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Validation of the Method for Determination of Melamine and Investigation its Trace in Milk from Vietnam by LC-MS/MS

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

This work describes a rapid, selective, and sensitive method by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to detect melamine (MEL) in milk and dairy products. The optimal conditions of liquid chromatographic separation extraction and mass spectroscopy of MEL have also been examined. The linear range for analyte detected by the method was 0.5÷100.0 ng/mL, with correlation coefficients was 0.999. Mean recoveries of the method in the real samples at three spike levels (low, medium, and high) were within the range of 98.5% ÷102.5% (n =7). LOD, LOQ values of the method were 10 and 30 ng/mL, respectively. The influence of the matrix effect on the accuracy, repeatability, and recovery of the process was insignificant. The proposed method was used to quantify the content of this compound in various real samples, which were collected in Ho Chi Minh City-Vietnam in 2020.

Keywords: LC-MS/MS; tandem; melamine; matrix effect.

1. INTRODUCTION

Melamine (MEL) is a synthesis organic most commonly observed in the form of white crystals rich in nitrogen (named 2,4,6-triamino-1,3,5-triazine, $C_3H_6N_6$). It is created in large amounts fundamentally for use in the synthesis of melamine-formaldehyde resins to produce

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laminates, plastics, coatings, commercial filters, glues or adhesives, and dishware and kitchenware [1] (WHO, 2009). MEL matured a topic of discussion in 2007 when veterinary scientists confirmed that pet food infection of melamine was the cause of hundreds of pet deaths [2]. The ingestion of MEL may lead to renal failure, kidney stones, and other health problems [3]. U.S. Food and Drug Administration (FDA) described that MEL and cyanuric acid concentrate and interact in the urine-filled renal microtubules when they are absorbed into the bloodstream. Then, they crystallize and procedure numerous round, yellow crystals, which in turn block and damage the renal cells that line the tubes causing the kidneys to malfunction [4]. In 2008, high concentrations of MEL were announced in contaminated Chinese infant formula. More than 51,900 infants and young children in China were hospitalized for urinary problems, possible kidney stones, possible renal tube blockages, and related to the consumption of melamine-contaminated infant formula and related dairy products [5]. After that time, melamine was detected in liquid milk and yogurts, powdered milk, cereal products, confectionaries, cakes and biscuits, protein powders, frozen desserts, and some processed foodstuffs. These foods included ammonium bicarbonate, dried whole egg, fresh hen eggs, nondairy creamer, animal feed, and animal feed ingredients [6]. Many countries have set maximum residue limits (MRL) for melamine in various products to protect public health and food safety. For example, the US FDA set the MRL of MEL in milk and dairy products and milk foods as 0.25 mg/kg and stressed that infant formula sold to US consumers must be utterly free of MEL. The European Union (EU) set the MRL of MEL in dairy products and high-protein foods at 2.5 mg/kg. The Ministry of Health of China published new dairy safety standards and emphasized that food should not be tainted with MEL. Ministry of Health of Vietnam announced and stressed that food should not be contaminated with MEL [7]. In Vietnam, the maximum limit of melamine cross-contamination in food is regulated as follows: MEL content must not exceed 1.0 mg/kg in food for children under 36 months old and not exceed 2.5 mg/kg in other foods [8].

Many determination methods for MEL have been developed, such as liquid chromatography [9], immunoassay, GC-MS, ELISA test, and liquid chromatography-MS [10].

The MEL-contaminated milk scandal has happened over the past ten years, but to strengthen the control from the state management agencies and raise the awareness of the Vietnamese people about the quality of milk in general and contaminated milk MEL in particular, the testing and analysis of this banned substance is necessary. Therefore, we have validated the method for analyzing MEL by reversed-phase LC-MS/MS in milk and dairy products. The procedure was then used to analyze 16 samples of dairy milk purchased from major retailers in Vietnam.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All reagents were of analytical grade. Melamine (99%) standard was purchased from Sigma Andrich (USA). Acetonitrile (ACN), formic acid (FA), HCl (36-38%), Amoniac 30%, and Dichloromethane (CH_2Cl_2) were supplied from Merck (Darmstadt, Germany). Methanol was of HPLC grade and acquired from J. T. Baker (Phillipsburg, USA). $MgSO_4$, NaCl, and C_{18} powder were obtained from Waters (Milford, MA, USA).

2.2 Instrumentation

The method validation was conducted on liquid chromatography (LC) system, including the column ZORBAX 300SB-C reversed polarized phase inversion liquid chromatography column, 250mm x 4.6mm; particle size 5µm and thermostat autosampler (TSQ 7000, Thermo Quest Finnigan Bremen, Germany). The equipment is combined with the Waters TQD three quadrupole mass spectrometers with APCI ion source. Selected reaction monitoring (SRM) was applied for data acquisition.

2.3 Chromatographic and MS Conditions

For liquid chromatographic separation, the binary mobile phases were A(H_2O- HCOOH 0.1%) B (ACN: H_2O (40:60)). Table 1 illustrates the mobile phase program for the loading pumps. The flow rate was maintained at 0.6 mL/min during the whole chromatographic analysis process. Both standard and sample solutions were held at 10°C in the sample tray. A 5.0 µL of standard or samples was injected into the apparatus system via an autosampler. Using the MeOH and deionized water (1:1,v:v), cleaned triplicate the needle and the sample loop in the autosampler. Selected Reaction Monitoring (SRM) spectra obtained in positive ion mode were applied to identify the specified analyte.

Table 1. The mobile phase gradient program

Time (min)	Flow rate (mL/min)	A(%)	B(%)
0.00	0.6	90	10
0.20	0.6	90	10
3.00	0.6	0	100
4.50	0.6	0	100
5.00	0.6	90	10
7.00	0.6	90	10

2.4 Sample Treatment

2.4.1 Sample collection

Samples of powdered milk, pasteurized liquid milk, sweetened condensed milk were collected from supermarkets in Ho Chi Minh City, Vietnam, from October to November 2020.

2.4.2 Sample extraction

0.5 g of the homogenized sample was placed in a 10 mL test tube. 3 mL of distilled water were added to a test tube and shaken well with a vortex machine for 3 minutes. Then, the sample was ultrasound for 5 minutes. 6 mL of acetonitrile was added and shaken vigorously for 3 minutes, followed by ultrasound for 5 minutes. 1 mL of 1M HCl was added and shaken vigorously for 3 minutes, using ultrasound for 5 minutes. The sample was centrifuged at 4000 rpm for 10 minutes. 5.0 mL of solution was placed in a 30 mL test tube with a lid. Next, 3 mL of water and 15 mL of dichloromethane were added and shaken thoroughly for 2 minutes using a vortex machine. The test tubes were centrifuged at 2000 rpm for 5 minutes. The upper layer was withdrawn and transferred to a 10-mL test tube. 2.5 mL dichloromethane and 0.1M HCl were added. The sample was shaken well for 2 minutes and centrifuged at 2000 rpm for 5 minutes. Finally, the supernatant was removed by pipette and pooled into the original 10 mL test tube.

2.4.3 Solid-phase extraction

SPE Strata Screen-C 200mg/3mL (Phenomenex) column was connected to SPE extractor. 3.0 mL of methanol and 3 mL H_2O were passed through the column. The extract is then passed through the column and eluted with 1.0 mL (x2) 0.1 N HCl and 0.5 mL (x 2) methanol. The MEL was eluted with 5 ml of 5% ammonia in methanol in a 50 ml flask. The sample was then vacuum-evaporated at 45-50 ° C to dryness. The residue was then dissolved with 1.0 mL of acetonitrile: water / 1: 4 (v / v) mixture. Finally, the solution was filtered to a 0.45 μm membrane filter into a vial for quantification.

2.5 Method Validation

2.5.1 Standards preparation

A stock solution of 100 µg/ml concentration of MEL was set in DIW, from which a standard solution 10 µg/ml was obtained in DIW by dilution and kept at 4°C. Calibration mixtures of concentration levels 5.0, 10.0, 20, 50.0, 100.0 and 200.0 ng/mL were freshly made in ACN/DIW (8:2). Standard solutions were injected into the LC-MS / MS machine under the optimum conditions selected in order of low to a high concentration. The calibration curve equation was established by the relationship between the area of the peaks and the concentration of standards.

MEL is considered positive in the test sample if it fully meets the criteria of Commission Decision 2002/657/EC [11]. Analyte signal with two mass transfer modes for each substance analysis and two mass transfer modes for the corresponding internal standards must have appeared with a signal-to-noise ratio per ion must be ≥ 3:1. The analyte's relative retention time corresponds to the mean relative retention time of the calibration solution within ± 2,5% tolerance. The peak area ratio between the various mass transfer reactions of each analyte is within the permissible range specified in Commission Decision 2002/657/EC.

2.5.2 LOD and LOQ

The detection limit (LOD) of an analytical procedure is the smallest analyte content that this procedure can detect with the statistical confidence that the sensitivity of the procedure can be assessed. Likewise, the quantitative limit (LOQ) is the minimum content of the analyte that can be quantified using this procedure [12]. The test was carried out as follows: MEL standard solutions were added to the blank milk sample so that the concentration of these substances in the sample was relatively low. Samples were treated and performed analysis on the equipment under the conditions in section 2.3 to find a sample solution with a known minimum standard concentration Cmin giving a signal: 3 <T = S / N <10.

LOD and LOQ were determined by the following formula: LOD = 3.S / N and LOQ = 3.LOD

2.5.3 Recovery (R%)

The recovery of the method is determined by the standard addition technique [13]. MEL concentration was added to the sample at three levels of 15, 30, and 100 ng / L. Samples were processed and measured in section 2.3. Recovery (R%) is determined by the formula:

$$\%R = \frac{C_{\mathrm{Re}} - C_m}{C_c} * 100$$

Where:

C_{Re}: total concentration of the sample and concentration of the added standard
C_m: concentration of the sample analyzed
C_c: concentration of the added standard

2.5.4 Repeatability

The evaluation of the Intra-assay imprecision and inaccuracy was conducted using an experimental model of previous works [14]. These tests were evaluated by examining three quality controls 15, 30, and 100 ng/L of MEL as six replicates during a single day. The mean, standard deviation, and coefficient of variation values were measured for each quality control. The inaccuracy of the assessments for each quality control was determined as the distinction between the mean measured concentration and the nominal concentration as a percentage of the nominal concentration. Inter-assay imprecision was evaluated in six assays run on separate days with two quality controls containing MEL concentrations within the operating range. This data was again shown as the coefficient of variation.

2.5.5 Matrix effect

The evaluation of the matrix effect was conducted using an experimental model of previous works [15,16]. The matrix effect was examined by measuring the analyte's analytical signal in the postextraction spiked solution and the analyte standard in a neat solution. The test was conducted on three samples with the protocol as mentioned above. The final solution was spiked at three levels: 15.0, 30.0, and 100 ng/L f the standard MLN. The matrix effect was defined by the following equation:

ME (%) = X/Y×100

Where: X, Y are the chromatographic peak area of the standard in neat solution and peak area of the standard spiked into sample solution after extraction, respectively.

3. RESULTS AND DISCUSSION

3.1 Chromatographic and MS Conditions

The conditions for LC chromatography have been established with the C18 ZORBAX 300SB-CN column (250 x 4.6 mm, particle size 5.0 μm), mobile phase A (H_2O- HCOOH 0.1%), and B (ACN- HCOOH 0.1%) and 5.0 µL injection volume. The flow rate was set at 0.6 mL/min, MEL retention times were 4.6 min (RSD= 1.21%). This result is entirely consistent with the previous works [17].

Mass spectrometric data were acquired in atmospheric pressure chemical ionization (APCI), a widely used ionization technique in mass spectrometry, positive mode, using the selected reaction monitoring (SRM) function. The instrument operates with the following parameters: curtain gas, 25 (manufacturers units); source gas 1, 45; source gas 2, 45; CAD gas pressure high; and nebulizer current, 3.0. Two SRM transitions were monitored for MEL. Collision energy and collision exit potential settings were optimized for each transition during infusion of the MEL solution. The optimization results were as follows: original ion of m/z 127 and product ions of m/z 85, m/z 68. The m/z of 85 was chosen for the quantitation analysis. This data is entirely related to previous studies (Fig. 1). This result is quite similar to the earlier researches [9,17].

3.2 Method Validation

3.2.1 Linearity

Linearity test solutions were adjusted from the stock MEL solution at six concentration levels ranging from 5.0, 10.0, 20, 50.0, 100.0 and 200.0 ng/mL. A calibration curve was received by plotting the peak area vs. concentration. A linear calibration plot was obtained over the calibration range of 5.0-200 ng/L with regression equation y = $1.344.10^4$ x + $1.74.10^3$ and correlation coefficient (r) of 0.9997. The RSD% values of the repeated injections are < 5% within each level.

3.2.2 LOD and LOQ

LOD and LOQ of the method were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known concentrations. A precision

study was also carried at the LOQ level by injecting six individual preparations, and RSD% of the peak area was calculated. The LOD was determined to be 1.0 ng/mL and 1.5 ng/mL for baby milk and liquid milk, respectively, and the LOQ was settled to be 3.0 mg/kg and 4.5 ng/mL for baby milk and fluid milk, respectively. The RSD% of the precision study conducted at the LOQ level was within 5%.

3.2.3 Percentage recovery, matrix effect

The percent recovery was expressed as the percentage of the standard recovered from the sample matrix. All concentrations used in the examination were within the limits of the analytical curve because samples all contained MEL. Table 2 shows that the percent recoveries were higher than 90%, which designated that this method is reliable. Table 2 shows that at three sample backgrounds with different fat content, ME values of MEL ranged from 88.06% to 109.82%, which were in an agreeable range. RSD values at various concentrations were suitable values. These values proved that the influence of the matrix sample on selectivity and recovery was negligible.

3.2.4 Repeatability

The RSD precision within-day was between 1.45 and 4.63%. The accuracy of the method within-day was between 95.0 and 103.5%. The accuracy of the day-to-day data of this study was from 95.1 to 103.8%. The stability of the samples was found to be at least 7 days.

The LOD, LOQ, recovery, ME, and precision of this method for the measurement of MEL in some dairy products were compared with other published techniques [9,10]. These data indicated that a sensitive and verified HPLC-MS/MS method for determining melamine residue in milk and dairy products was developed. The recommended approach was sensitive, reliable, and accurate and allowed the detection of melamine residues at levels as low as 10.1 to 12.3 µg/ kg in various dairy products. The method can be used for the routine judgment of melamine residues in various dairy products.

3.3 Application to Actual Samples

The proposed validated LC-MS/MS method was applied to the determination of MEL in three different batches of the compound solution. Satisfactory results were obtained as shown in Table 3; The results showed that MEL was not present in the samples analyzed. These data prove that the management of milk quality in Vietnam has been much better than ten years ago. Or, fraudulent manufacturing firms no longer exist in the dairy market.

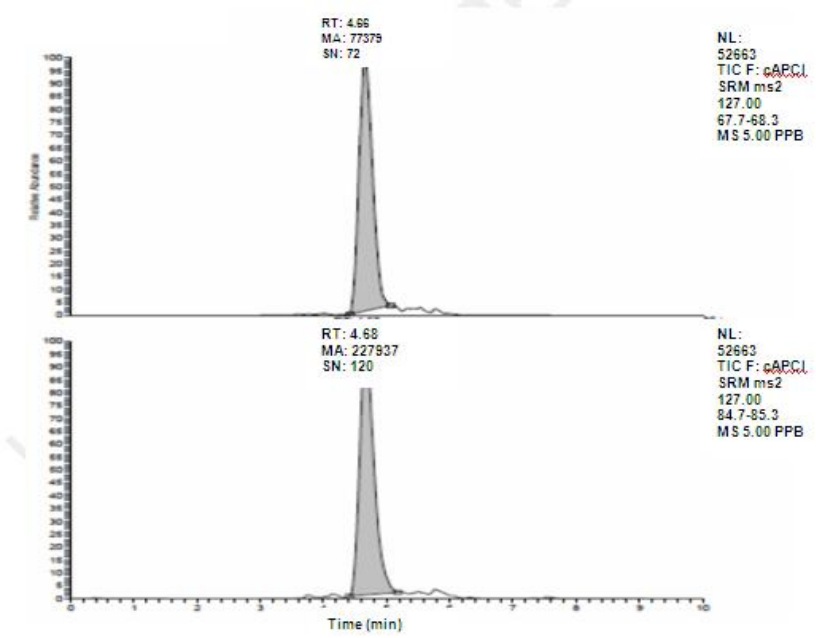

Fig. 1. LC-MS/MS chromatogram of MEL its production: 127.0/85 and 127.0/68

Table 2. The percentage recovery and matrix effect of the method at three levels of concentration (n=6)

Samples	Spiked(ng/mL)	Recoveries		Matrix match	
		Mean (%)	RSD%	Mean ME (%)	RSD (%)
Children Powder Milk	15.00	106.31	6.12	105.31	2.11
	30.00	95.07	6.01	88.06	8.01
	100.00	106.21	5.31	101.31	6.31
Sweeten Milk	15.00	103.41	8.99	105.41	3.91
	30.00	96.51	6.71	99.58	6.71
	100.00	93.11	6.48	93.11	4.29
Pasteurised milk	15.00	103.381	5.12	107.31	7.12
	30.00	99.81	6.89	109.82	6.81
	100.00	92.98	5.15	103.91	5.16

Table 3. Result of MEL in milk samples analyses

Samples	Found*
Children Powder milk 1	ND
Children Powder milk 2	ND
Children Powder milk 3	ND
Children Powder milk 4	ND
Children Powder milk 5	ND
Children Powder milk 6	ND
Children Powder milk 7	ND
Sweeten milk 1	ND
Sweeten milk 2	ND
Sweeten milk 3	ND
Sweeten milk 4	ND
Pasteurized milk 1	ND
Pasteurized milk 2	ND
Pasteurized milk 3	ND
Pasteurized milk 4	ND
Pasteurized milk 5	ND

*ND: none detection, * values are expressed as the mean ± SD (n = 3)*

4. CONCLUSIONS

In summary, this work evaluated the analytical method for MEL. The optimization conditions of HPLC-MS / MS machine using APCI ionization source, mobile phase composition, milk sample preparation have been thoroughly investigated. This method showed advantages such as good qualitative and quantitative, wide linear range, high sensitivity and good selectivity, high recovery efficiency, and relatively low detection limit and quantification limit. The method has also been applied to analyze 20 types of milk in the Vietnamese market. The results showed that these samples did not contain melamine.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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