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An optimized method for simultaneous determination of Melamine and Cyanuric Acid in animal tissues by Liquid Chromatography-Triple Quadrupole Mass Spectrometry

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Abstract

An optimized method was developed for simultaneous determination of melamine (MEL) and cyanuric Acid (CYA) in meat, liver and kidney tissues of five kinds of animals (cattle, goat, pig, chicken and fish). MEL and CYA were extracted with acetonitrile/water (70/30) by shaking. After being simply defatted with *n*-hexane, both analytes were detected within 12 min using liquid chromatography-triple quadrupole mass spectrometry (LC/MS/MS) on Waters Atlantis HILIC columns. The optimized method using isotope-labeled analytes as internal standard was linear over a range of concentrations with 2-500 ng/mL for MEL and 4-500 ng/mL for CYA. The mean recoveries of MEL and CYA in tissues respectively ranged from 83-108% and 75-102% with RSD < 11%. The limits of detection (LOD) for MEL and CYA respectively were 0.01 and 0.02 μg/g of tissues. The results showed that the optimized method have a simple procedure of sample preparation, a wide linear range and higher sensitivity, and therefore suit for determination of MEL and CYA in animal tissues especially when analyzing big batch of samples from pharmacokinetic studies of MEL and CYA in animal.

Keywords Melamine; Cyanuric Acid; Animal Tissue; Liquid Chromatography-Triple Quadrupole Mass Spectrometry; Determination

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Introduction

As a nitrogen-based industrial chemical, Melamine (MEL) was generally used in the manufacture of plastics and flame retardants but not approved as a food additive for use in

human or animal feed. However, aim to improve nitrogen content it was illegally added in protein-based food commodities such as milk, infant formula, pet food and wheat flour, and therefore caused serious healthy danger. In 2008, the Chinese milk incident caused an estimated 300,000 victims up to November, and some infants were diagnosed with kidney stones because of being fed on milk powder contaminated with MEL [1]. In 2007, a major renal disease and associated deaths in cats and dogs broke out in USA because of being fed on pet food ingredients contaminated with MEL and its analogues [2]. The studies found that MEL in combination with cyanuric acid (CYA) would form insoluble melamine-cyanurate (MEL-CYA) crystals in kidneys, and so block and damage renal tubules leading to renal failure in infants and pets [3,4].

Previous reports found presence of MEL in animal including chickens, hogs, and fish etc. Some methods were also developed for determination of MEL and analogues in animal tissues and feed. Those included enzyme-linked immunosorbent assay (ELISA) [5,6], gas chromatography/mass spectrometry (GC/MS) in pet food [7-9] and milk products [10], liquid chromatography [11,12], sweeping-micellar electrokinetic chromatography [13], and Liquid chromatography/tandem mass spectrometry (LC/MS/MS) in aquatic products [14], kidney [15], meat [16], rat plasma and tissues [17], feed [18], pet food [19,20], infant Formula [21] and food [22]. Aim to shorten analytical time and improve determination efficiency especially when analyzing large batch of samples, this study developed a simple, rapid and sensitive analytical method for simultaneously extraction and quantitative determination of MEL and CYA in animal tissues including meat, liver and kidney of cattle, goat, pig, chicken and fish.

Results and Discussion

Method optimization

Generally, solid phase extraction (SPE) was used to clean of sample extracts in previous studies, MCX and MAX SPE cartridge were respectively used for MEL and CYA [14,23]. Filigenzi etc [16] used 10 g of samples of pork tissues to homogenize with acetonitrile and water, and then extracts were acidified hydrochloric acid and subsequently cleaned up with liquid-liquid and SPE. Andersen etc [14] optimized this method to detect MEL in aquatic product, and used a smaller sample size (5g) and internal standard. However, SPE method was not preponderant in analysis speed and quickly to process a big batch of samples. Especially, the sample extracts must be separated to two parts and respectively used to clean for MEL and CYA because of their different chemical characters, which can increase double time to prepare samples.

Present optimized method used a simple sample preparation to simultaneously extract MEL and CYA from tissues sample. This reduces the probability of analytes losses and to avoid lot-to-lot variations with SPE, and greatly improves analysis speed. A smaller sample size (2g) was used and correspondingly decreases solvent volume. In extract solvent, the ratio (70/30) of acetonitrile and water was proved be an good effect including cleaner sample and higher signal compared with other ratios (50/50 and 30/70). At the same time, MEL and CYA were stable in this solvent for a week because their signals have not significant decrease during this period. Two milliliters of *n*-hexane was use to get rid of fat from extracts, which can improve clean effect and response signal. Some clean materials including C_{18} and graphite powder were also used to try to obtain

further clean effect. However, the results showed that the clean of samples not only have apparent improvement but response signal decrease a little. In addition, the increase of acetonitrile in final sample solution and gradient elution in HPLC was help to obtain better peak shape and higher signal for both analytes. So finally, 0.5 mL of the extract was mixed with 0.5 mL of acetonitrile, and centrifuged at 13000 rpm for 10 min at 4°C, which improves percentage of acetonitrile to result in precipitation of much protein in extract at high speed centrifugation, and simultaneously improve response signal. The typical chromatogram of spiked tissue sample obtained under above optimized conditions was shown in Fig.1.

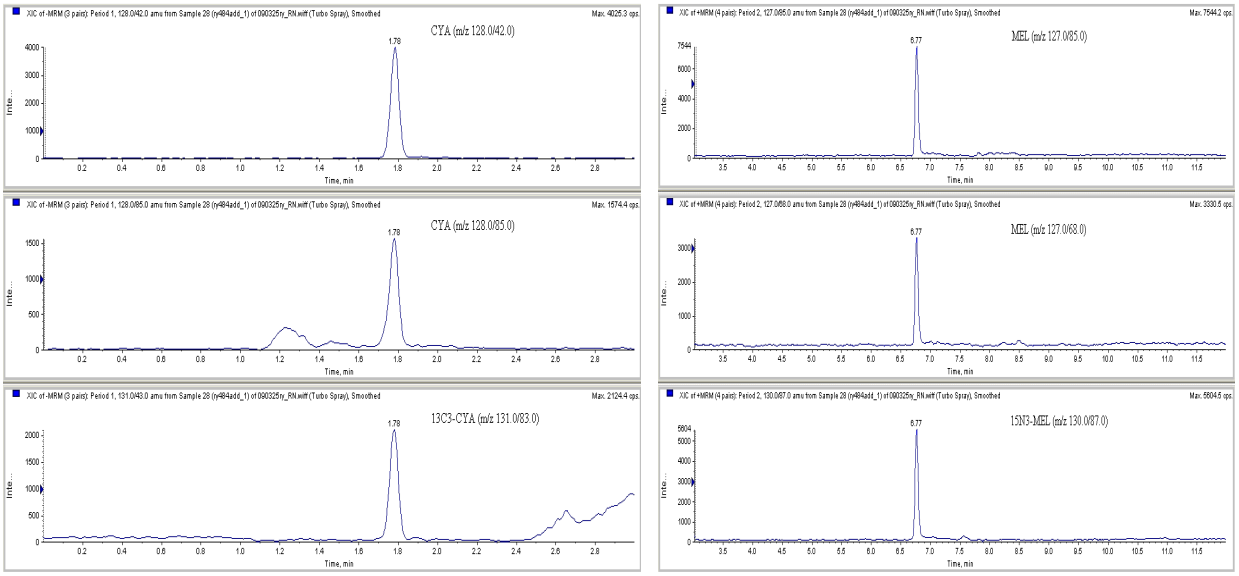

Fig. 1 Chromatograms of spiked meat sample with MEL at 0.2 μg/g and CYA at 0.4 μg/g

Method performance

Linear calibration curves were obtained over the concentration range of 2-500 ng/mL for MEL and 4-500 ng/mL for CYA, which are equivalent to 0.02-5.0 μg/g of MEL and 0.04-5.0 μg/g of CYA in animal tissues. The mean regression equations were y = 0.0679x - 0.0417 for MEL and y = 0.0393x + 0.00195 for CYA, and both correlation coefficients were > 0.999. The closeness of each standard response relative to the back-calculated concentration using the curve was summarized with accuracy by Analyst 1.4.2 software, the accuracies of MEL and CYA were respectively range from 93% to 105% and 95 to 108%, and indicated a good linearity.

The method was applied to three kinds of tissues including meat, liver and kidney, and five types of animals including pig, goat, cattle, chicken and fish. A series of spiked samples was prepared at three levels (Mel at 0.05, 0.2 and 2.0 μg/g, and CYA at 0.1, 0.4 and 4.0 μg/g) and analyzed to evaluate recovery and matrix effects in each type of animal tissues. The results were summarized in Table 1. The recovery of MEL and CYA in all animal tissues respectively ranged from 83-108% and 75-102% with RSD < 11%.

The LOQ for MEL and CYA in all animal tissues, defined as concentration produced a signal-to-noise ration of 10, respectively were 0.02 and 0.04 μg/g. The LOD for MEL and CYA, defined

as concentration produced a signal-to-noise ration of 3, respectively were 0.01 and 0.02 μg/g of animal tissues.

Table 1 Recoveries for tissues of five sorts of animals (n=6)

Animal	Tissue	Analyte	Level(μg/g)	%Recovery	SD	%RSD
Cattle	Meat	MEL	0.05	101	9.3	9.2
			0.2	105	3.6	3.4
			2.0	102	7.0	6.9
		CYA	0.1	80	7.8	9.8
			0.4	89	6.6	7.4
			4.0	85	7.6	8.9
	Liver	MEL	0.05	102	10.6	10.4
			0.2	108	4.2	3.9
			2.0	104	3.2	3.1
		CYA	0.1	88	5.1	5.8
			0.4	96	6.4	6.7
			4.0	97	4.5	4.6
	Kidney	MEL	0.05	91	4.9	5.4
			0.2	90	3.0	3.3
			2.0	107	4.7	4.4
		CYA	0.1	86	7.4	8.6
			0.4	101	2.5	2.5
			4.0	102	3.8	3.7
Goat	Meat	MEL	0.05	105	8.4	8.0
			0.2	98	7.0	7.1
			2.0	104	4.9	4.7
		CYA	0.1	84	5.4	6.4
			0.4	93	8.6	9.2
			4.0	92	6.8	7.4
	Liver	MEL	0.05	89	5.9	6.6
			0.2	99	7.3	7.4
			2.0	97	6.7	6.9
		CYA	0.1	81	6.9	8.5
			0.4	85	8.2	9.6
			4.0	101	7.8	7.7
	Kidney	MEL	0.05	88	3.5	4.0
			0.2	93	5.5	5.9
			2.0	87	5.6	6.4
		CYA	0.1	76	4.4	5.8
			0.4	81	3.5	4.3
			4.0	79	3.3	4.2
Pig	Meat	MEL	0.05	84	5.6	6.7
			0.2	90	7.2	8.0

			2.0	97	3.9	4.0
		CYA	0.1	78	4.9	6.3
			0.4	93	9.0	9.7
			4.0	82	6.8	8.3
	Liver	MEL	0.05	95	5.8	6.1
			0.2	102	5.4	5.3
			2.0	91	7.4	8.1
		CYA	0.1	75	4.3	5.7
			0.4	83	6.1	7.3
			4.0	77	4.0	5.2
	Kidney	MEL	0.05	98	3.5	3.6
			0.2	107	5.1	4.8
			2.0	103	4.7	4.6
		CYA	0.1	78	3.9	5.0
			0.4	89	6.7	7.5
			4.0	80	3.3	4.1
Chicken	Meat	MEL	0.05	101	7.7	7.7
			0.2	107	5.4	5.0
			2.0	102	4.3	4.2
		CYA	0.1	86	5.1	5.9
			0.4	98	5.6	5.7
			4.0	95	3.8	4.0
	Liver	MEL	0.05	94	8.1	8.6
			0.2	105	8.0	7.6
			2.0	103	5.5	5.3
		CYA	0.1	75	5.7	7.6
			0.4	79	4.5	5.7
			4.0	82	4.2	5.1
	Kidney	MEL	0.05	83	6.3	7.5
			0.2	94	3.9	4.1
			2.0	97	4.6	4.7
		CYA	0.1	78	4.1	5.3
			0.4	85	3.3	3.9
			4.0	81	2.9	3.6
Fish	Meat	MEL	0.05	96	9.5	9.9
			0.2	101	5.2	5.1
			2.0	104	3.7	3.6
		CYA	0.1	79	4.9	6.2
			0.4	78	6.9	8.8
			4.0	89	5.4	6.1
	Liver	MEL	0.05	106	5.3	5.0
			0.2	103	9.0	8.7
			2.0	102	3.0	2.9
		CYA	0.1	89	5.8	6.5

			0.4	81	6.4	7.9
			4.0	83	3.2	3.9
	Kidney	MEL	0.05	102	5.7	5.6
			0.2	107	4.2	3.9
			2.0	104	5.6	5.4
		CYA	0.1	78	5.5	7.0
			0.4	96	8.3	8.6
			4.0	84	4.1	4.9

Materials and Methods

Chemicals

Melamine (MEL, 99.5% purity) and Cyanuric acid (CYA, 99.0% purity) standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). $^{15}N_3$-MEL(98% chemical purity, 98% isotopic purity) and $^{13}C_3$-CYA(98% chemical purity, 99% isotopic purity) standards were obtained from Toronto Research Chemicals Inc. (North York, Canada) and used as internal standards. Acetonitrile and n-hexane (HPLC grade) was obtained from Fisher. Water was purified using a Millipore Milli-Q system. Ammonium acetate, formic acid and other chemicals and solvents were HPLC grade and purchased from commercial sources.

Preparation of standard solutions

All stock solutions were separately prepared by dissolving 10 mg of analytes in acetonitrile/water/formic acid (80/20/0.1%, v/v/v), and stable for up to 3 months when being stored at 4°C. Work standard solutions of 10.0 and 1.0 μg/mL of MEL, $^{15}N_3$-MEL, CYA and $^{13}C_3$-CYA were separately made by taking appropriate aliquots of the stock solution and diluting to volume with acetonitrile. Seven-point calibration curves were prepared for MEL at 2, 5, 10, 50, 125, 250, 500 ng/mL and CYA at 4, 8, 20, 50, 125, 250 and 500 ng/mL, by adding aliquots of work standard solutions of MEL, CYA, $^{15}N_3$-MEL and $^{13}C_3$-CYA in same tube and diluting to 1 mL. The final concentrations of $^{15}N_3$-MEL and $^{13}C_3$-CYA in each calibration standard were 20 ng/mL and 40 ng/mL, respectively.

Sample preparation

Animal tissues including liver, kidney and meat were previously homogenized by an Ultra-Turrax T-18 basic tissue homogenizer (IKA-Labortechnik/Tekmar Company, Cincinnati, OH, USA). Homogenized Samples (2 g) were weighed into 50-mL polypropylene tubes, then internal standards including 40 μL of $^{15}N_3$-MEL (10 μg/mL) and 80 μL of $^{13}C_3$-CYA standard solution (10 μg/mL) were added to sample. After this 10 mL of acetonitrile/water (70/30, v/v) were added in tubes. The tubes were capped, vortex-mixed for 1 min, shook for 30 min and centrifuged at 4500 rpm for 10 min. An aliquot (3 mL) of upper extract was transferred to 10-mL tubes, vortex-mixed for 1 min with 2 mL of n-hexane and centrifuged at 4500 rpm for 2 min. The n-hexane layer was discarded and 0.5 mL of the extract was vortex-mixed for 1 min with 0.5 mL of acetonitrile, and centrifuged at 13000 rpm for 10 min at 4°C. Finally the extract was filtered through 0.22μm PVDF syringe filter into a small volume autosampler vial for analysis. A 10 μL-

aliquot of each sample was then injected into LC/MS/MS system in replicates. All control and fortified samples were prepared in the same method.

LC/MS/MS analysis

Liquid chromatography was carried out using an Agilent 1200 series HPLC equipped with a G1322A degasser, G1311A quatpump, G1316B column compartment, G1329A autosampler and a 20-µl sample loop (Wilmington, DE, USA). The separation was performed on a Waters Atlantis HILIC silica column (3µm, 150×2.1 mm) with an HILIC silica guard column cartridge (3µm, 10×2.1 mm) at 30°C. The mobile phase consisted of (A) acetonitrile/5mM ammonium acetate/formic acid (95/5/0.1, v/v/v) and (B) acetonitrile/5mM ammonium acetate/formic acid (5/95/0.1, v/v) and a flow rate at 0.3 mL/min. Injection volume was set at 10 µl. The gradient elution conditions were listed in Table 2.

Table 2 The Gradient Elution Conditions

Time (min)	A (%)	B (%)	Flow rate (mL/min)	Comment
0	100	0	0.3	CYA elution
3.0	40	60	0.3	
5.0	40	60	0.3	MEL elution
5.1	100	0	0.3	
12.0	100	0	0.3	

Table 3 MRM conditions of MEL and CYA

Compound	Retention time (min)	Q1 Mass (amu)	Q3 Mass (amu)	DP (V)	CE (V)
CYA	1.78	128.0	42.0*	-23	-23
			85.0	-20	-12
$^{13}C_3$-CYA	1.78	131.0	43.0*	-37	-26
MEL	6.77	127.0	85.0*	22	28
			68.0	24	43
$^{15}N_3$-MEL	6.77	130.0	87.0*	23	29

* MRM used for quantification

Mass Spectrometry was carried out using an API 2000 LC/MS/MS System (AB Sciex Instruments) equipped with Turbo Electrospray Ionization (ESI) probe in the multiple reaction monitoring (MRM) scan mode. LC/MS/MS operation parameters were obtained by tuning MS using separate solution of MEL and CYA (10 µg/mL) at 10 µL/min into Mobile Phase A at 0.3 mL/min. The optimized LC/MS/MS parameters were as follows: Curtain gas (CUR) 30, Collision Gas (CAD) 3, Ion Source Temperature (Tem) 450°C, Ion Source Gas 1 (GS1) 70 and

Ion Source Gas 2 (GS2) 60 for both analytes, Ion Spray Voltage (IS) -4500 (CYA) and 2000 (MEL), Focusing Potential (FP) -270 (CYA) and 400 (MEL), Entrance Potential (EP) -10 (CYA) and 10 (MEL), Collision Cell Exit Potential (CXP) -4 (CYA) and 4 (MEL). Precursor and product ions of MEL and CYA with corresponding Declustering Potentials (DP) and Collision Energies (CE) in positive or negative polarity were shown in Table 3. The first period (1-3 min) of the LC-MS/MS analysis was done by negative ion for CYA, and then the polarity was switched (3-12 min) to positive ion for MEL analysis. All signals were received and processed by Analyst 1.4.2 software.

Assay validation

The calibration curves were generated by plotting the peak area ratio of each analyte to internal standard versus concentration of corresponding analyte. Linear regression analysis was performed using Analyst 1.4.2 Software. The assay was validated by analyzing control tissue samples (n=6) spiked with MEL at 0.05, 0.2 and 2.0 µg/g, and CYA at 0.1, 0.4 and 4.0 µg/g. The fortified samples were prepared by adding appropriate amount of MEL and CYA standard solutions to 2 g of blank animal tissue and processing with method described above. The limit of detection (LOD) and limit of quantification (LOQ) for each analyte were considered to be concentrations in tissues sample that produced a signal-to-noise (S/N) ratio of 3 and 10, respectively.

Conclusion

Present study developed an optimized method for simultaneous determination of MEL and CYA in meat, liver and kidney tissues of cattle, goat, pig, chicken and fish. The method adopted simple procedure of sample preparation to shorten analysis time, had a broad linear range and higher sensitivity, and can be widely applied to qualititatively and quantitatively detect MEL and CYA in animal tissues of different types of animals, especially suited for analysis of big batch of samples from pharmacokinetic studies of MEL in animal.

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