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Identification and Determination of Melamine in Milk by High Performance Liquid Chromatography – UV Detector

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ABSTRACT

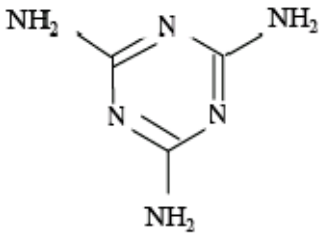
Melamine is a small polar compound which is very rich in nitrogen (67% by mass). It is recently been found in milk products and animal food, where it possibly have been added to give a false impression of high protein content. It is believed that melamine combined with cyanuric acid can cause fatal kidney stone due to the formation of an insoluble melamine-cyanurate complex. The consequences are fatal for the youngest, the babies and small kids. The reason for adding Melamine to milk to make it appear in protein in order to achieve a better price for the milk. The protein content of the milk is measured non-specifically as cumulative parameter with nitrogen compound determination by Kjehldahl so that the addition of Melamine was not detected. So determination of Melamine and other small nitrogen-rich- compounds (ammeline, ammelide and cyanuric acid) is there for of large important to ensure food safety. In this project a suitable pretreatment of the milk product and determination of Melamine with the other nitrogen compounds performed using High Performance Liquid Chromatography-UV Detector techniques. The project determined the best way meet the various method criteria (suitable procedures to handling the sample, preparation standard).

Keywords: Milk, HPLC-UV detector, Melamine, Ion pair chromatography, Symmetry C_{18} column.

INTRODUCTION

Melamine is a polar organic compound with a 1,2,3- triazine skeleton see scheme one, is commonly used for its fire retardant properties and Is often combined with formaldehyde in molding of plastics [1& 2]. Since melamine contains 66% nitrogen by mass, the addition of melamine to food boost the apparent protein content. A driving force for the adulteration of a food product with melamine is that it's high nitrogen content increases the apparent content measured by standard protein analysis tests, such as Kjeldahl or Dumas [3]. Melamine contamination has been reported in products such as milk, infant formula, frozen yogurt, pet food, biscuits, candy, and coffee drinks [4]. However, unfortunately, melamine can result in the formation of insoluble melamine cyanyrate crystals in kidneys thus causing renal failure [5]. In early 2007, it became a topic of much of hundreds of pet deaths due to pet food contamination [6]. In September 2008, infant formulas that were illegally adulterated with melamine led to health problems for thousands of infants in China, and this accident captured the attention from all over the world. A more

recent incident of contamination of milk with melamine has resulted in numerous cases of renal complications in children and six deaths have been attributed to consumption of tainted product [7&8].



Scheme 1 Structure of melamine.

To ensure that the food supply in not affected by melamine adulterated products, several countries and organization have taken proactive steps by increasing sampling and testing of imported milk-derived ingredients and finished products. Recently , a threshold of 1 ug/ml for melamine in infant formula was set by Food and Drug Administration (FDA) in the USA [7&10]. In December 2008, WHO (Word Health Organization) expert meeting concluded that the limit of melamine content in infant formula should be 1.0 mg/ kg [9]. At the same time, a national standard method GB/T 22400-2008) for rapid determination of melamine in raw milk using LC has been issued in China with the limit of detection of 50 ug/Kg (China National Standardizing Committee, 2008a, 2008b). Following these, detection of low levels of melamine in infant formula or milk-based products has become critical [11&12]. A safety limit of melamine ingestion has been officially set at 2.5 ppm for adult food and 1 ppm for infant formula by the USA food and Drug Administration [13]. The maximum residue level of melamine in infant formula is legally regulated at 1 ppm by Chinese government after the melamine accident [14].

Gopalakrishnan Venkatasami and John R. Sowa Jr [2] developed HPLC method to determinate of melamine in infant formula acetonitrile free. The mobile phase was 0.1% Trifluroacetic acid/ methanol 90:10 is pumped at a flow rate of 0.3 ml/min and C18 as analytical column . Hanwen Sun et. al. [15] determination of melamine residue in liquid milk by reversed phase high- performance liquid chromatography with solid-phase extraction. They used ion pair chromatography by using sodium n-heptaneaulfonate-acetonitrile as a mobile phase in addition they used PCX-SPE cartridge to clean-up milk samples. Yongqiang Tian et al [16] studied the comparison between the chromatographic methods such as HPLC-Uv and non- chromatographic methods such as enzyme–Link immunoassay (ELISA) and the new rapid colorimetric assay (RCA). They reported different of the results and the advantages and disadvantages of each methods. Fengxia Sun et. al. [17] review the recent developments in the analytical method in the detection of melamine in the milk powder, infant formula and pet food. The presented and discussed the advantage, disadvantage and the applicability of chromatographic and non chromatographic methods. In the recent years, gold nanoparticle (Au NPs) and silver nanoparticles (Ag NPs) have been widely used as colorimetric probes for chemical sensing and biosensing of various substance such as melamine in milk [18&19]. Xiao-Lin Zheng et. al. [20] presented hydrophilic interaction chromatography (HILIC) using NH2 column combined with ultraviolet detector to determined of melamine in dairy product.

several analytical methods High performance liquid chromatography (HPLC) is a common for detecting melamine and several HPLC methods have been proposed for quantitative determination of melamine [2,21-25]. HPLC techniques have been applied to the simultaneous detection of melamine, ammeline, ammelide, and cyanuric acide in rice, wheat, and corn flours by Ehling et al. [26] liquid chromatography/mass spectrometry (LC/MS) [22,27-29], gas chromatography (GC) [30], gas chromatography/ mass spectrometry (GC/MS) [31] and capillary electrophpresis (CE) [9,32], have been developed for the detection of melamine in infant formula and milk-base products. However, most of the above methods required expensive and complicated instruments (such as MS) and time consuming sample pretreatment (such as derivatisation or extraction), which made high throughput.

In recent years, HPLC has been proven to be one of the most powerful techniques for the anaylsis biological matrices, pharmaceutical preparations, due to its a high efficiency and good reproducibility. The ideal analytical technique for melamine detection should possess several features. It should be suitable for a variety to complex food or environmental material and have high sensitivity, high specificity, short detection time, low cast, and require and minimal sample preparation [33].

In this work , a symmetry C_{18} column form waters was used to identification and separate of melamine in powder milk, after clean-up by solid phase extraction SPX cartridge . Buffer of citric Acid and sodium 1-octane sulfonate acetonitrile was used as mobile phase component. Factors affecting retention of melamine were explored, including buffer concentration, pH, and percentage of organic solvent in the mobile phase. The optimal UV detection wavelength was selected to be 240 nm. Satisfactory retention of melamine, good peak shape, and high sensitivity were obtained under the chosen conditions. The propose was to find a condition under which, the strong polar melamine can be detect directly without dervatization.

MATERIALS AND METHODS

2.1 Reagents

Melamine , sodium 1- octane sulfonate HPLC grade, from Acros organic, new jersey, USA, Methanol HPLC grade ,Acetonitrle HPLC, Trichloroacetic acid, Citric acid monohydrate, sodium 1- octane sulfonate, Ammonia solution, from Sigma-Aldrich, Germany.

2.2 Preparation of standards of Melamine

A 1000 ug/ml melamine stock standards was prepared by accurately weighing 100 mg of melamine into a 100 ml volumetric flask . Melamine was dissolved with aqueous methanol (50% v/v). The stock standard was diluted appropriately to prepare working standards with concentration of 0.01, 1.00, 2.00, 5.00, 10.00, 50.00 ug/ml for calibration curve.

2.3 HPLC system of Analysis

Melamine composition determined by HPLC Waters system equipped with 515 pump from (Milford, MA, USA) stainless steel filter, guard column Symmetry C_{18} (5 um, 3.9x 20 mm) , Symmetry C_{18} column (5 um, 250x 4.5 mm) was used through this study Waters column heater module with Waters Temperature control module, Waters 486 Tunable Absorbance Detector , Waters Automated Gradient Controller associate with Dell computer system using Empower software to run and control all the calculations for the instrument. The temperature of the oven was set up to 50 ^{o}C. The loop of the injection was set up to 20 ul with Rheodyne injector (Rohnert Park, USA) model 712 . The concentration of the products were determined from the peak areas under the curve using Empower software for instrument control and data collection and processing. Solid phase extraction was performed using SCX SPE cartilage 3cc 60mg supplied from Algint Technolgy. Centrifuge kind Mikro 22R was supplied from Hettich, Republic of Germany. Water purification system and the ultrasonic bath supplied by Barnstead International.

Before the quantitative and qualitative determination of melamine in the milk (infant milk samples, we prepared standard solutions of different standards concentrations. With those standard solutions of different melamine we made calibration lines for each one of the melamine, which later used for assessing the concentration corresponding to the different peaks in the chromatograms.

2.4 Chromatographic conditions

Guard Column: Symmetry C_{18} (5 um, 3.9X 20 mm) Guard Column
Analytical Column: Symmetry C_{18} (5 um, 4.6X 250 mm) Column

Preparation of mobile Phase : Buffer of citric Acid 2.10 gm and 2.16 gm sodium 1-octane sulfonate were dissolved in 980 ml dionized water and two ranges of pH were tested 4.5 and 3.0 the volume brought to one litter after the pH adjust by sodium hydroxide. Then 920 ml of the list solution was taken and mixed with 80 ml Acetonitrile. So the ratio is 92:8 volume to volume used for the isocratic separation mode for melamine. In the same manner another ratio of the mobile phase buffer solution to acetonitrile (85:15) was prepared and studied too.
Column Temp: 50 ^{o}C
Flow Rate: One ml per minute
Injection Volume: 20 ul
UV Detection: 240 nm

2.5 Sample Collection

There are different brands of powdered milk samples available in KSA markets which are imported from different milk producing countries in the world. For our experiment we used seven different varieties of powdered milk samples collected from local market of Taif.

2.6 Sample preparation

Food samples are typically complex matrices that are difficult to analyze because of the abundance of proteins and carbohydrates. Effective isolation and extraction of melamine and analogs from complex matrices is necessary prior to melamine determination. The main objectives of sample treatment, including extraction, preconcentration, and derivatization, are to achieve lower limits of detection by removing matrix constituent that may affect detection or enrichment of analytes [35]. However, because of the complexity of the matrices, most sample-preparation procedures require extraction followed by one or more clean-up steps that can take from ten minutes to hours.

Milk powder samples blank control free from any addition of melamine and the second sample is spike sample with 20 ul from the stock standard solution were accurately weighed into 50-ml centrifuge tubes, and 15 ml of aqueous trichloroacetic acid and 5 ml acetonitrile were added to each. After one minute of vortex shaking samples were placed in an ultrasonic bath for 30 minutes, and then shaking for 15 minutes. The tubes were transferred to centrifugation (setting rpm to 6000 for 30 minutes) the supernatants were passed through filter paper into 25 ml volumetric flasks. Samples were brought to volume with DI water.

2.7 Fortified Sample preparation

Milk powder sample around one gram was accurately weighed into 50-ml centrifuge tubes then fortified with 0.001 mg and the final weighed one gram exactly , and 5 ml of HPLC methanol and 5 ml of deionized water were added. After one minute of vortex shaking, each was placed in an ultrasonic bath for 30 minutes. To each was added 10 ml of 1% trichloroacetic acid (1 gram in 100 ml dionized water). Vortex shaking was carried out for five minutes and sonication for 15 minutes . After 30 minutes of centrifugation (setting rpm 6000), supernatants transferred to 25 ml volumetric flasks and the brought to volume with DI water if needed. Prior to passed it on the activated SPE cartilage. The solutions were filtered through a 0.45 um desk filter. Same dilution was made to brought the concentration of the sample within the range of the calibration curve.

2.8 Solid-phase extraction (SPE) for milk samples

Prior to use the SPE column was activated by passing 3 ml of methanol and 5ml water in turn. The mixture of 5 ml of sample extract and 5 ml of water was passed through the activated SPE. Then the SPE washed with 3 ml methanol and 3 ml water respectively. The elution carried out with 6 ml of aminated methanol solution freshly papered by mixing of 5 ml ammonia solution and 95 ml methanol. The eluent collected and dry at 50 ^{O}C and re-dissolved in 1 ml of the mobile phase. Prior to injection the samples filtered with 0.45 um desk filter.

RESULTS AND DISCUSSION

Peak identification was based on the retention times t_R, identification of the melamine was confirmed with known standards injected individually through the HPLC and the retention time for melamine was 19.78 minutes. Figure 1 shows the peak identification and a typical chromatogram for 100 ug/ml melamine.

The mobile phase of the buffer and acetonitrile as described elsewhere above is the most common used eluent in RP-HPLC ion paring chromatography. The retention time for melamine decrease with the percentage of the acetonitrile in the mobile phase decrease. The pH of the mobile phase was a prominent factor of determining the retention of melamine. Within the pH range of 3.0-4.5, the retention time of melamine decreasing with the mobile phase pH get closer to 4.5. So the buffer solution and acetonitrile at 92:8 ratio and pH 4.5 was most likely optimum conditions for the system we finally chosen as the mobile phase for separation melamine in this work. The shorter retention time, nice and symmetry peaks were achieved as shown in figure 1. Linearity for calibration curve of melamine under the chosen condition, the calibration curve for standard solutions at a series of concentrations as described elsewhere above shown in figure 2.

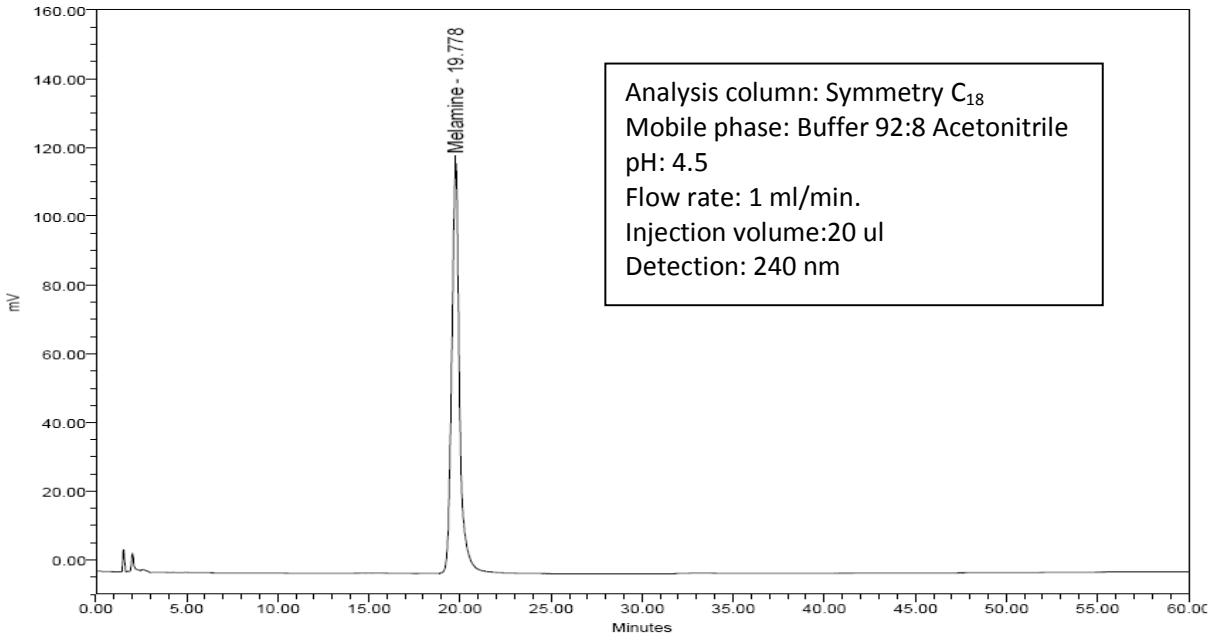


Figure 1 Peak Identification for the retention time for 100 ppm melamine

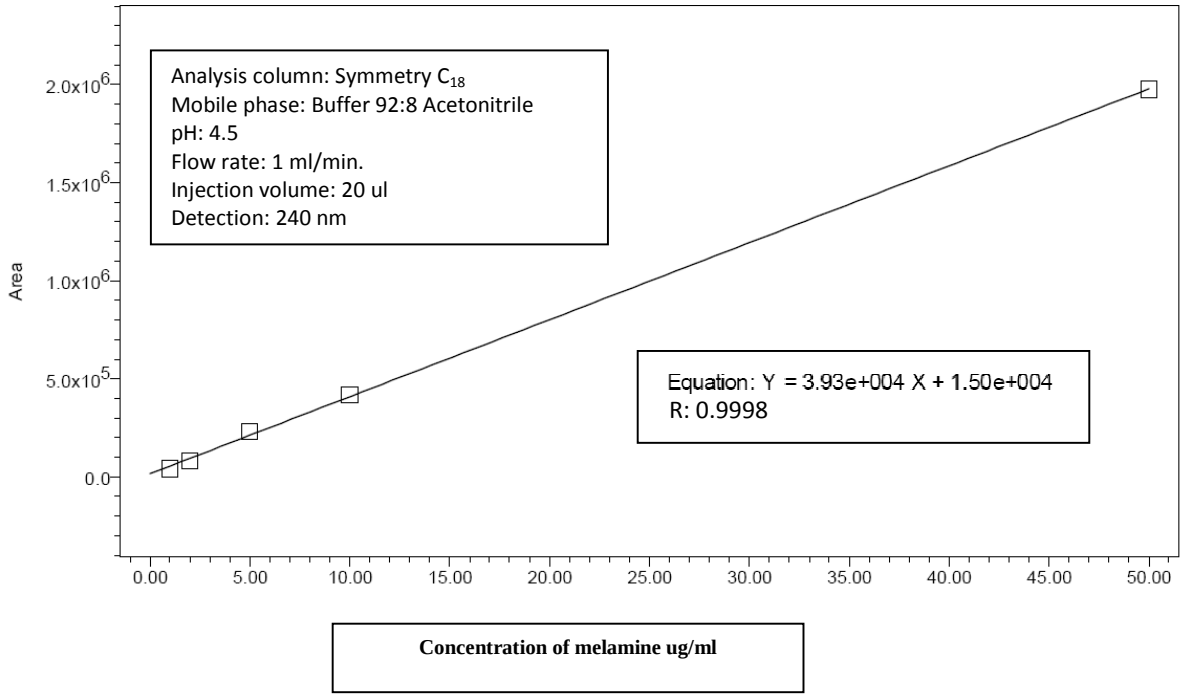


Figure 2 Calibration curve for melamine.

Excellent linearity between the peak area and melamine concentrations was obtained in the tested melamine concentrations rang of 0.01-50 ug/ml. It is covers the concentration for the powder milk samples. The regression equation was as shown in figure 2. Where Y is the peak area and X is the concentration of melamine ug/ml. Figure

5 demonstrated the measurement for melamine in this method was provide and cleared to determine 0.01 ug/ml or less. Figure 3 showed a typical overlay chromatograms for standards of melamine was injected separately to confirm the proper check for the retention time as well as presented very good reproducibility of the retention time.

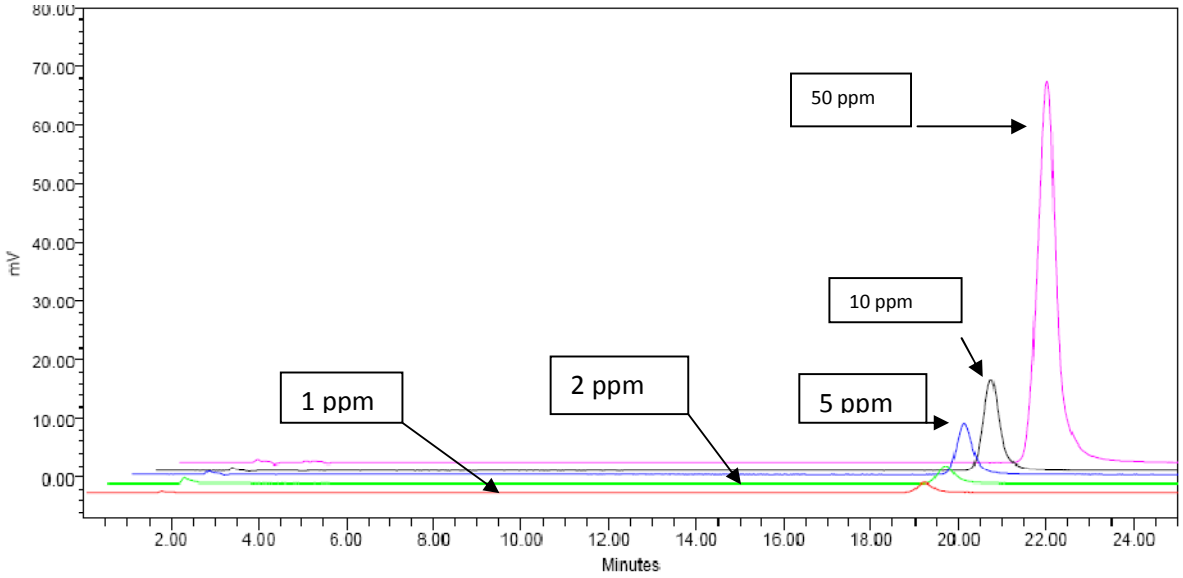


Figure 3 Overlay chromatograms on individual standard for melamine.

Figure 4 Illustrated the typical chromatographic profiles for the mobile phase only. It is appeared very nice and clear back ground without any peaks in the rang of the appearance of melamine.

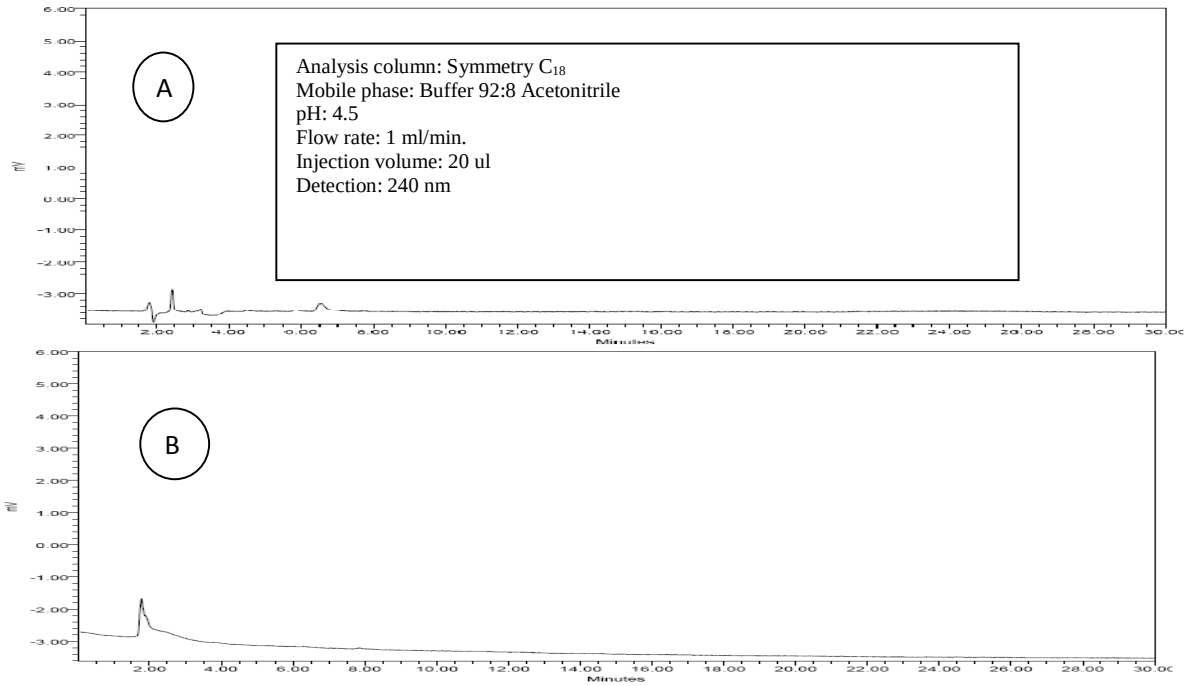


Figure 4 A typical chromatogram for the mobile phase only.

Figure 5 gave a list of chromatogram for 0.01 ug/ml of melamine three repeated injection represented the reproducibility and sensitivity of the method for very low level of melamine .

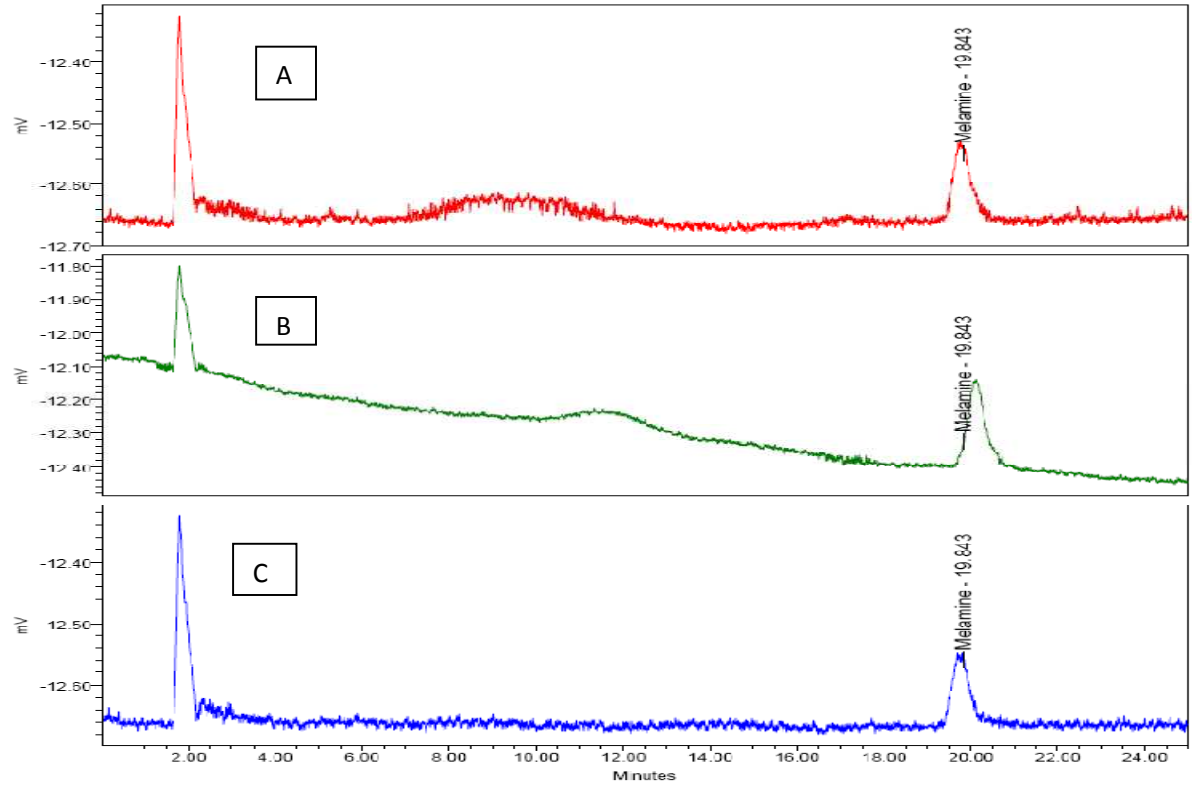


Figure 5 Triple injections chromatograms for 0.01 ug/ml for melamine

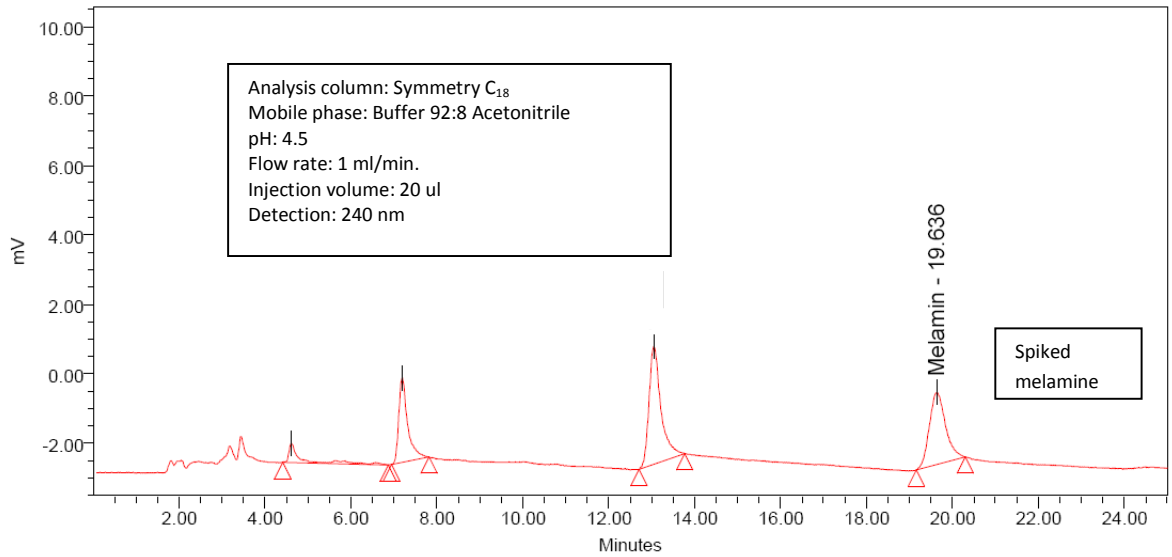


Figure 6 Chromatogram for infant formula powder milk spiked with 20 ul of melamine stock solution

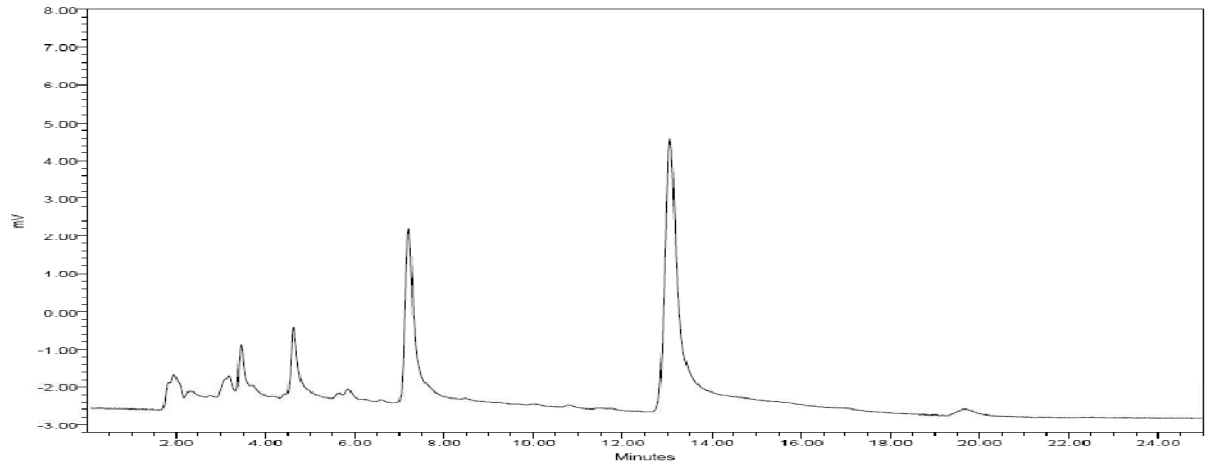


Figure 7 Chromatogram of infant formula powder milk as blank control (without any addition).

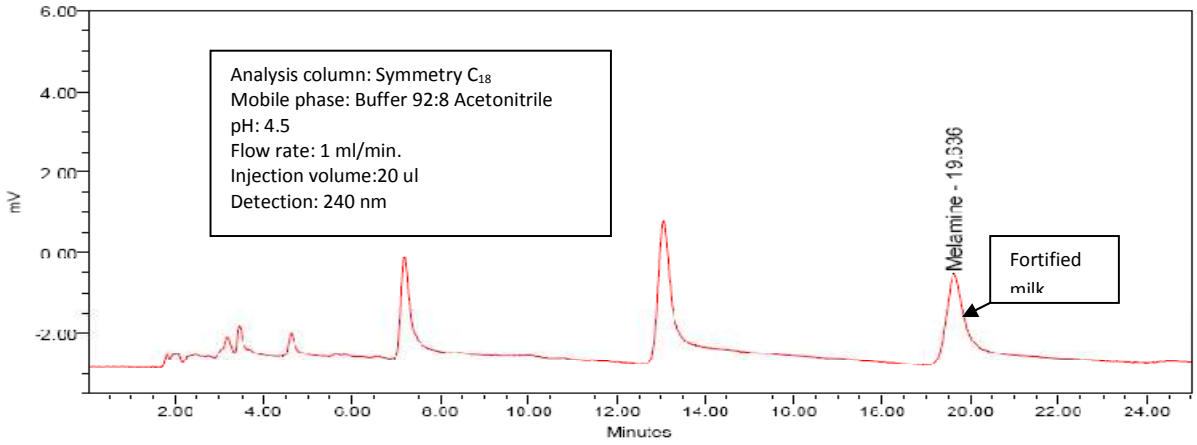
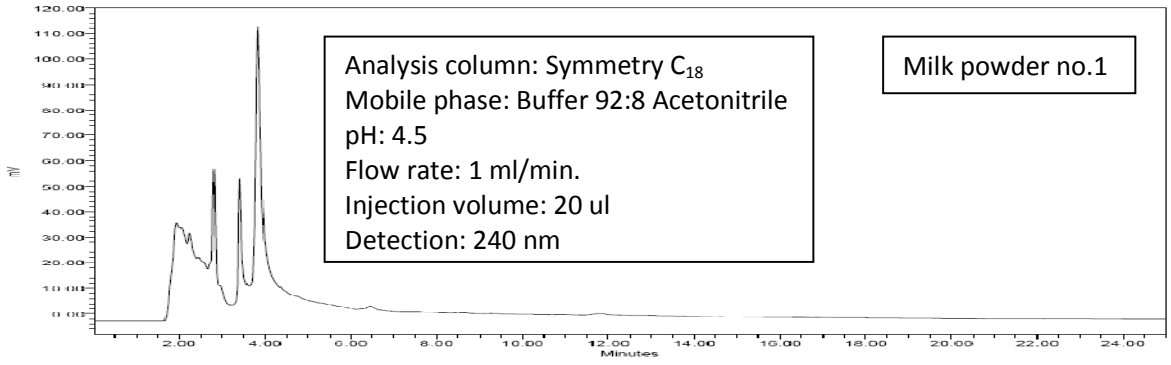
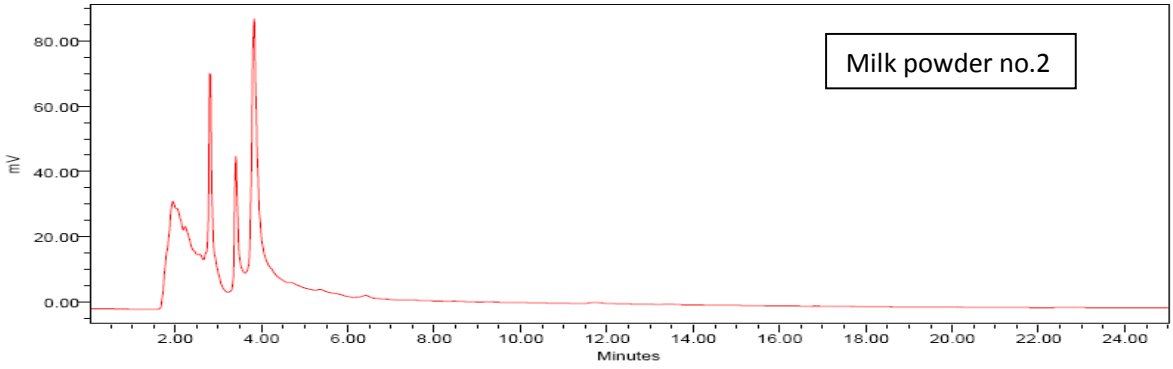


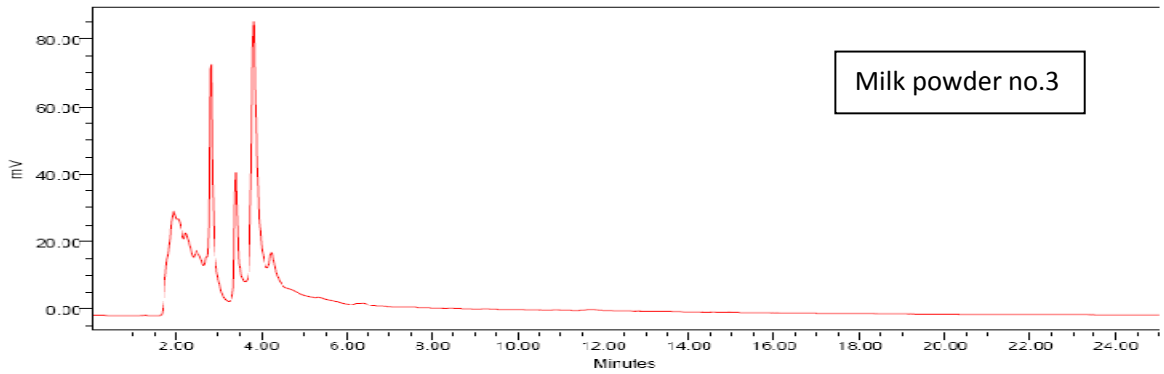
Figure 8 Chromatogram for infant formula powder milk fortified with a 0.001 mg melamine powder

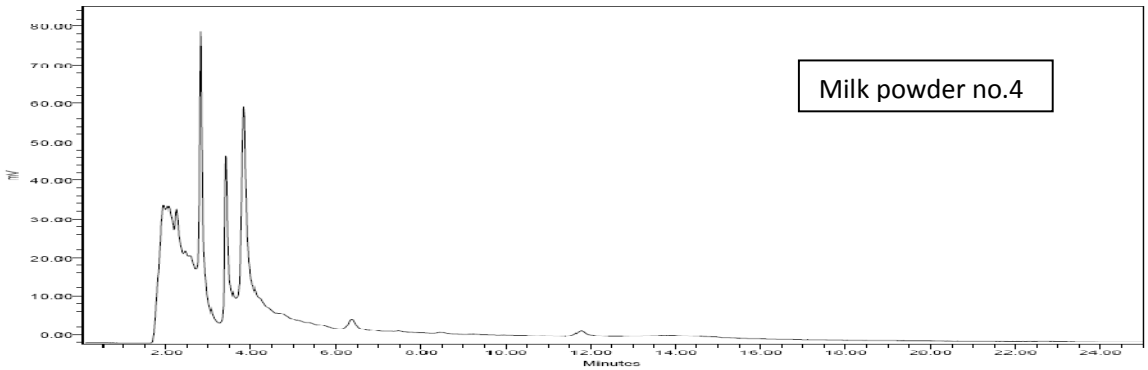
The mobile phase and the infant formula milk powder was first injected to assure that it contained no melamine and there are not any peaks in the retention time of the melamine as we located before this can be seen in figures 4&7 no melamine was detected. The HPLC method showed essentially no interference by matrices (the protein and carbohydrate) on the appearance of melamine . So this method is powerful to separate of melamine for subsequent identification and quantification using Symmetry C_{18} column. Figure 6 Showed the chromatogram for the infant formula milk powder spiked with 20 ul from the stock solution (1000 ug/ml of melamine). Melamine is detected as can seen at 19.63 minutes retention time. Figure 9 demonstrate the chromatograms of a infant formula milk powder fortified with 0.0010 gram melamine. A large peak was observed for melamine due to the high concentration of melamine in the sample and this can be avoidable. The dilution was carried out to reduce band broadening in the peak of melamine, the result shown in chromatogram 8.

Six milk powder samples (1-6) obtained from market were analysis. Figure 9 is illustrated chromatograms of these sample in addition to the mobile phase only. The results showed all the sample free from melamine. In order to ensure and identified that in Figure 10 sample no. 6a that the peak at 12 minute in figure 9 sample no. 6 is not melamine by spike the sample no. 6 with know amount of melamine as the peak of melamine it appear later on in the regular position.


Analysis column: Symmetry C_{18}
Mobile phase: Buffer 92:8 Acetonitrile
pH: 4.5
Flow rate: 1 ml/min.
Injection volume: 20 ul
Detection: 240 nm
Milk powder no.1
mV
Minutes


Milk powder no.2
mV
Minutes


Milk powder no.3
mV
Minutes


Milk powder no.4
mV
Minutes

No.6

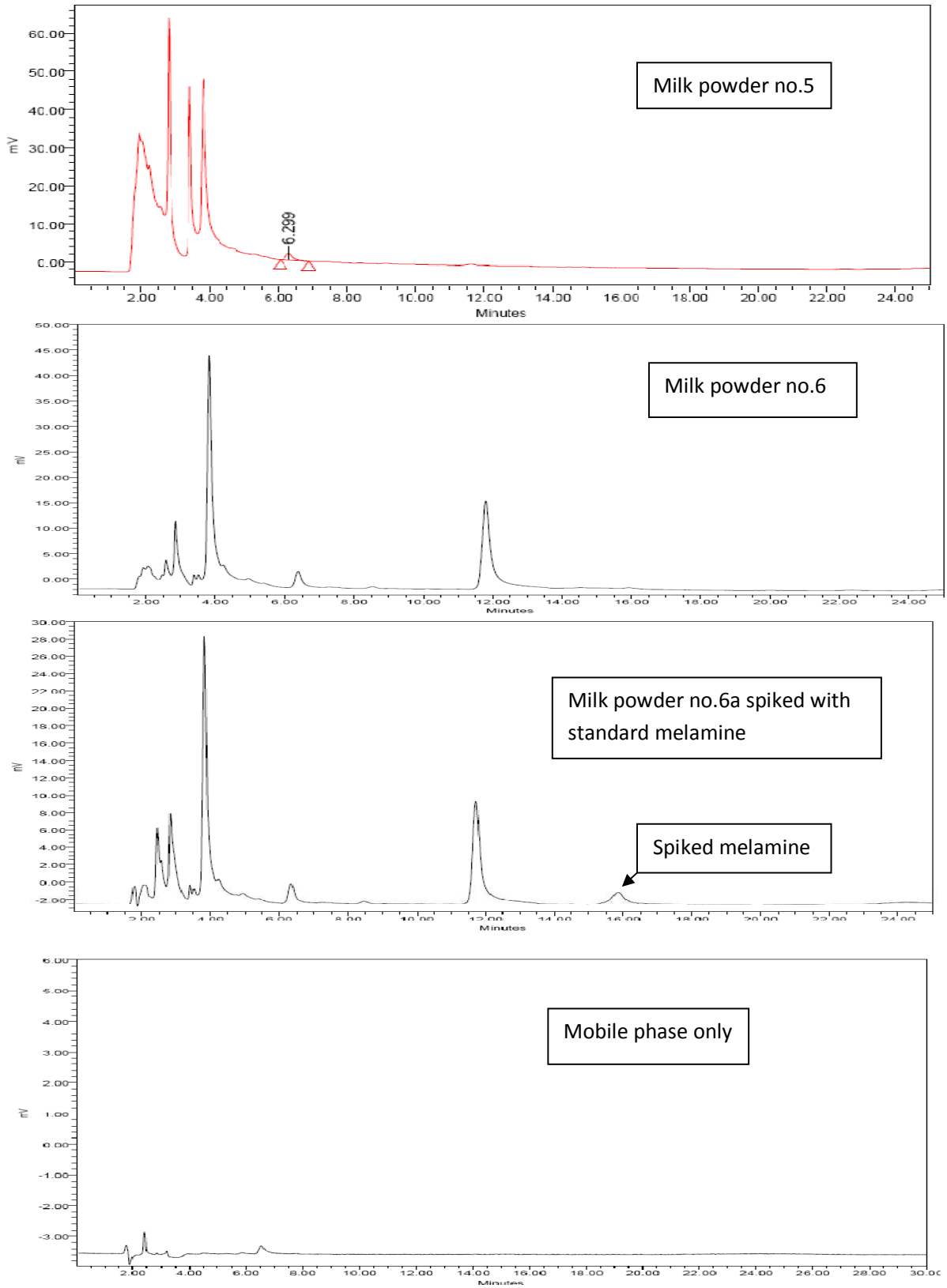

Milk powder no.5
6.299
mV
Minutes
Milk powder no.6
mV
Minutes
Milk powder no.6a spiked with standard melamine
Spiked melamine
mV
Minutes
Mobile phase only
mV
Minutes

Figure 9 chromatograms of six milk powder samples in addition to the mobile phase

CONCLUSION

Recent product recalls and food safety incidents due to melamine adulteration or contamination have caused a worldwide food security concern. This has led to many methods being developed to detect melamine in foods, but few methods haves been reported that can rapidly and reliably measure melamine in environmental samples. To meet the need, a RP- HPLC ion pairing chromatography with UV detection method it used in this study. It is demonstrated that melamine is conveniently detected at very low concentrations 0.01 ug/ml. Using Waters HPLC with symmetry C_{18} as analytical column and SCX as SPE for clean-up the samples. It is possible to detect and quantify melamine at concentrations relevant to food authorities in less than 25 minutes with relatively with low cost. It is simple , sensitive and robust and allows for analysis of large number of samples, without degradation in column performance. Very low noise is observed, emphasizing the effectiveness of the clean-up procedure for complicated matrix

Because of the simplicity and the sensitivity, this propose method has the potential to be a useful tool for the routine melamine monitoring in real-time.

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