

ORIGINAL ARTICLE

Sensitive Detection of Melamine in Infant Milk and Coffee Mate by a Buffer Mediated Extraction and HPLC-PDA Analytical Method

Maryam Rezai, Behrouz Akbari-adergani, Maryam Shekarchi*

Food & Drug Control Laboratories and Food & Drug Laboratory Research Center, Food and Drug Organization, Ministry Of Health and Medical Education, Tehran, Iran

(Received: 13 July 2014 Accepted: 16 September 2014)

KEYWORDS

Melamine
Coffee mate
Infant milk
Protein content

ABSTRACT: Melamine is a potentially hazardous compound and one of the major concerns especially in dairy products and pet foods. In the present study a sensitive, simple and reliable method for extraction and determination of melamine in infant milk and coffee mate has been developed. This method consists of an initial extraction in buffer media prepared by formic acid and sodium formate, followed by protein precipitation by acetonitrile and dichloromethane. The chromatographic separation was carried out on a 100-Nucleosil -NH₂ column with an optimized acetonitrile-water (80:20 v/v) as a mobile phase and with a photodiode-array detector. The analytical method was validated according to the validation parameters, such as, selectivity, linearity (0.08-10 µg/mL, with $r^2 = 0.9998$ and 0.05-10 µg/mL with $r^2 = 0.9997$), precision (intra-day 0.52-2.66%, 0.78-1.20; inter-day 2.96-4.20%, 2.80-3.00%) and accuracy (92-102%, 92-100%) for powdered milk and coffee mates respectively. The limits of detection and quantization were 0.02, 0.08 µg/mL for powdered milk and 0.01, 0.05 µg/mL for coffee mate, respectively.

INTRODUCTION

Melamine discovered in baby formula and products containing milk has led to one of the largest worldwide

food recalls in history. Some manufacturers illegally added melamine, an ingredient used in plastics

* Corresponding author: mshekarchim@yahoo.com (M. Shekarchi).

manufacturing, to increase the apparent protein content of the food. The effect of this addition caused the death of both pets and babies that consumed these fraud products. Melamine can form an insoluble compound cyanuric acid in the body, causing kidney stones that can be deadly in infants [1-2]. The structure of melamine and its related compound cyanuric acid are illustrated in Figure 1.

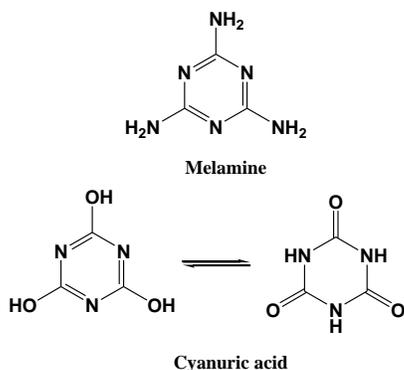


Figure 1. Chemical structure of melamine and cyanuric acid

The world health organization (WHO) has recommended the tolerable daily intake (TDI) for melamine to be 0.2 mg/kg of body mass. In China and the US, the maximum residue levels (MRLs) for infant formula has been set at 1.0 mg/kg and at 2.5 mg/kg for milk and other milk products, while in Europe, the food safety authority has set the limit to 2.5 mg/kg for all products containing greater than 15% milk [3]. As a result, there is growing government and consumer concern towards the presence melamine in food products.

Therefore, there is a need for a sensitive, simple and reliable test for determination of melamine in food products. Several analytical methods have been established for analysis of melamine in different matrices using enzyme immunoassay [4-7], GC-MS [8-11], LC-MS [12-15], HPLC with UV detection [16-21] and electrochemical method [22]. Electrochemical techniques are very rapid and economical for

determination of many organic compounds and even for heavy metals in aqueous systems [23-25] but for analysis of melamine mainly need modifying surface electrode with lower reproducibility for electrode preparation. In other methods many sample preparation steps is necessary for the analysis of melamine. Some of these methods involve many derivatization and pretreatment procedures such as mixed-mode ion exchange solid phase extraction [13-16, 19] prior to final analysis, which is very time consuming, and need expensive and sophisticated instrumentation and highly skilled personnel restrict for their use in routine analysis. Protein precipitation is nowadays routinely established as clean up techniques for the target enrichment and clarification to assist analytical quantification. Liquid chromatography determination can be carried out through ion exchange, ion-pairing and hydrophilic interaction chromatography being the most common method (HILIC) [26]. Most of these methods have used ion pair reagents and complex sample preparation. Reverse phase liquid chromatography (RP-LC) with ion pair reagents has been also reported but the complex mobile phases associated to these method make the column equilibration time to be late [20] and are detrimental to the column life time and the low volatility also obstructs the compatibility with mass detection [15, 19 and 27]. There are just a few published methods that have used non-polar column such as -C18 [28] or more polar column such as -CN [29] and -NH₂ [30] functional groups in a bonded phase chromatography technique for better separation of chemicals in dairy products.

Extraction in formate buffer and protein precipitation by acetonitrile and dichloromethane is very clean, simple and economical for the determination of melamine in such a complex food matrices. Indeed, the application of such technique, coupled with HPLC due to the cleaner extraction solutions resulting quite separated peaks in the chromatogram, enhances background signal and improve the sensitivity for determination of melamine.

The complexity of the sample matrix and the low concentration levels are two main drawbacks in the analysis of melamine in infant milk and coffee mate samples.

In this study, for the first time, we report a sensitive method for determination of melamine in infant milk and coffee mate by a buffer mediated extraction and HPLC-PDA analytical method having sensitivity range sufficiently below the maximum residual level in the range of parts-per-million. We used a sensitive, simple and efficient pre clean-up procedure which is thoroughly compatible with the proposed high performance liquid chromatography with photo diode array detection method. To the best of our knowledge, this is the first report on the detection of melamine by the HPLC-PDA with such an efficient extraction method.

MATERIALES AND METHODS

Chemicals and Reagents

Distilled water was purified using a Milli-Q system from Millipore (Le montsur-Lausanne, Switzerland). All solvents used in chromatography were HPLC grade and obtained from Merck (Darmstadt, Germany). Melamine reagent (99.0%) was acquired from Fluka (Milwaukee, WI, USA). Buffer solution was prepared by dissolving 136 mg of sodium formate in water in a 1.0-liter volumetric flask and make to volume. Then the pH was adjusted to 3.7 with formic acid. Four various brand of powdered milk (Humana®, Nan®, Multi® and Biomile®) and four coffee mate with a same brand Nestle® (Serial no.: 13160523Lk, 13150523LK, 13130523UP and 13130523UW) were collected from Iranian retail market.

Instrumentation

In all solutions, the pH was adjusted by digital Metrohm pH meter (model 744) equipped with a combined glass-calomel electrode. The HPLC experiment was performed using a Waters Alliance system equipped with a vacuum degasser, quaternary solvent mixer and a

2998 Alliance photodiode array detector. The UV spectra were collected across the range of 200-900 nm with a PDA detector, extracting 220 nm for chromatograms. Empower software Ver.1 was utilized for instrument control, data collection and data processing, which was supplied by Waters Company. The analytical column was a 100-Nucleosil-NH₂ (4.6 mm×250 mm). The mobile phase was an isocratic mode of acetonitrile-water (80:20 v/v) at flow rate of 0.8 ml/min. The injection volume for all samples and standards was 20 µL.

Standard preparation

The melamine stock solution with a concentration of 1000 µg/mL was prepared in formic acid 2.5%. Intermediate standard solutions (100 and 20 µg/mL) were prepared by dilution of stock solutions by 50:50 acetonitrile/ water in volumetric flask. Working standard solutions in different concentrations (0.05-10 µg/mL) were obtained by diluting the intermediate standard solution with 50:50 acetonitrile / water.

Sample preparation

In all cases, samples were taken thoroughly homogenized material. Exactly 0.2 gram of milk powder or coffee mate was transferred to 10 ml volumetric flask and 10 ml formate buffer (0.02M, pH=3.7) was added following shaking well during 30 min. The mixture was centrifuged in 4 °C at 11000 rpm for 10 min. After that, 4 ml of supernatant was transferred to 10 ml test tube and centrifuged at 11000 rpm in 4 °C for 10 min. About six ml acetonitrile was added to 3 mL of extract and centrifuged in 4 °C at 11000 rpm for 10 min. Then 12 ml dichloromethane was added and centrifuged in the same condition for 10 min. The upper layer (water) was filtered through 0.45 µm PTFE filter and 20 µl of the final solutions were injected into the HPLC system.

Validation

The reliability of the HPLC-method for analysis of melamine was validated through its selectivity, linearity,

precision, and recovery, limit of detection and limit of quantization [31].

Selectivity

Selectivity is the ability of the method to measure accurately the analyte response in the presence of all interferences. Therefore, the extraction mixtures obtained from the sample preparation were analyzed and the melamine peak was evaluated for peak purity and resolution from the nearest eluting peaks.

Linearity

Linearity was evaluated through the relationship between the concentration of melamine and absorbance obtained from the UV-HPLC detector in the base of verification of the normal distribution of results. The determination coefficient (r^2) was calculated by means of the least-square analysis [32-33]. The calibration line was achieved through two replicates of each concentration of melamine (0.05-10 mg/l), to identify the extent of the total variability of the response that could be explained by the linear regression model.

Precision

The precision of each method indicates the degree of dispersion within a series on the determination of the same sample. Three samples in three levels (0.5, 1, 1.5) mg/l were analyzed on the same day (intra-day) and three for consecutive days (inter-day), and then the relative standard deviations (RSDs %) were calculated. Each sample was injected to HPLC thrice.

Recovery

This parameter shows the proximity between the experimental values and the real ones. It ensures that no loss or uptake occurred during the process. The determination of this parameter was performed during the method by studying the recovery after a standard addition procedure, with two additional levels. Three replicate amounts of milk (3×0.6 g) were weighted and each of them was divided into three equal portions (0.2 g). One part was used as the real sample and others had

been spiked with melamine standard solution (1.0, 2.0) $\mu\text{g/mL}$ in two levels. In each additional level, three determinations were carried out and the recovery percentage was calculated in every case. Each sample was injected into HPLC three times.

RESULTS AND DISCUSSION

One of the challenging aspects of method development in quantitative analysis of milk products is the complexity of these matrices. The simpler the method, the better conduction by different operators and in different labs with lower bias.

Extraction procedure

Extraction is the main step for the recovery and isolation of melamine from powdered milk and coffee mates. It is influenced by chemical nature and interfering substances. Melamine (2, 4, 6-triamino-1, 3, 5-triazine) is a small polar molecule which is slightly soluble in water and ethanol. The sample pretreatment for melamine usually involves liquid extraction by a polar solvent, and some complex matrices require further clean up with solid-phase extraction. Commonly used solvent for melamine extraction from different matrices includes a mixture of acetonitrile/water/ diethylamine, trichloroacetic acid solution (precipitant agent), methanol and hydrochloric acid. Several buffer solutions with various pHs were evaluated for extraction of melamine. A citrate (pH= 3.1), formate (pH= 3.7), oxalate (pH=4.2) and acetate (pH=4.7) buffer solutions were evaluated. The results revealed that a buffer mediated solvent with formic acid and sodium formate (0.02M, pH= 3.7) had an excellent choice for extraction of melamine from such a complex media (Figure 2).

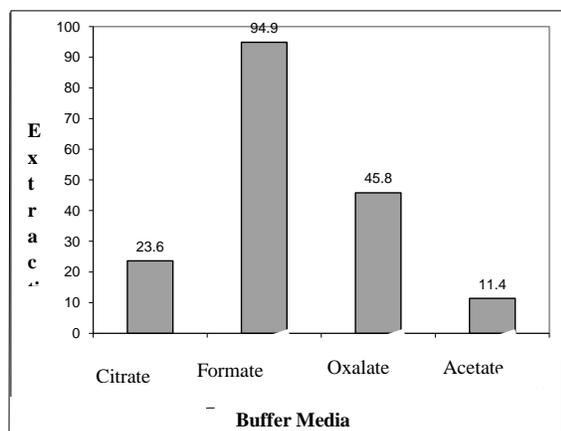


Figure 2. Effect of buffer media on the extraction of melamine

It was selected from several mixtures of solvents for extraction of melamine from powdered milk and coffee mates, for purification purposes and preparation of standard materials. Besides the high recovery, it is less interfering in comparison with other solvents, making it a suitable solvent for extraction of melamine and preparation of standard materials.

For further purification and precipitation of proteins, acetonitrile has been used following by addition of dichloromethane, which help the separation of water acetonitrile layers. Comparison between two experiments showed that the smaller sample size appeared to have a significant effect on the accuracy of melamine analysis. In general using the 0.2 g powdered milk and coffee mate with 0.02M formate buffer was selected as the best method for melamine extraction.

Method development and validation results

The main objective of this study was to develop a sufficient HPLC- UV method for identification and determination of trace amount of the melamine in powdered milk and coffee mates. As is shown in Figure 3, the matrix of sample was complicated therefore; it was difficult to separate melamine from the interferences

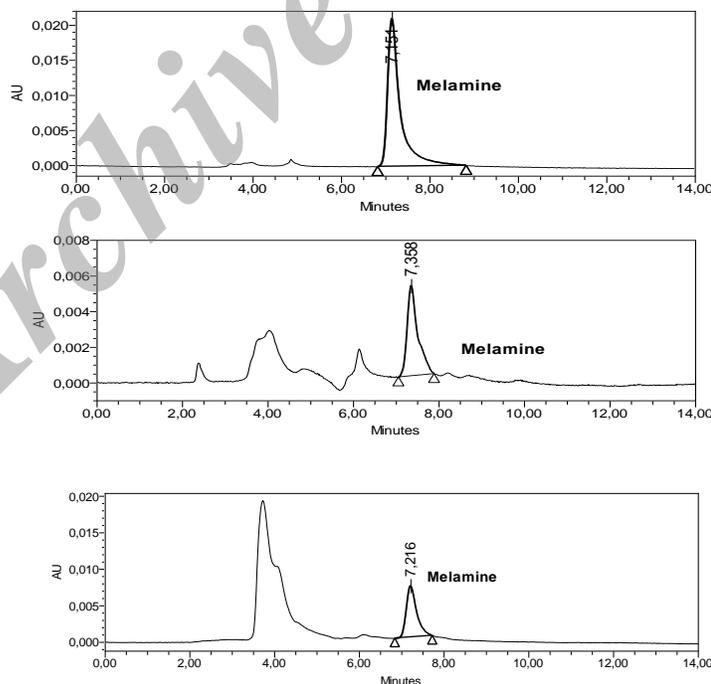


Figure 3. Typical HPLC chromatogram of (a) A standard solution containing 4.0 $\mu\text{g/mL}$ of melamine, (b) After percolation of 1.0 gr powdered milk spiked with 10.0 $\mu\text{g/mL}$ of melamine and (c) After percolation of 1.0 gr coffee mate spiked with 10.0 $\mu\text{g/mL}$ of melamine. Conditions: column 100-Nucleosil-NH₂ (4.6 mm \times 250 mm), eluted with a mixture of acetonitrile-water (80:20 v/v) as described in instrumentation section with a flow rate of 0.8 mL.min⁻¹ and detected at 220 nm

After comparison between the different columns such as, C18, CN, and NH₂, the best separation efficiency was obtained by using the NH₂ column. The mobile phase investigations showed that the ratio of organic modifiers, such as the acetonitrile in the mobile phase, was the key to achieve good separation. So isocratic mode of acetonitrile-water (80:20 v/v) at flow rate of 0.8 ml/min was used for obtaining the best selectivity of the method. The pH value did not play an important role in separation. Comparison between the purity threshold and purity angle reported in the empower software showed that the method was specific for the melamine

and the reported peak was completely separated from the other interfering compounds (Figure 2). The linear relationship between the detector response and different concentrations of melamine was confirmed by regression equation $y=113918x-19699$ and $r^2=0.9998$ in the range of 0.08-10 mg/L. The overall limit of detection (LOD) was 0.02, 0.01 mg/L and limit of quantification (LOQ) 0.08, 0.05 mg/L for powdered milk and coffee mate, respectively. To verify the precision and repeatability of the method, the relative standard deviations (RSDs %) of the intra-day and inter-day have been shown in Table 1.

Table1: Method validation parameters for detection of melamine in powdered milk and coffee mate

Validation parameter	Results	
	Powdered milk	Coffee mate
LOD& LOQ (µg/mL)	0.02 & 0.08	0.01 & 0.05
Selectivity	Selective	Selective
Linearity(r^2)	0.9998	0.9997
Range (µg/mL)	0.08-10	0.05-10
Intraday precision 0.5, 1.0, 1.5(µg/mL) (n=3, RSD%)	2.6, 0.52, 1.8	1.2, 1.5, 0.78
Interday precision 0.5, 1.0, 1.5(µg/mL) (n=3, RSD%)	4.2, 2.9, 3.4	3.6, 2.8, 3.1

The results of intermediate precision using different analysts, different instruments, and on different days, showed that these parameters did not have any significant effect on the variation of results. After these validation studies, the method's ability to provide good quantization in our laboratory was confirmed. The final step in the precision assessment would be our next target. In this step it is focused more on the bias in results, rather than on determining the differences in precision alone, as inter-laboratory crossover studies. Accuracy, which was evaluated as recovery, after spiking the milk and coffee mate samples with standards

at three concentration levels have been shown in Table 2. As the careful optimization of extraction conditions caused the good recovery for melamine. The present method was utilized to determine melamine in several powdered milk product and coffee mates. The obtained results of melamine contents in those dairy products are listed in Table 2. The results obtained from the method validation according to linearity, selectivity, accuracy and precision showed that the proposed method was suitable for the analysis of melamine.

Table 2. Assay of melamine in different brands of powdered milk and coffee mate by means of the described buffer mediated clean-up, solvent protein precipitation and HPLC-PDA analytical method

Sample Name	Content ($\mu\text{g/mL}$)	Added ($\mu\text{g/mL}$)	Determined* ($\mu\text{g/mL}$)	Recovery (%)
Humana	No detected	1.0	0.98 \pm 0.8	98.0
		2.0	1.81 \pm 0.1	99.5
Multi	No detected	1.0	1.02 \pm 1.2	102.0
		2.0	1.92 \pm 2.2	96.3
Nan	No detected	1.0	0.99 \pm 0.9	99.0
		2.0	1.85 \pm 2.2	92.5
Biomile	No detected	1.0	1.01 \pm 0.8	101.0
		2.0	1.88 \pm 1.5	94.0
Nestle A	No detected	1.0	0.95 \pm 0.4	95.0
		2.0	1.96 \pm 0.8	98.0
Nestle B	No detected	1.0	0.95 \pm 0.02	95.0
		2.0	1.92 \pm 0.6	96.0
Nestle C	No detected	1.0	1.00 \pm 1.2	100.0
		2.0	1.79 \pm 0.7	89.0
Nestle D	No detected	1.0	0.93 \pm 0.2	93.0
		2.0	1.84 \pm 0.9	92.0

CONCLUSIONS

In the present study, the LC determination of melamine on an aminated stationary phase has been achieved in only about 7 minutes with a combination of solvents acetonitrile/water (80:20 v/v) in an isocratic mode. All factors affecting the retention time of melamine and selectivity of the method, including pH and percentage of acetonitrile were studied. The detection has been optimized using a photodiode-array detector at a wavelength of 220 nm. This method can separate melamine from interferences with good resolution without using ion pair reagent or solid phase extraction

by only clean up in a formic acid buffer solution and using protein precipitation technique. By this study, we can propose a validated method for analysis of melamine in complex food matrices with an amino analytical column and simple clean up in buffer media and precipitation of proteins using acetonitrile and dichloromethane instead of expensive ion-exchange solid phase extraction methods. It is a sensitive, simple, fast and reliable in both preparation with minimum use of solvents and reagents. Low limits of detection and quantization comparing to other analytical methods

makes it a good choice for detection and determination of melamine in such a complex samples. The analytical procedures are suitable for quality control of powdered milk and coffee mates in food control laboratories.

ACKNOWLEDGMENTS

The general manager of food and drug control laboratories due his laboratory facilities support in this work is acknowledged. Special Thanks are forwarded to the Food and Drug Laboratory Research Center in Food and Drug Organization for financial support. The authors declare that there is no conflict of interests.

REFERENCES

1. Tyan Y.C., Yang M.H., Jong S.B., Wang C.K., Shiea J., 2009. Melamine contamination. *Anal Bioanal Chem.* 395(3), 729-35.
2. Mast R.W., Jeffcoat A.R., Sadler B.M., Kraska R.C., Friedman M.A., 1983. Metabolism, disposition and excretion of melamine in male Fischer 344 rats. *Food Chem Toxicol.* 21, 807-810.
3. WHO (World Health Organization), 2009. Toxicological and health aspects of melamine and cyanuric acid, Available from <http://www.who.int/topics/melamine/en> [accessed on May 12, 2011].
4. Garber E.A., 2008. Detection of melamine using commercial enzyme-linked immunosorbent assay technology. *J Food Prot.* 71, 590-594.
5. Lei H.T., Shen Y.D., Song L.J., Yang J.Y., Chevallier O.P., Haughey S.A., Wang H., Sun Y.M., Elliott C.T., 2010. Hapten synthesis and antibody production for the development of a melamine immunoassay. *Anal Chim Acta.* 665, 84-90.
6. Liu J.X., Zhong Y.B., Liu J., 2010. An enzyme linked immune sorbent assay for the determination of cyromazine and melamine residues in animal muscle tissues. *Food Control.* 21, 1482-1487.
7. Wang Q., Haughey S.A., Sun Y.M., Eremin S.A., Li Z.F., Liu H., Xu Z.L., Shen Y., Lei, H.T., 2011. Development of a fluorescence polarization immunoassay for the detection of melamine in milk and milk powder. *Anal Bioanal Chem.* 399, 2275-2284.
8. Li J., Qia H.Y., Shi Y.P., 2009. Determination of melamine residues in milk products by zirconia hollow fiber sorptive microextraction and gas chromatography-mass spectrometry. *J Chromatogr A.* 1216, 5467-5471.
9. Miao H., Fan S., Wu Y.N., Zhang L., Zhou P.P., Li J.G., Chen H.J., Zhao Y.F., 2009. Simultaneous determination of melamine, ammeline, and cyanuric acid in milk and milk products by gas chromatography-tandem mass spectrometry. *Biomed Environ Sci.* 22, 87-94.
10. Xia X., Ding S.Y., Li X.W., Gong X., Zhang S.X., Jiang H.Y., Li J.C., Shen J.Z., 2009. Validation of a confirmatory method for the determination of melamine in egg by gas chromatography-mass spectrometry and ultra-performance liquid chromatography-tandem mass spectrometry. *Anal Chim Acta.* 651,196-200.
11. Xu X.M., Ren Y.P., Zhu Y., Cai Z.X., Han J.L., Huang B.F., Zhu Y., 2009. Direct determination of melamine in dairy products by gas chromatography/mass spectrometry with coupled column separation. *Anal Chim Acta.* 650, 39-43.
12. Feng J.W., Cai Q.R., Liu X.C., Yu Y.G., Peng Y.F., Zhang Y. 2008. Determination of melamine and cyromazine in milk and dairy products by LC-MS/MS. *Mod Food Sci Technol.* 24, 1058-1060.
13. Ibáñez M., Sancho J.V., Hernández F., 2009. Determination of melamine in milk-based products and other food and beverage products by ion-pair liquid chromatography tandem mass spectrometry. *Anal Chim Acta.* 649, 91-97.
14. Panuwet P., Nguyen J.V., Wade E.L., D'Souza P.E., Ryan P.B., Barr, D.B., 2012. Quantification of melamine in human urine using cation-exchange based

high performance liquid chromatography tandem mass spectrometry. *J Chromatogr B*. 887-888, 48-54.

15. Wu Y.T., Huang C.M., Lina C.C., Ho W.A., Lin L.C., Chiu T.F., Tarn D.C., Lin C.H., Tsai T.H., 2009. Determination of melamine in rat plasma, liver, kidney, spleen, bladder and brain by liquid chromatography-tandem mass spectrometry. *J Chromatogr A*. 1216, 7595-7601.

16. Ehling S., Tefera S., Ho I., 2007. High-performance liquid chromatographic method for the simultaneous detection of the adulteration of cereal flours with melamine and related triazine by-products ammeline, ammelide, and cyanuric acid. *Food Additives and Contaminants*. 24, 1319-1325.

17. Filazi A., Sireli U.T., Ekici H., Can H.Y., Karagoz A., 2012. Determination of melamine in milk and dairy products by high performance liquid chromatography. *J Dairy Sci*. 95, 602-608.

18. Muñiz-Valencia R., Ceballos-Magaña S.G., Rosales-Martinez D., Gonzalo-Lumbreras R., Santos-Montes A., Cubedo-Fernandez-Trapiella A. Izquierdo-Hornillos R.C., 2008. Method development and validation for melamine and its derivatives in rice concentrates by liquid chromatography. Application to animal feed samples. *Anal Bioanal Chem*. 92, 523-531.

19. Sun H.W., Wang L.X., Ai L.F., Liang S.X., 2010. A sensitive and validated method for determination of melamine residue in liquid milk by reversed phase high performance liquid chromatography with solid-phase extraction. *Food Control*. 21, 686-691.

20. Tittlemier S.A., 2010. Methods for the analysis of melamine and related compounds in foods: a review. *Food Additives and Contaminants: Part A, Chemical Analysis Control Expose Risk Assess*. 27,129-145.

21. Venkatasami G., Sowa R.J., 2010. A rapid, acetonitrile-free, HPLC method for determination of melamine in infant formula. *Anal Chim Acta*. 665, 227-230.

22. Qian C., Hong Z., Lixi Z., Jian W., Rui W., Xiaohui Q., Yujian H., 2009. Electrochemical determination of melamine using oligonucleotides modified gold electrodes. *Talanta*. 80, 484-488.

23. Akbari-adergani B., Norouzi P., Ganjali M.R., Dinarvand R., 2010. Ultrasensitive flow-injection electrochemical method for determination of histamine in tuna fish samples. *Int J Food Properties*. 43, 1116-1122.

24. Ganjali M.R., Norouzi P., Akbari-adergani B., 2007. Thulium (III) Ions Monitoring by a Novel Thulium(III) Microelectrode Based on a S-N Schiff Base. *Electroanalysis*. 11, 1145-1151.

25. Ganjali M.R., Norouzi P., Akbari-adergani B., Riahi S., Larijani B. 2007. An Asymmetric Lutetium (III) Microsensor Based on N-(2-Furylmethylene) Pyridine-2,6-Diamine for Determination of Lutetium(III) Ions. *Analytical Letters*. 40, 1923-1938.

26. Dejaegher B., Mangelings D., Vander Heyden Y., 2008. Method development for HILIC assays. *J Sep Sci*. 31, 1438-1448.

27. Lin M., 2009. A review of traditional and novel detection techniques for melamine and its analogues in foods and animal feed. *Frontiers of Chemical Engineering in China*. 3, 427-435.

28. Akbari-adergani B., Eskandari S., Bahremand N., 2013. Determination of Sodium Benzoate and Potassium Sorbate in “Doogh” Samples in Post Market Surveillance in Iran 2012. *Journal of chemical Health Risk*. 3(1), 63-69.

29. Sugita T., Ishiwata H., Yoshihira K., Maekawa A., 1990. Determination of melamine and three hydrolytic products by liquid chromatography. *Bull Environ Contam Toxicol*. 44, 567-571.

30. Wei R., Wang R., Zeng Q., Chen M., Liu T., 2009. High-performance liquid chromatographic method for the determination of cyromazine and melamine residues in milk and pork. *J Chromatogr Sci*. 47, 581-584.

31. ICH Q₂B. 1996. Validation of analytical procedures. London: methodology (CPMP/ICH/ 281/ 95).

32. Hajimehdipoor H., Shekarchi M., Khanavi M., Adib N., Amri M., 2010. A validated high performance liquid chromatography method for the analysis of thymol and carvacrol in *Thymus vulgaris* L. volatile oil. Pharmacognozy Magazine. 6,154-158.

33. Shekarchi M., Hajimehdipoor H., Khanavi M., Adib N., Bozorgi M., Akbari-adergani B., 2010. A validated method for analysis of Swerchirin in *Swertia longifolia* by high performance liquid chromatography. Pharmacognozy Magazine. 6, 13-18.

Archive of SID