World Journal of
Chemical and Pharmaceutical Sciences

Journal homepage: https://zealjournals.com/wjcps/

ISSN: 2945-3186 (Online)

(RESEARCH ARTICLE)

Check for updates

Pipetting sample preparation with water eluent followed by water mobile phase HPLC analysis for residual monitoring of melamine in milk

Naoto Furusawa *

Graduate School of Human Life and Ecology, Department of Nutrition, Osaka Metropolitan University, Osaka 558-8585, Japan.

World Journal of Chemical and Pharmaceutical Sciences, 2023, 02(01), 017–022

Publication history: Received on 20 November 2022; revised on 10 January 2023; accepted on 13 January 2023

Article DOI: https://doi.org/10.53346/wjcps.2023.2.1.0032

Abstract

The author introduces a small-scale, time shortening, economical sample preparation with water eluent followed by an isocratic water phase HPLC system for quantifying melamine in cow's milk. Sample preparation is achieved by homogenization using a handheld ultrasonic-homogenizer with water followed by MonoTip®C18 pipette tip contains silica monolith bonded with octadecyl group with water eluent. For determination and identification of analyte, the HPLC uses an analytical C18 column, an isocratic 100% water, and a photo-diode array detector. The procedure, performed under a 100% water conditions, uses no organic solvents and poisonous reagents at all and is, therefore, harmless to both humans and the environment. The method validation data were well within the international method acceptance criteria. The total analytical time and quantitation limit were < 6 min/sample and 0.24 µg/mL, respectively. The present technique may be proposed as an international harmonized analytical method for routine residue monitoring for melamine in milk.

Keywords: International harmonized analytical method; Pipette tip; Water mobile phase HPLC; Melamine; Residue monitoring

1 Introduction

Melamine [1,3,5-triazine -2,4,6-triamine (C3H6N6)] is an inexpensive nitrogen-containing industrial chemical (melamine contains 66.6% N/weight) [1] that is widely used in the manufacture of plastics and adhesives, but may be used to falsify test s to check the protein content in milk products.

The 2008 melamine-tainted milk scandal resulted from the mixing of melamine in diluted milk to increase shipments because the addition of melamine increases apparent protein content, and reports of melamine-contaminated milk and milk-based foods in many countries [2-3]. To prevent a repeat of the scandal caused by melamine, the food residue monitoring for melamine has been strengthened.

The Codex Alimentarius Commission has set the maximum levels of melamine allowed in food products containing milk to 2.5 µg/g (or µg/mL) and the amount of the chemical allowed in powdered infant formula to 1 µg/g [4], equivalent to the FDA's safety limit [3]. Milk is an indispensable food and a base raw material for a wide variety of foods because it is highly nutritious, inexpensive and readily available. Strict monitoring of the presence of melamine in milk is, therefore, an important activity as a means of guaranteeing food safety.

The development of international standardized methods to determine chemical residues in foods is essential to guarantee equitable international trade in these foods and ensure food safety for consumers. Whether in industrial

* Corresponding author: Naoto Furusawa

nations or developing countries, an internal harmonized method for residue monitoring in foods is needed. The optimal method for the routine monitoring of chemicals in foods should be simple, quick, economical in time and cost and, importantly, cause no harm to the environment and analyst. Especially, eliminating the use of organic solvents and reagents is an important goal in terms of environmental conservation, human health and the economy [5].

The reported methods for quantification of melamine in milk include HPLC–UV [6]/photodiode array detector (PDAD) [7]/fluorescence detection [9] and LC–MS–MS [9 -11]. The FDA issued new methods for the analysis of melamine in liquid formula, based on LC–MS–MS detection [12]. The direct analysis of liquid milk by low-temperature plasma MS (LTP–MS) [13] and ultrasound-assisted extractive electrospray ionization (EESI–MS) [14] have been proposed. The facilities which LC–MS–MS, LTP–MS or EESI–MS system are available are limited because these operations are complex and require skill to perform accurate and robust analysis, and these instruments are extremely expensive. These are unavailable in many analytical institutions and laboratories for routine work, particularly in laboratories in developing countries and/or in local health centers in developed countries. All of the methods mentioned above consume organic solvents in the HPLC and LC–mass spectrometry (MS)-MS mobile phases as well as for extraction and deproteinization in sample preparation. The risk associated with these solvents extends beyond direct implications to human health by affecting the ecosystem in which we all reside. Furthermore, the disposal (combustion) of large amounts of waste organic solvents used in these instrumental analyses is very costly. No optimal method that satisfies the requirements for melamine analysis previously described has yet been established.

This article describes a safe, economical and time shortening method to monitor melamine residue in milk using 100% water in sample preparation and 100% water mobile phase HPLC separation without using organic solvent and reagents.

2 Material and methods

2.1 Reagents and apparatus

All chemicals including melamine standard were purchased from FUJIFILM Wako Pure Chem. Ltd. (Osaka, Japan). Distilled water was of HPLC grade. The following apparatuses were used in the sample preparation: handheld ultrasonic-homogenizer (model HOM-100, 2 mm ID probe, Iwaki Glass Co., Ltd., Funabashi, Japan); micro-centrifuge (Biofuge® fresco, Kendo Lab. Products, Hanau, Germany); MonoTip® C18 pipette tip (packed with silica monolith that consists of continuous through-pores and octadecyl bonding); sample throughput volume ≤ 200 µL; through-pore diameter of 10 - 20 µm; meso-pores of 20 nm; surface area of 200 m^2/g (GL Sciences, Inc., Tokyo, Japan). The HPLC system, controlled with *ChromNAV*® chromatography data system, included a model PU-4180 pump and DG-4580 degasser (Jasco Corp., Tokyo, Japan) equipped with a model CTO-10AS *VP* column oven (Shimadzu Scientific Instruments, Kyoto, Japan), as well as a model MD-4017 photodiode-array detector (PDAD) connected with a model LC-Net II/AD interface box (Jasco). The following five types of TMS (trimethylsilyl group), C4, C8, and C18 non-polar sorbent (the highly purified silica-based) columns (5 µm d_P; 4.6 mm i.d.; 150 mm length) for HPLC analysis were used: Inertsil® TMS; Inertsil WP300 C4; Inertsil WP300 C8; Inertsil ODS-4; InertSustain® C18 (all GL Sciences Inc., Tokyo, Japan). Table 1 lists physical/chemical specifications of particles packed into the columns.

2.2 Pipette tip operating procedure

After attaching a MonoTip C18 pipette tip to a micro pipette (20 – 200 µL volume type), preconditioning of the tip was carried out by drawing and ejecting (to waste) 50 µL of distilled water to reduce background noise. A 50 µL aliquot of the sample was drew into the conditioned MonoTip C18 tip, and ejected back into another sample tube. This series of IN and OUT operations was defined as one pipetting operation in this study.

2.3 HPLC operating conditions

The analytical column was an InertSustain® C18 (150 × 4.6 mm, 5 µm) column using an isocratic mobile phase of water at a flow rate of 1.0 mL/min at 30 °C. PDAD was operated at 190 – 300 nm: the monitoring wavelength was adjusted to 202 nm which represent maximum for melamine. The injection volumes were 10 µL.

2.4 Preparation of stock standards and working solutions

Stock standard solution of melamine was prepared by dissolving melamine in water followed by water to a concentration of 50 µg/mL. This solution was stored at -20 °C. Working standard solutions of this compound were freshly prepared by suitably diluting the stock solutions with water on the day of the analysis.

2.5 Sample preparation

An accurate 50 µL milk sample was taken into a 1.5 mL micro-centrifuge tube and homogenized with 750 µL of water with a handheld ultrasonic-homogenizer for 20 s. The mixture was filtered through a 0.2-µm disposable syringe filter unit. A 50 µL aliquot of the filtrate was aspirated into the conditioned MonoTip C18 pipette tip and dispensed back into the sample tube. The eluate was injected into the HPLC-PDAD system.

2.6 Method validation

The performance of the developed method was validated in terms of many parameters from the international guidelines for method validation of analytical procedure [15-17].

3 Results and discussion

3.1 Sample preparation

The advantage of the present procedure is that melamine in milk is pretreated quickly, economically, and environment-friendly on small-scale, requiring only water as the analytical reagent. The ultrasonic-homogenization enabled the satisfactory extraction of melamine from a milk sample of 0.05 mL with water of 0.75 mL without bumping. The extract from milk did not form an emulsion that would hinder melamine recoveries. After being homogenized, the extract obtained was filtered through a disposable filter unit and purified by the monolithic pipetting tip, MonoTip C18. The MonoTip C18 treatment is not only used for deproteinization but can also be used for defatting. As a preliminary study, a 50 µL of melamine standard solution (5 µ/mL in water) was applied to the MonoTip C18 and the recovery of melamine from the pipette tip was examined. The MonoTip C18 gave a satisfactory recovery (average 97.6 %) and repeatability (RSD 3.1 %) for melamine. The extract obtained above was easily purified by the pipette tip, which was performed by one pipetting operation. The quick and easy procedure resulted in high recovery and reproducibility with great saving time and cost. The resulting extract was free from interference, as can be seen in HPLC traces of blank (Figure 1-a) and spiked milk sample (Figure 1-b). These findings demonstrate that the extraction and purification worked well.

3.2 HPLC conditions

To achieve the separation with an isocratic 100% water mobile phase and optimize faster separation, the author tested 5 types of revised phase columns (Table 1). This study used water as the isocratic mobile phase and examined column temperatures ≥ 25 °C, HPLC flow rates ≥ 0.5 mL/min, and HPLC retention times ≤ 5 min: because the HPLC separations were performed serially, the time/run was critical for routine residue monitoring. The short run time not only increased sample throughout for analysis but also affected the method-development time. The five columns were compared regard to 1) the quantifiable/identifiable separation between melamine and its interfering peaks; 2) sharpness of peaks obtained upon injection of equal amounts. The resulting chromatographic separation and peak form profiles within the conditioning ranges examined are also presented in Table 1.

Table 1 Physical/chemical specifications of the less-retentive reversed-phase columnsa used and resulting chromatographic melamine separation obtained under the HPLC condition ranges examined[b]

Column[a]		Pore diameter (nm)	Surface area (m^2/g)	Carbon content (%)	Chromatographic melamine peak	
Trade name	bonded group				Peak form	Separation
Inertsil®TMS	trimethylsilyl	10	450	4	Rounding	Overlapped[c]
Inertsil® WP300 C4	butyl	30	150	3	Sharp	Overlapped
Inertsil® WP300 C8	octyl	30	150	4	Sharp	Overlapped
Inertsil® ODS-4	octadecyl	10	450	11	Sharp	Slightly overlapped[d]
InertsilSustain® C18	octadecyl	10	350	14	Sharp	Completely separated

[a] d*p* = 5 µm; i.d. = 4.6 mm; lengnth = 150 mm.; [b] Mobile phase of water, column temperatures ≥ 25 °C, flow rates ≥ 0.5 mL/min and retention times ≤ 5 min; [c] Melamine was overlapped with the interfering milk extract; [d] Overlapped below spiked levels of 2.5 µg/mL.

Columns A, B, and C had difficulty separating melamine and the interfering milk extract throughout the examined condition ranges. In column D, melamine overlapped with the interference peak when the spiked levels were less than 2.5 µg/mL. The complete separation of melamine and interference peaks, a symmetrical peak, and a short retention time were achieved with Column E using an isocratic water mobile phase with a flow rate of 1.0 mL/min and a column temperature of 30 °C. Figure 1 displays typical chromatograms for a blank milk sample and for a spiked milk sample obtained under the procedure developed here, with the PDAD set at 202 nm (giving maximum absorption spectrum for melamine). The present isocratic HPLC-PDAD system achieved reproducible separation in < 3.5 min without the need for a gradient system with poor reproducibility and requiring time for conditioning during continuous analysis.

3.3 Method validation

Table 2 summarizes the main method validation parameters. The quantifying limit in milk samples was 0.24 µg/mL for melamine. The value is less than the Codex maximum level (2.5 µg/mL for milk) [5]. The system-suitability evaluation is an essential parameter of HPLC determination, and it ascertains the strictness of the system used. The suitability was evaluated as the relative standard deviations of peak area and retention time calculated for 10 replicate injections of a spiked milk sample with melamine (5 µg/mL). The values were estimated to be 0.22% for retention time and 0.35 % for peak area, respectively. Including this system-suitability, the linearity, accuracy, and precision are within the international acceptance criteria (Table 2) [15-17]. In terms of selectivity, the present HPLC-PDAD system easily confirmed the peak identity of target compound. The analyte was identified in a milk sample by its retention time and absorption spectrum. In Figure 1-b, the melamine spectra obtained from the milk sample was practically identical to that of the standard: spectral search processing of the chromatography data system calculated that the correlation coefficient between the two absorption spectrum was 0.9969 (identical evaluation criterion >0.98). The system did not require the use of MS or MS/MS, which is very expensive and is not widely available for routine work.

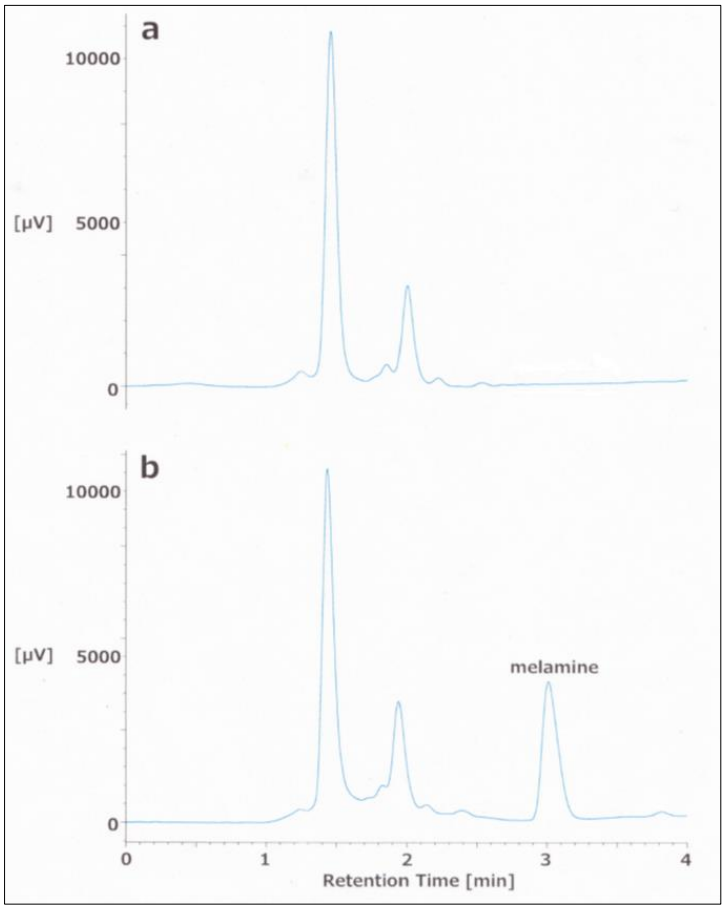

Figure 1 Chromatograms obtained from the present HPLC system for a blank milk samples (a) and a milk sample spiked with melamine (5 µg/mL) (b). PDAD set at 202 nm. Retention time for melamine = 3.0 min

Table 2 Method validation data for melamine-fortified milk samples

		Melamine	Codex acceptance criterion[a]	FDA recommendation[b]
Linearity (r)[c]		0.9996		≥ 0.999
Accuracy[d]		95.6	80 - 100	
Presision[e]		2.9	≤15	
Sensitivity (QL[f])		0.24	2.5[g]	
System suitability: Injection repeatability[h] (RSD, %)	Retention time	0.22		≤ 1
	Peak area	0.35		≤ 1

[a] [15,16]; [b] [17]; [c] r is the correlation coefficient ($p < 0.01$): Mean of three determinations using spiked milk samples for calibration curves; range of concentration was 0.5 - 10 μg/mL; [d] Average recovery (%, n = 10) from five replicates at two different spiked levels (2.5 and 5 μg/mL); [e] Value is R.S.D. (%, n = 10); [f] Quantification limit (μg/mL), QL as the concentration of analyte giving a signal-to-noise ratio = 10.; [g] Codex maximum level for melamine in milk: 2.5 μ g /mL; [h] Data as the relative standard deviations calculated for 10 replicate HPLC injections (10 μL) of the prepared eluate for milk sample spiked with melamine (5 μg/mL).

3.4 Cost and time performances

The total time and budget required for the analysis of a single sample were less than 6 min and approximately USD 6.45 (as of 17 November 2022), respectively. These findings became term required for the routine assay. The short time and low-cost quantitative method increased the sample throughput for actual routine monitoring work.

3.5 Application to real milk samples

Twenty samples of commercial raw milk purchased from convenience stores in Osaka, Japan were analyzed using the present method. No samples contained detectable concentrations of melamine. All chromatograms were free from interferences.

4 Conclusion

A pipetting sample preparation with water eluent followed by an isocratic water mobile phase HPLC-PDAD method for quantification of residual melamine in milk has been successfully established. The method validation data were well within the international method acceptance criteria. The present procedure provided an easy-to-use, fast, and environment/analyst-friendly and resulted in high recovery and repeatability with considerable saving of analysis time/cost. In particular, the present technique may be proposed as an international harmonized method for routine residue monitoring melamine in milk.

Compliance with ethical standards

Acknowledgments

The present study was carried out at public expense (by the author's university department). Gratefulness.

Disclosure of conflict of interest

There is not any conflict of interest.

References

[1] S. Ehling S,Tefera S, Ho IP. Food Addit. Contam., 24, 1319-1325, 2007.

[2] World Health Organization. Disease Outbreak News, 2008 - China, 29 September 2008.

[3] World Health Organization. Melamine and Cyanuric acid: Toxicity, Preliminary Risk Assessment and Guidance on Levels in Food, 25 September 2008.

[4] Codex alimentarius, General Standard for Contaminants and toxins in Food and Feed, CXS 193-1995, Adopted in 1995, Revised in 1997, 2006, 2008, 2009.

[5] Anastas PT and Warner JC, Green Chemistry: Theory and Practice, Oxford University Press, Oxford, United Kingdom, 1998.

[6] Venkatasami G and Sowa Jr. JR. Anal. Chim. Acta, 665, 227–230, 2010.

[7] Rambla-Alegre M, Peris-Vicente J, Marco-Perio S, Beltran-Martinavarro B, Esteve-Romero J. Talanta, 81, 894–900, 2010.

[8] de Lourdes V et al.. Food Control, 51, 402-407, 2015.

[9] Mi J et al.. Agilent Technologies Application Note 5989-9950EN, Oct 2008.

[10] Tran B et al.. J. Agric. Food. Chem., 58, 101–107, 2010.

[11] Chen D et al.. Talanta, 134, 144-152, 2015.

[12] U.S. FDA Laboratory Information Bulletin No 4421.

[13] Huang G et al.. Chem Commun., 5, 556–558, 2009.

[14] Zhu L et al.. Chem. Commun., 5, 559-561, 2009.

[15] Codex alimentarius commission, ALINORM 01/23, Codex Committee on Methods of Analysis and Sampling, Joint FAO/WHO Food Standards Programme, the 23nd Section of the Codex Committee on Method of Analysis and Sampling.

[16] Huber L. Validation and Quantification in Analytical Laboratories, 2nd ed., Chapter 11, Validation of Analytical Methods, CRC Press, FL, USA, 2007.

[17] United States Food and Drug Administration, Guidelines for Submitting Samples and Analytical Data for Methods Validation, Rockville, Maryland, 1987.