3 hours. Finally, NGO powder in brown color was prepared and ready

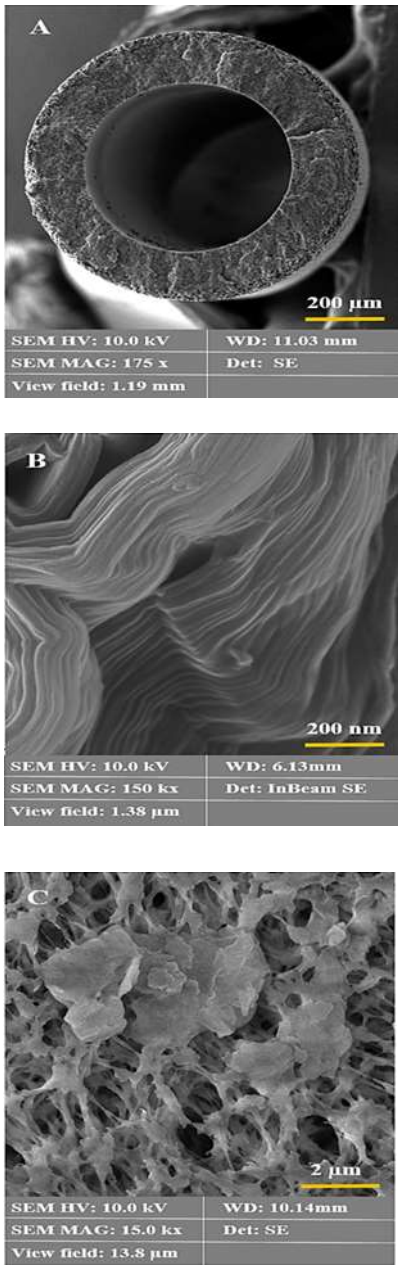

Fig. 2: SEM images of (A) Cross-section of the hollow fiber only (B) NGO powder (C) NGO immobilized in HF porous.

For further processes. Scanning Electron Microscopy (SEM) (LEO, Model 1450VP, Germany), and EDS were used to characterize the NGO (see Figs. 2, 3).

Preparation NGO immobilized membrane

To prepare the NGO immobilized membrane, the polypropylene hollow fibers were cut into 5 cm pieces

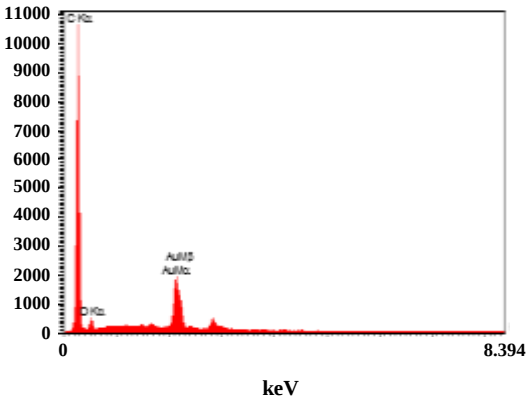

Fig. 3: EDS of NGO.

and were cleaned in acetone to remove any pollutants. After that, 20 µL of dispersed NGO in 1-octanol was injected into the lumen of the hollow fiber with a micro-syringe. The hollow fiber was reinforced by NGO and used for the immobilization of the SLM (see Fig. 2). Finally, one end of the hollow fibers was closed by using a hot surface before sample preparation.

Extraction of MEL by EME

Twenty milliliters of the sample solution containing the interested analyte in pure water was transferred into the sample vial. Then pH was adjusted to 3.0 with 10 mM HCl as the donor solution. The prepared solution in the donor phase was ready for extraction. For preparing the EME cell, the hollow fibers including the SLM (1-octanol with NGO) were placed into donor solution. 20 µL as acceptor solution (HCl pH 2.0) was then introduced into the lumen of the hollow fiber manually by using an HPLC syringe. platinum electrodes with 0.25 mm diameter were placed in acceptor solution (cathode) and into donor solution (anode) then connected to DC power supply (Biorad® with voltage range between 0-600) with a voltage of 70 V and the extraction was continued by applying a magnetic stirrer for 10 min at 750 rpm. After completing the extraction, 20 µL of the acceptor solution was injected into HPLC for further analysis.

Analytical conditions

Melamine was determined by HPLC DIONEX equipped with ULTIMADE 3000 PUMPE, UVD170U detector UV-Vis, and a six-port Rheodyne valve with a 20 µL sample loop, NH_2 column (WATERS, 3 µm, and 15 mm 4.6). The mobile phase includes an acetonitrile and water volume ratio 80:20 and the flow rate was set at 1mL per min. The detection was performed at a wavelength of 220 nm [6].

RESULTS AND DISCUSSION

Optimization of the EME method

To obtain high enrichment and extraction efficiency of the analytes using this technique (NGO/EME), the main parameters were optimized. The chemical properties of the organic solvents used as SLM are important in extraction efficiency, and some are critical for success and reliable electro-kinetic cross-membrane extraction [26]. In common, the SLM should be chosen based on some parameters such as well compatibility with both GO and HF, low aqueous solution (donor phase) solubility, proper partition coefficient for the analyte, and non–volatile property [27]. The organic solvent used as SLM should fulfill a few requirements, such as very low water solubility, low volatility, and low viscosity so that the SLM will not partly dissolve in the two aqueous phases and not evaporate during EME [28]. Also, GO must be well dispersed in the organic solvent [27]. In this study, we experienced some different nitro-aromatic solvents consisting of 2-Nitrophenyl octyl ether, di (2-ethyl hexyl) phosphate, Bis 2-ethyl hexyl) phosphate, and mix nitro-aromatic solvent and 1-octanol as SLM. Among tested solvents, GO was well dispersed in 1-octanol and it was found to be more effective for the extraction of MEL.

In EME, the applied voltage has been proven to act as the dominant driving force which is an important principle for the efficient extraction of the analytes. When the extraction is performed above a certain optimal voltage, the EME efficiency may also decrease. This might be because the current across the SLM may be relatively high with higher voltage, and electrolysis may occur at the electrodes. To achieve the optimum condition for electric potential was applied the potential was in the range of 0-90 V. The results are shown in Fig. 4. Thus, the selected voltage was considered 70 V for the study.

Extraction time and stirring rate are influencing factors in increasing the kinetics and the yield of extraction. The stirring rate enhances the mass transfer of the analyte and reduces the thickness of the double layer around the interface on both sides of the SLM. The applied stirring rate for extraction of MEL was between 220-1000 rpm and the optimum rate was identified at 750 rpm (Fig. 5).

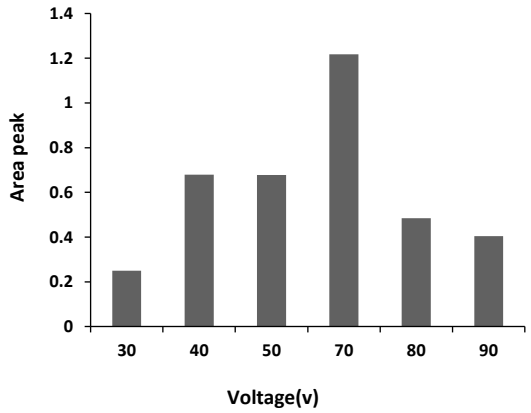

Fig. 4: Effect of applied potential on the peak area of 2 mg/mL NGO in 1-octanol, 10 mL HCl with pH=5 as donor phase, 700 rpm stirring rate, 15 min extraction time.

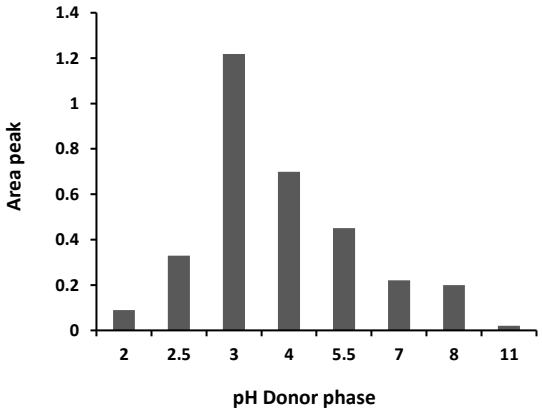

Fig. 6: Effect of pH donor phase on the peak area of 2 mg/mL NGO in 1-octanol, 700 rpm stirring rate, 15 min extraction time, applied 70 v.

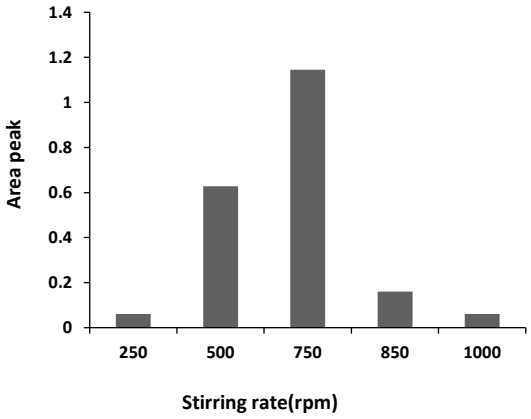

Fig. 5: Effect of Stirring rate on the peak area of 2 mg/mL NGO in 1-octanol, 10 mL HCl with pH=5 as donor phase, 15 min extraction time, applied 70 v.

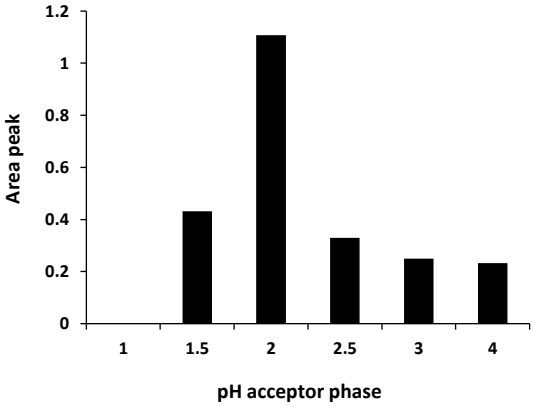

Fig. 7: Effect of pH acceptor phase on the peak area of 2 mg/mL NGO in 1-octanol, 10 mL HCl with pH=5 as donor phase, 700 rpm stirring rate, 15 min extraction time, applied 70 v.

The results confirmed that the agitation of the sample enhances extraction. However, higher stirring rates (>750 rpm) resulted in massive air bubbles and decreased pre-concentration factors.

Time is the other variable that can influence the flux of the analyte in the electrokinetic cross-membrane extraction. To evaluate the role of time in the electrokinetic migration of the analyte, the extraction efficiency was studied between 5 and 30 min. In this study, the optimum extraction time was determined at 15 min (Fig. 8). EME recoveries may increase slightly with increasing stirring rate and extraction time but may cause double layer formation on hollow fiber surface due to the formation of attached air bubbles to surface. Applying NGO-EME with reduced extraction time and stirring rate (5 min and 750 rpm) is a useful strategy for overcoming this problem, consequently reinforced HF enhances mass transfer in a very short time and lower stirring rate, and as a result, decreasing the double-layer intense ionization of the analyses in the sample solution was essential for achieving electrokinetic migration [29].

The donor phase pH is another key parameter for the optimization of the process. Therefore, a range of 2 to 11 was considered for the pH of the sample solution to find the optimum pH of the donor phase. The results illustrate that the extraction of the analyte would be more efficient at pH 3 in the donor solution (Fig. 6). The pH of the acceptor phase should have a pH of 2 for obtaining the maximum extraction efficiencies (Fig. 7). In the extraction system using EME, the pH of the acceptor phase has more

Table1: Quantitative result of NGO-EME from melamine in the aqueous, powdered coffee creamer, and infant formula samples.

Matrix	LR[a]	Equation	R^{2b}	LOD[c]	LOQ[d]	RSD[e]%
Water sample	0.02-14000	y = 1.1924x - 0.028	0.998	0.01	0.031	1.024
Infant formula	0.04-2500	y = 4.7464x - 1.1539	0.997	0.03	0.1	4.85
Powdered coffee creamer	0.09-18000	y = 5.0352x + 0.4589	0.995	0.07	0.19	2.52

a) Liner range (ppb), b) Correlation coefficient, c) Limit of Detection (ppb), d) Limit of Quantitation (ppb), e) Relative standard deviation

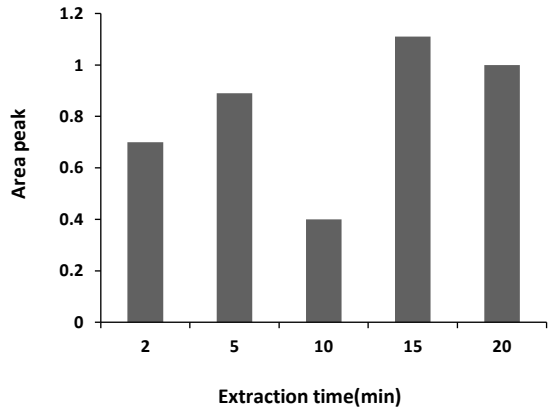

Fig. 8: Effect of extraction time on the peak area of 2 mg/mL NGO in 1-octanol, 10 mL HCl with pH=5 as donor phase, 700 rpm stirring rate, applied 70 v.

important than the donor phase. The Presence of MEL as ion form (cation) is critical to achieving efficient transfer from the donor phase into the SLM [30].

Analytical performance

Fig. 9, shows the HPLC-UV chromatograms obtained from the analysis of standard MEL, the matrix, and the spiked sample with 600 (ppb) of MEL in infant formula after extraction by the proposed method.

The analytical method validation parameters under optimized extraction conditions including the Linear dynamic Range (LR), Limit of Detection (LOD), Limit of Quantitation(LOQ), Relative Standard Deviations (RSDs), correlation of coefficient(r^2), and Equation were evaluated in water, powdered coffee creamer and infant formula samples. These results were shown in Table 1. LOD in water, powdered coffee creamer, and infant formula were detected according to a Signal-to-Noise (S/N) ratio of 3. The reproducibility was calculated by performing a 5 replicates analysis.

The method showed good linear ranges of 0.02-18000 (ppb) with correlation coefficients greater than 0.995-0.99 (ppb) for water samples, infant formula, and powdered coffee creamer. The limit of quantification (LOD) value of the present method for a water sample, infant formula, and powdered coffee creamer was 0.01-0.07(ppb).

Method performance for MEL was determined in several matrices. For recovery determination (accuracy) the spiked samples in different levels were applied for each matrix. Repeatability of the NGO-EME measurements reported as RSD values in aqueous, powdered coffee creamer, and infant formula samples, varied between 1.02 and 4.85% for inter-day and 3.16 to 3.32% for intra-day (Table 2).

In comparison with the other conventional sample preparation methods, the developed method has the merits of considerable analysis speed, appropriate separation efficiency, notable precision, and high sensitivity. The method was compared with the other previous works. As shown in Table 3, the LOQ of this method is lower than other conventional methods. Also, it is evident that the proposed method has a wide dynamic linear range. Regarding the running cost and complication of the instrument, this method has a moderate running cost by using the common instrument of HPLC-UV-Vis which could be applied in routine MEL analysis in food control laboratories.

Moreover, the concomitant use of hollow fibers and graphene oxide nanoparticles increases electrical conductivity and accelerates MEL migration in a short time, for different samples.

CONCLUSIONS

The present study proposed a rapid and reliable method for extraction and detection of MEL in infant formulae matrix by NGO-EME. The electro-membrane extraction was performed by hollow fiber which was reinforced by nano-graphene oxide. The LOD and LOQ of the proposed method were improved in comparison with other studied methods. The extraction procedure was followed by high-performance liquid chromatography coupled with an ultraviolet detector (HPLC-UV). It is concluded that

Table 2: The accuracy of NGO-EME method in the aqueous, powdered coffee creamer, and infant formula.

	Infant formula			Powdered coffee creamer			Water sample		
	Concentration (ppb)	%RSD[a]	RR[b]	Concentration (ppb)	%RSD	RR	Concentration (ppb)	%RSD	RR
Inter day	600	4.83	109	250	3.16	109	700	3.9	93.1
intra-day	600	5.31	106	250	3.32	105.9	700	4.1	101.4

[a] *Relative standard deviation (n=5).* [b] *Relative recovery after the spiked amount of analyte.*

Table 3: The efficiency of the NGO-EME developed method in comparison with the last reported methods.

Method	Matrix	Calibration range	R^2	LOQ	Ref
HF-LPME	fresh milk	0.1-50 mg/kg	0.9993	0.01 mg/kg	[31]
DLLME[a]	Infant formula	1.0 -500 µg/L	0.9993	0.3 µg/L	[32]
Acetonitrile-free	infant formula	1.0–80 µg/ mL	0.9993	0.2 µg/ml	[33]
Buffer method extraction	milk products	0.05-10 µg/ml	0.9994	0.08 µg/ml	[6]
NGOs/EME	Infant formula	0.04-2500 ppb	0. 997	0.1 ppb	This study

[a] *Disperse Liquid-Liquid Microextraction*

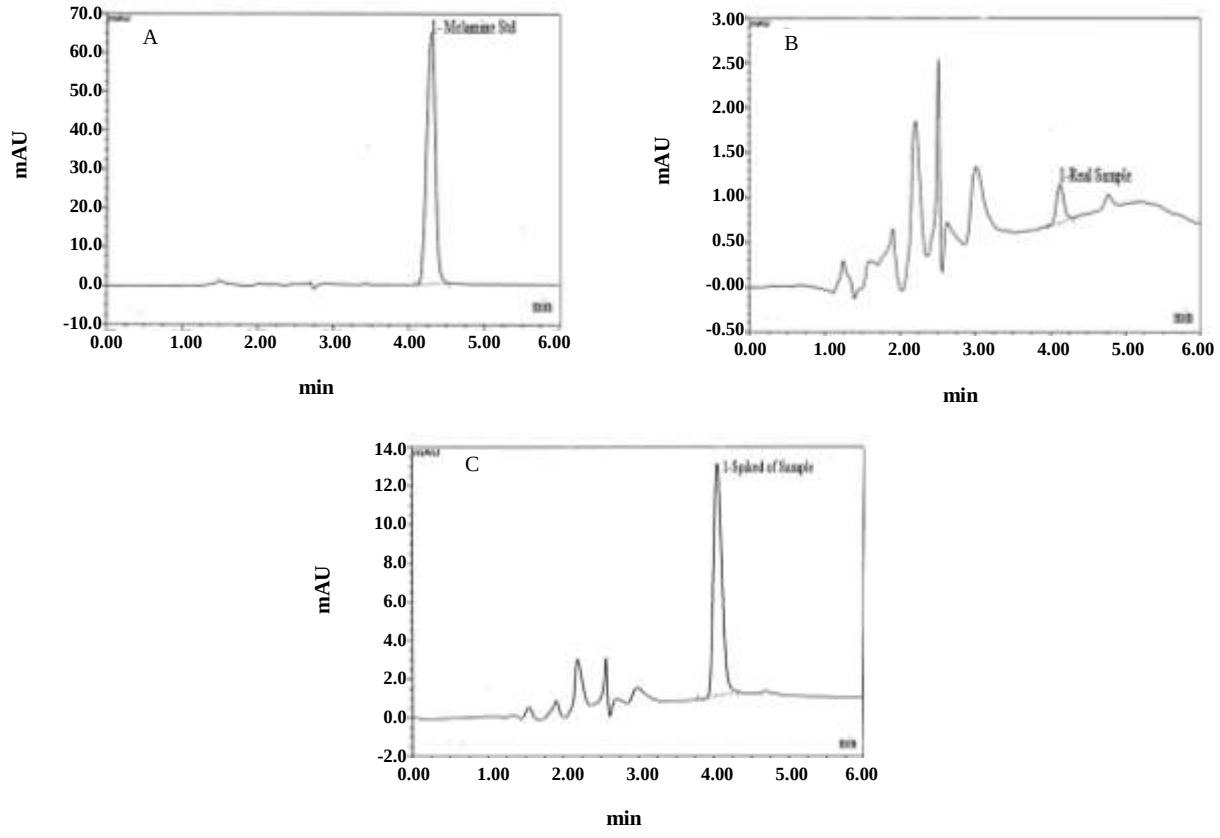

Fig. 9: Chromatogram of standard MEL (A), real sample (B), spiked of MEL in the real sample extracted by the NGO-EME method.

this method is sensitive, cleanup performance, cost-effective, and environment-friendly compared with the conventional LPME method. Finally, the present method is considered for the routine analysis of MEL added in infant formula or other similar matrices like powdered coffee creamer.

Abbreviations

ELISA	Enzyme-Linked Immunosorbent Assay
EME	Electromembrane Extraction
FTIR	Fourier-Transform Infrared
GO	Graphene Oxide
HF-LPME	Hollow Fiber Liquid-Phase Microextraction
LLE	Liquid-Liquid Extraction
MEL	Melamine
MRL	Maximum Residue Level
NGO	Nanographene Oxide
SEM	Scanning Electron Microscope
SPE	Solid-Phase Extraction
SLM	Supported Liquid Membrane

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