Journal of Chemical Health Risks

www.jchr.org

ORIGINAL ARTICLE

Application of Graphene Oxide Reinforced Hollow Fibers as a Novel Electromembrane Extraction Method for Quantitative Analysis of Dicyandiamide in Infant Formula

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(Received: 15 September 2018 Accepted: 13 December 2018)

KEYWORDS

Dicyandiamide;
Electromembrane;
Graphen oxide;
Infant formula

ABSTRACT: Dicyandiamide (DCD) is a nitrogenous compound which is generally used as a fertilizer, antimicrobial and nitrogen enrichment agent in soil. Grass and drinking water contamination with DCD may lead to presence of the chemical residues in milk and infant formula. The aim of this study was to investigate a rapid, simple and accurate method for quantitation of DCD in infant formula using electromemebrane extraction with reinforced hallow fiber by graphene oxide (GO-EME). The extraction method was optimized by solvent, graphene oxide concentration, voltage, stirring speed, pH and time parameters. After extraction, DCD was analyzed by HPLC coupled with UV/Vis detector. The analytical method validation parameters including accuracy, precision, LOD and LOQ were determined to ensure the method's validity.The calibration curve with a correlation ratio of 0.999 was obtained. The recoveries were from 78.0% to 80.0%. The LOD and LOQ were 0.04 mg L^{-1} and 0.1 mg L^{-1}. This study indicates that the graphen oxide-electromembrane extraction is a simple, accurate and rapid method for determination of DCD in infant formula.

INTRODUCTION

Dicyandiamide (DCD) is a chemical compound, widely-applied as a soil fertilizer and prevent nitrogen loss in soil (Figure 1) [1, 2]. It has been also used in cow pastures to increase the growth of grass. However, grass and drinking water contamination with DCD may lead to presence of the chemical residues in milk products. In 2012, DCD residue contamination has been reported in dairy products of New Zealand [3].

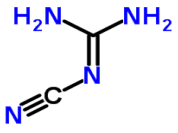

Figure 1. Structure of dicyandiamid.

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DOI: 10.22034/jchr.2018.545323

Measurement of nitrogen content of food product, as an index of protein content, is a common test in food control laboratories. The addition of chemical compounds with high nitrogen content, lead to increase in apparent total protein content which is a common adulteration in food product [4, 5]. Melamine is one of the chemical compounds with 66 percent nitrogen content which was added to water-diluted milk to increase the apparent protein content. This adulteration led to kidney problems and three infant's death [6, 7]. DCD is also a nitrogen-rich compound which can be used to increase false protein in food.

However, both migration and intentional using of DCD residue to milk and dairy products has become a severe concern because of the possible risk to children's health. Methemoglobinemia and eczema have been reported as some DCD-related diseases [8].

Titration, calorimetric, weighting, polarography, IR (Infra-red) absorption, Raman and chromatography are the most common analytical methods in identification of DCD [9]. Although there are a wide range of methods for analysis of DCD, some of them have disadvantages. In the titration technique, amino guanidine is an interferential agent in the silver nitrate titration. The polarography method is highly time-consuming. Gravimetric technique has also a number of measurement difficulties. One of the other proposed techniques is IR spectroscopy, which is suitable for non-aqueous samples such as medicines [9, 10]. Moreover, high performance liquid chromatography techniques [11], liquid chromatography with mass spectroscopy detector (LC-MS/MS) [12, 13], direct analysis in real time (DART) ionization source coupled with Quadrupole Time-of-Flight Tandem Mass Spectrometry (Q-TOF MS/MS) [14] are other technique for DCD analysis.

Liquid-liquid extraction, solid-phase extraction (SPE) and microwave techniques are used as the extraction method for DCD analysis [15, 16].

In the present study, we decided to apply a rapid, simple, accurate and environment friendly analysis method for quantitation of DCD in infant formula using electromemebrane extraction with reinforced hallow fiber by graphene oxide (GO-EME).

MATERIALS AND METHODS

Chemicals and reagents

DCD reference standard was obtained from Sigma-Aldrich (Germany). Graphite, Sodium hydroxide, 1-Octanol, hydrochloric acid, acetonitrile and acetone were obtained from Merck, Darmstadt, Germany. Ultra-pure water was used from a Millipore system (Le Montsur-Lausanne), Switzerland. All reagents and solvents were analytical grade. PPQ3/2 polypropylene hollow fibers were purchase from Membrane Co. (Wuppertal, Germany) with an inner diameter of 0.6 mm, a thickness of 200 μm, and a pore size of 0.2 μm.

Standards and sample preparation

Due to good solubility of DCD in polar solvents, the standard stock solution containing 1 mg L^{-1} of DCD was provided in deionized distilled water. 10 mM of hydrochloric acid solution was used to prepare working standards for drawing the calibration curve. Spiked samples were prepared at the concentration of 1 to 5 mg L^{-1} by adding DCD standard solution to infant formula.

Hollow fibers preparation

The hollow fibers were cut into a length of 5 cm pieces, dipped in acetone for 10 minutes and placed in an ultrasound bath for 10 seconds to remove the impurities and contaminants of the hollow fibers. Hollow fibers were dried in air. The graphene oxide (GO) synthesis was based on the Hummer method [17]. The quality of synthesis was confirmed by FT-IR, as well as Scanning Electron Microscope (SEM; Tescan; Czech Republic).

Briefly, 2 mg of GO in 1 ml 1-octanol was sonicated in an ultrasonic bath for 90 minutes to disperse GO in solvent. Then, 20 μl of dispersed GO was injected into hollow fibers with Hamilton syringe. The excess dispersed GO was gently washed to remove from the fiber lumen. The excess

GO may results in damage of chromatography column during HPLC analysis. The end of the hollow fibers was closed using heat. Finally, 20 µl of the acceptor phase containing 10 mM HCl was injected to the hollow fiber lumens using the microsyringe (Hamilton).

EME set-up and procedure

The electromemebrane extraction (EME) method and the preparation of hollow fiber-based EME tubes have been explained by P.-Bjergaard and A.Gjelstad [18]. In this study, 15 µl Supported Liquid Membrane (SLM) was used for each extraction. One platinum electrode was placed inside the lumen as cathode electrode and another one was placed in the sample solution as anode electrode. Then, both electrodes were connected to a POWER-PAC-3000 power supply (BIO-RAD), and the extraction process was performed by applying an optimum voltage of 70 V between the electrodes and the rotation simultaneously with magnet stirrer (Figure 2). Following the end of extraction, the power supply and magnetic stirrer were turned off. The acceptor solution was immediately collected by Hamilton syringe and analyzed using HPLC-UV.

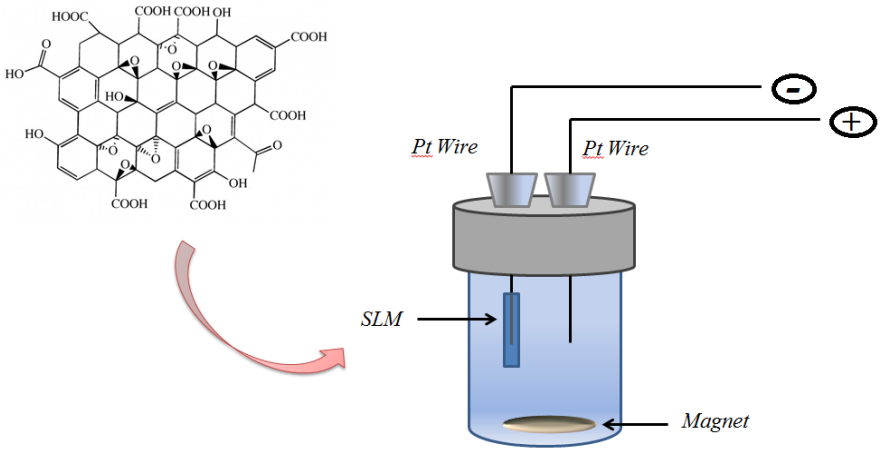

Figure 2.

The equipment used for EME.

Analytical conditions

The analysis conducted using a Dionex Ultimate 3000 HPLC system from American company Dionex, including the Ultimate 3000 pump. Chromeleon software from Dionex was used to control tools and data processing. The analyte detected with UV/VIS detector (VWD-3400) with a wavelength of 220 nm. The temperature of the column oven (Tcc-100 from Dionex Corporation) was set at 30 ° C. Weston C18 column (250 mm × 4.6 mm, 3 µm) was selected from the Water Companies for conducting the separation. The volume of injection was 20 µl with the flow rate of 0.5 ml/min. The mobile phases containing phosphate buffer (50 mM and potassium dihydrogen phosphate) and acetonitrile with a ratio of 80 and 20 (V/V) were used due to good separation of DCD.

Method validation

The analytical method validation parameters including accuracy, precision, LOD and LOQ were determined.

RESULTS AND DISCUSSION

Characterization of GO

GO synthetize was done according to Hummer method [17]. The quality of synthetized GO was evaluated by FT-

IR and SEM. FT-IR shows the functional groups in a molecule over the wave number range of 4000-500 cm^{-1}. The oxidation of the geraphite was confirmed by FT-IR spectrum. Various types of oxygen functionalities in graphene oxide at broad and wide peak at 3418 cm^{-1} were ascribed to the O-H stretching vibrations of the C-OH groups and water [19, 20].

The stretching vibrations at 1726 and 1048 ascribed to C=O and C-O respectively. The absorption band at 1624 attributed to the C=C of un-oxidized graphitic domains, confirmed the success of geraphene oxide synthetize (Figure 3). The surface morphology of the prepared GO was confirmed by SEM. Figure 4A and 4B showed the sheet-like structure of GO.

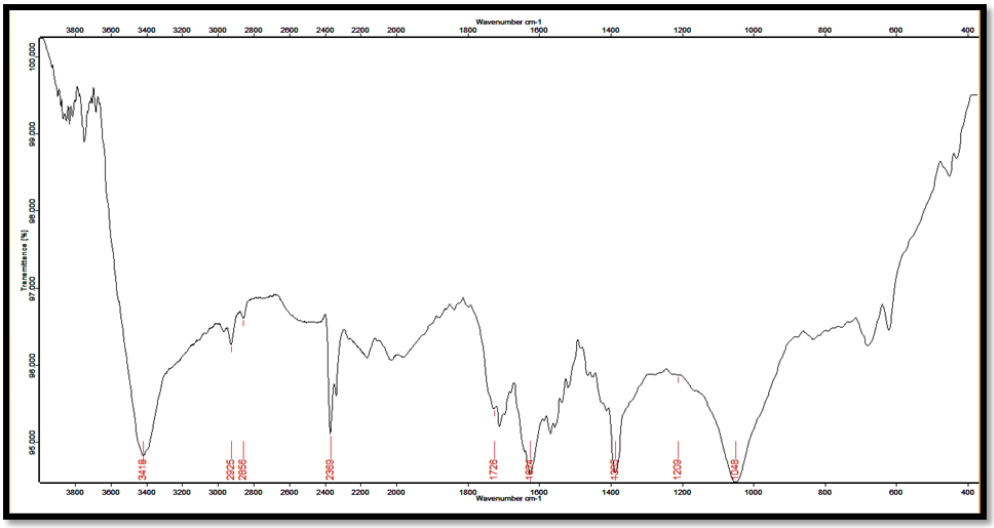

Figure 3. The FTIR spectrum of GO.

Optimization of SLM composition

In order to obtain the most efficient extraction, the different parameters affecting the extraction procedure were optimized. The results indicated that the extraction of analyte was influenced by voltage and concentration of GO, stirring speed time and pH of donor phase. GO should be dispersed in the appropriate organic solvent. Three common organic solvents including 1-octanol, n-hexane and ethyl acetate were investigated. The best dispersion of GO was in 1-octanol.

GO concentration was also evaluated on extraction efficiency in the range of 0.2 to 3 mg/ml. GO, at the concentration more than 3 mg/ml, was not well dispersed in 1-octanol. As shown in Figure 5-F, the results showed that the optimal concentration of the GO in 1-octanol was 2 mg/ml in SLM, in terms of peak analyte area. Finally, the reinforced graphene oxide hollow fiber was confirmed by SEM. Figure 4C and 4D illustrates that GO was successfully immobilized in the wall pores of hallow fibers.

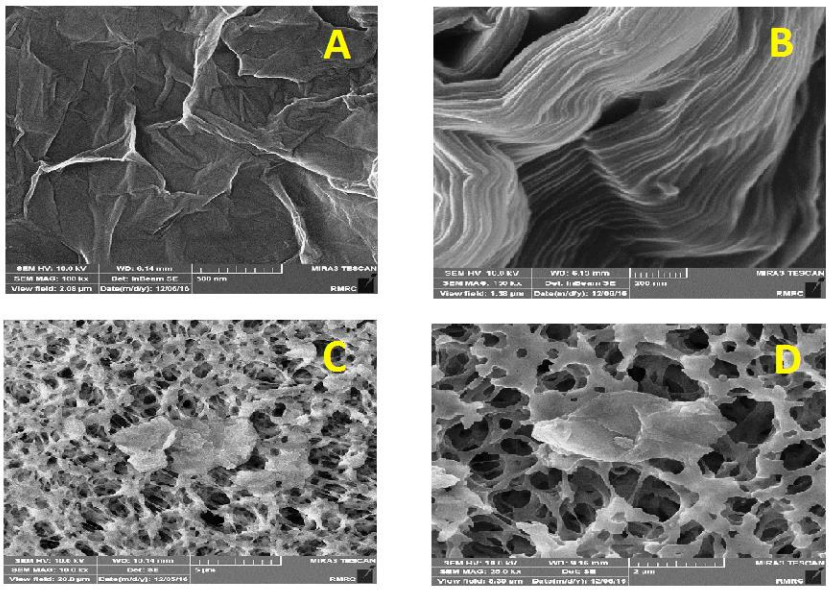

Figure 4. Scanning electron microscope of GO (A and B) and GO-HF (C and D).

Optimization of EME Method

Stirring speed is one of the important factors in the EME method. Low steering speed can lead to low extraction efficiency and reduced accuracy and reproducibility [21]. However, the magnetic stirrer with a high speed can cause air bubbles and also solvent evaporation. The optimum stirring speed was evaluated in the range of 250-1000 rpm. As shown in Figure 5D, the optimum speed for the extraction was at 750 rpm.

In general, EME is based on the balance of analytes between the sample solution (donor) and the organic solvent (acceptor) in the hollow fibers [22]. Therefore, migration of the analytes across the SLM into the organic solvent is dependent upon the time. For this purpose, a series of extraction times between 3 to 20 min was investigated. Our results showed that the best extraction efficacy was occurred at 15 min (Figure 5E). However, due to the presence of air bubbles, organic solvent evaporation and/or dissolution in sample solution, the increasing time will reduce DCD extraction efficiency [23].

The pH of the donor phase was considered as one of the important parameters following optimization process. In order to achieve efficient migration across the SLM in EME, it is necessary to change the basic analyte to positive form through the pH of donor solution [24]. A range of 2.0 to 7.0 was selected for the pH of sample solution to determine the optimum pH of donor phase. The results showed that the extraction of the analyte would be more efficient at pH 5.5 in the donor solution (Figure 5A). The pH of acceptor phase was also optimized at 2.0 (Figure 5B).

The electrokinetic migration of the analytes across the SLM is considerably dependent upon the voltage [25, 26]. The bubble formation at the electerodes and recovery value decrease occurs at high voltages [27]. In this study, we used GO as a SLM to increase the electrical conductivity leading to lower electrical potential. As shown in Figure 5C, the most efficient extraction was occurred at 70 v.

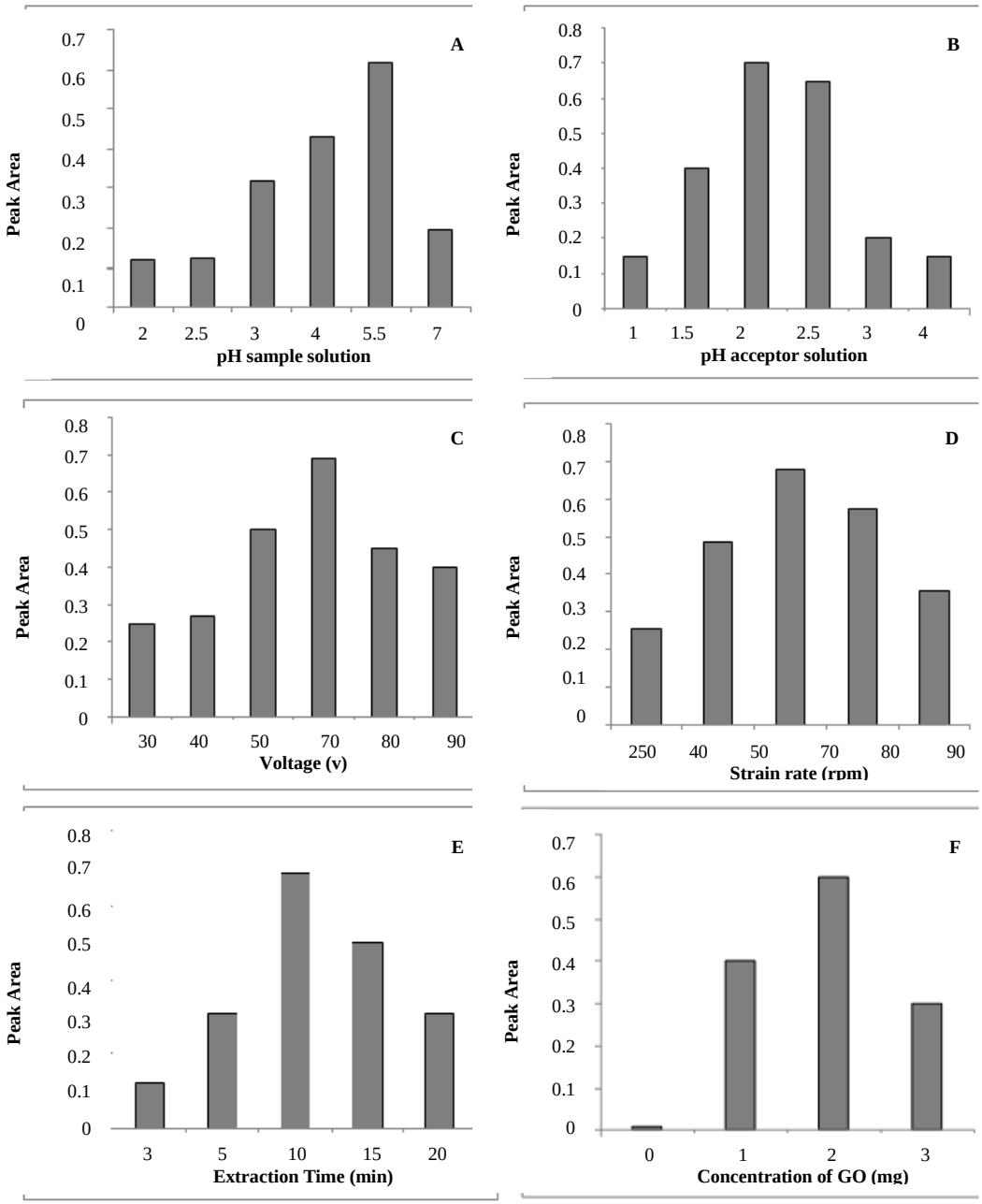

Figure 5. (A) Effect of pH of donor phase (B) Effect of pH acceptor phase (C) Effect of applied potential (D) Effect of stir-ring rate (E) Effect of extraction time (F) Effect of concentration of GO on GO-EME efficiency of DCD.

Analytical procedure and method validation

After optimization of extraction method, the analyte was detected by HPLC. Melamine, which is another adulteration, may be present in the infant formula. The retention time of melamine and DCD are the same. We set up a method of analysis to detect DCD and melamine simultaneously (Figure 6).

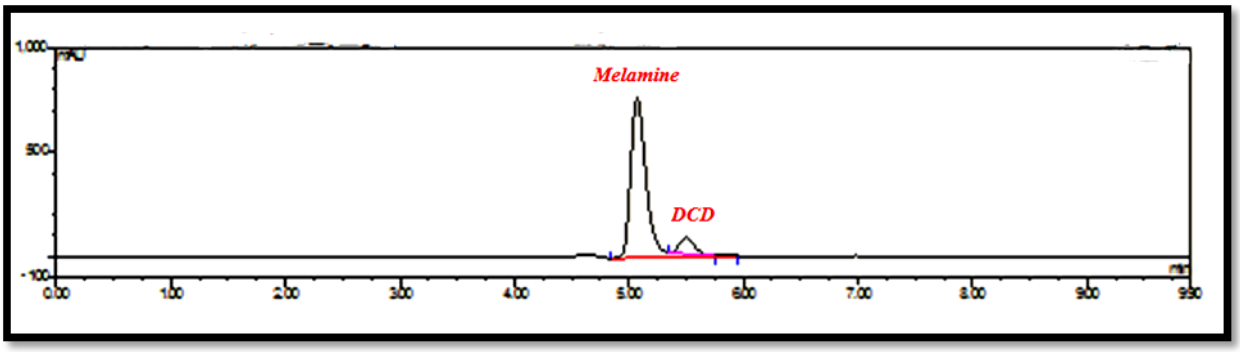

Figure 6. Chromatogram of melamine and DCD

To verify the efficacy of GO-EME procedure, method validation was also carried out with respect to the parameters including linearity, LOD, LOQ and precision. The results are shown in Table 1. The calibration curve with a correlation ratio of 0.999 was obtained. The recoveries were from 78.0% to 80.0% with the relative standard deviations in the range of 8.4% to 10.5% in metrics. The LOD and LOQ were 0.04 mg L^{-1} and 0.1 mg L^{-1} respectively.

These results showed that the extraction method is applicable for DCD analysis in milk samples. The method is rapid, simple, accurate and environment friendly. Here we also modified the extraction method by injection of dispersed graphene oxide solution to the hollow fibers which led to increase in electrical conductivity, migration and consequently extraction recovery.

Table 1. Recovery of the GO-EME method for DCD.

Analyte	Spiked (mg L^{-1})	Water			Infant formula		
		Found (mg L^{-1})	R^a (%)	RSDs (%)	Found (mg L^{-1})	R^a (%)	RSDs (%)
DCD	0.00	*nd*			*nd*		
	1	0.92	92.44	4.00	0.78	78	10.5
	5	4.97	99.49	1.64	3.99	79.8	8.43

nd: not detected
R^a: Recovery of the method

CONCLUSIONS

The migration of DCD residue to milk and dairy products has become a severe concern in worldwide because of the possible risk to children's health. Although there are a wide range of methods for analysis of DCD, some of them have disadvantages. In this study, we decided to apply a rapid, simple, accurate analysis method for quantitation of DCD in infant formula using electromemebrane extraction with reinforced hallow fiber by graphene oxide.

The use of EME extraction method based on reinforced graphene oxide hollow fiber with HPLC and UV detection was developed in the infant formulae matrix. In this method, based on the obtained results by injection of dispersed graphene oxide solution to the hollow fibers, electrical conductivity and absorption were increased. The analytical method validation parameters including accuracy, precision, LOD and LOQ were determined to ensure the method's validity. The calibration curve with a correlation ratio of 0.999 was obtained. The experimental results suggested that the method is reliable and consistent, as the method produced acceptable recoveries in the range

78.0% to 80.0%. The LOD and LOQ were 0.04 mg L^{-1} and 0.1 mg L^{-1}.

This developed method has several valuable advantages, such as low extraction time, low volume samples and organic solvent-free consumption. The method is so useful for complex matrices, such as food. It can be also detect by HPLC which is easier and cheaper than other analytical methods such as mass spectroscopy techniques.

ACKNOWLEDGEMENTS

The authors would appreciate Food and Drug Control Laboratories (FDCLs), Iran Food and Drug Administration (IFDA), Ministry of Health and Medical Education for supporting to prepare the chemicals and reagents and scientific comments.

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