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Original Article

Trace residue analysis of dicyandiamide, cyromazine, and melamine in animal tissue foods by ultra-performance liquid chromatography

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ABSTRACT

An effective sample preparation procedure using an accelerated solvent extraction (ASE) procedure, followed by cleaning with melamine molecularly imprinted polymers solid-phase extraction (MISPE) was developed. A novel and highly sensitive ASE–MISPE–ultra-performance liquid chromatography (UPLC) method was developed for effective separation and simultaneous determination of dicyandiamide (DCD), cyromazine (CYR), and melamine (MEL) in complex animal tissue foods. Under optimized conditions, good linearity was achieved with a correlation coefficient (r) of 0.9999 in the range of at least two orders of magnitude. The limit of quantification of the method was 1.7 μg/kg, 5.0 μg/kg, and 3.2 μg/kg for DCD, MEL, and CYR, which was three orders of magnitude smaller than the maximum residue limits (MRLs). The intra- and inter-day precisions (in terms of the relative standard deviation, RSD) of the three analytes were in the range of 1.7–3.1% and 3.1–6.3%, respectively. The average recoveries of analytes from blank chicken, beef, mutton, pork, and pig liver samples spiked with the three levels varied from 91.2% to 107% with RSD of 1.7–8.3% for DCD, 89.0–104% with RSD of 2.1–6.1% for CYR, and 94.8–105% with RSD of 1.1–6.6% for MEL. The proposed method has the characteristics of speed, sensitivity, and accuracy, and can be used for the routine determination of DCD, CYR, and MEL at the μg/kg level in complex animal tissue foods.

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1. Introduction

Dicyandiamide (DCD), cyromazine (CYR), and melamine (MEL) belong to nitrogen-rich chemical compounds containing cyanamide as the basic unit. DCD can result in some diseases, such as methemoglobinemia and eczema [1]. CYR is a triazine pesticide used for fly control in crop production and animal feed by inhibiting insect growth. In recent years, CYR use has caused actual environmental and human health problems [2]. MEL is a metabolite of CYR that is formed via dealkylation reactions in both plants and animals and might cause urolithiasis and bladder cancer [3]. These potential and economic adulterants were deliberately added to a number of different types of animal- and human-food sources to artificially enhance apparent protein content [4]. To ensure human food safety, China has set maximum residue limits (MRLs) for cyromazine residue in the range of 0.5 mg/kg to 1 mg/kg [5], and the World Health Organization has set the MRL for melamine in powdered infant formula and in other foods and animal feed at 1 mg/kg and 2.5 mg/kg, respectively [6]. During the monitoring of melamine in various foods in September 2012, traces of DCD were found in milk and milk products supplied by a manufacturer in New Zealand [7]. While there are no international standards for acceptable levels of DCD in food products, high doses of DCD are considered toxic to humans. Therefore, it is of great importance to develop a simple and sensitive method to monitor DCD, along with CYZ and MEL, in a wide variety of foods to ensure the health of customers.

After the melamine contamination incident, several papers reviewed the advancement in analytical methodology for MEL, CYR, and related analogs in foods [8–11]. After the DCD contamination incident, a high-performance liquid chromatography (HPLC) method was reported for the determination of DCD in dairy products, with the limit of quantification (LOQ) at 500 μg/kg [12]. Several LC tandem mass spectrometry (MS/MS) methods have been used for the determination of only target DCD in different dairy products, with reported LOQs of 10 μg/kg [13], 50 μg/L [14], 50 μg/kg [15], and 0.06 μg/kg [16]. The sensitivity of these LC-MS/MS methods is higher than HPLC-UV methods, but it is not widely available in general laboratories due to its high price.

Recently, LC-MS/MS methods for simultaneous measurement of nitrogen-rich compounds, including DCD in milk materials, were reported, such as adulterants containing nitrogen in spiked fresh milk without limit of detection (LOD)/LOQ information [17], six adulterations, including CYR and DCD, in skim milk by LC–MS/MS with LOQ of 180 μg/kg and 60 μg/kg for CYZ and DCD, respectively [4], and DCD, MEL, and cyanuric acid in milk and milk powder with LOQ of 20 μg/kg for milk samples and 50 μg/kg for milk powder samples [18]. We used HPLC-quadrupole time-of-flight mass spectrometry (QTOF-MS) for rapid screening and triple quadrupole mass spectrometry (TQ-MS) for quantification to analyze DCD and its five related compounds in infant formula, with LOQ of 66.4 μg/kg for DCD [19]. However, these methods were only used for analysis of milk and milk products. The simultaneous analysis of CYR, MEL, ammeline, ammelide, and cyanuric acid residues in complex tissue samples was developed by HPLC and LC-MS/MS, with LOQ of 40 μg/kg and 15 μg/kg, respectively [20]. To the best of our knowledge, there are few reports on the simultaneous determination of DCD, CYR, and MEL in animal tissue food.

Sample pretreatment is always a crucial step in deciding the LOD limits of the overall method, especially when large numbers of samples are involved and rapid extraction becomes even more essential. In the above reported methods, solid-phase extraction was used to treat milk sample for determination of CYR and MEL [9]. Microwave-assisted extraction [13], QuEChERs [15], and dispersive micro-solid-phase extraction [16] were used for analysis of DCD in milk powder. Accelerated solvent extraction (ASE) has the advantages of good recovery, rapidity, adequate precision, and less solvent use [21]. Pressurized liquid extraction (an ASE mode) was used for complex tissue sample preparation [20]; however, there is no report for the determination of DCD in meat samples using the ASE procedure. The classical solid-phase extraction (SPE) method has low selectivity and is time consuming, and molecularly imprinted polymers (MIPs) have been developed to improve selectivity. Coupling MIP with SPE combines the advantages of molecular recognition with traditional separation methods. We prepared melamine-MIPs and validated their performance [22]. After extraction with acetonitrile and centrifugation treatment for milk samples, the prepared melamine-MIP was used for SPE of MEL and CYR from milk and dairy products. However, these sample preparation procedures need to be improved for simultaneous determination of DCD, CYR, and MEL in animal food.

The main purpose of the present study was to develop a rapid and effective sample preparation procedure by coupling ASE with MISPE and to develop a sensitive HPLC method for simultaneous determination of DCD, CYR, and MEL in meat samples.

2. Methods

2.1. Chemicals and reagents

Dicyandiamide, cyromazine, and melamine (> 99.5% purity for each) were obtained from the Hebei Institute of Food Quality Supervision Inspection and Research (Shijiazhuang, China). Methanol (HPLC grade), acetonitrile (HPLC grade), ammonium acetate (analytical grade), and acetic acid (analytical grade, 36%) were purchased from Dikma Technologies Inc. (Tianjin, China).

Mobile Phase A was prepared by dissolving 0.77 g of ammonium acetate in 1000 mL of water, and adjusting the pH to 4.7 using acetic acid. Mobile Phase B was acetonitrile. DCD, CYR, and MEL stock standard solutions, 1000 mg/L, were prepared by dissolving the compounds, respectively, in a mixture of acetonitrile and water (8:2), and were stored at 4°C in amber glass bottles. A fresh working standard solution was prepared daily by diluting the stock solution with the mobile phase for different studies. The solution and methanol were filtered through 0.22-μm microporous polyvinylidene fluoride membranes before use. Distilled water was further purified by a Molelement 1820a ultrapure water apparatus (Molecular

Devices Limited, Shanghai, China) and then filtered through a 0.45-μm microporous membrane of mixed cellulose ester.

2.2. Instrumentation

The ultra-performance liquid chromatography (UPLC) equipment was an Acquity UPLC H-Class system (Waters, Milford, MA, USA), which consisted of an Acquity Quaternary Binary Solvent Manager, an Acquity Sample Manager-FTN, an Acquity diode array detector, and a high-temperature column heater. Empower III workstation was used as the data acquisition system. An Acquity UPLC bridged ethyl hybrid (BEH) hydrophilic interaction chromatography (HILIC) column (100 mm × 2.1 mm, 1.7 μm i.d.) was used as the analytical column and was connected to an inline precolumn. The extraction equipment was an APLE-2000 automatic accelerated solvent-extraction apparatus (Beijing Titan Instruments, Beijing, China) equipped with 11 mL stainless-steel extraction cells. A TGL-16M centrifuge (Xiangyi Centrifuge Co., Hunan, China) and RE-2000A rotary evaporator (Yarong Biochemistry Instrument Co., Shanghai, China), a MX-F Vortex mixer (Scilogex, LLC, Rocky Hill, CT, USA), and a PHS-3C pH meter (Shanghai Precision & Scientific Instrument Co., Shanghai, China) were used in sample preparation. SPE empty tubes and sieve plates were purchased from Bonna-Agela Technologies Inc. (Tianjin, China).

2.3. ASE procedure

Poultry muscle and pork liver were purchased fresh from a market (Baoding, China), cut into small pieces, and ground into a homogeneous sample using a mincer. This material was then kept frozen at −18°C. All samples were dried and kept in amber glass bottles at 4°C prior to analysis.

An ASE procedure was used in sample pretreatment. A cellulose filter was put in the bottom of an ASE extraction cell to prevent fine-powder breakthrough into the collection bottle. Homogenized powder sample (4 g) and diatomite (4 g) were mixed and transferred into an 11-mL extraction cell. Aqueous trifluoroacetic acid at pH 3.0 was selected as an extraction solvent. Conditions used in the extraction were: oven temperature of 70°C with a 3-minute heat-up time under a pressure of 10 MPa and two static cycles with a static time of 5 minutes. The flush volume was 40% of the extraction cell volume. The extract was purged from the sample cell using pressurized nitrogen for 1 minute. The raw extracts were transferred into 50-mL pear-shaped flasks and concentrated to 3 mL by using a rotary evaporator at 45°C for 15 minutes. All experiments were carried out in triplicate.

2.4. MISPE clean-up procedure

MIPs were synthesized with melamine as the template and methacrylic acid as the organic functional monomer by the procedure described in our previous work [22]. A MISPE column was prepared by packing the MEL-imprinted polymers (100 mg) into an empty SPE column (60 mm × 8.0 mm, i.d.). The cartridge was capped with two fritted polyethylene sieve plates at the bottom and top ends. The MISPE column (60 mg/3 mL) was preactivated with 3 mL methanol and equilibrated with 3 mL water. After the 3 mL concentrated extract was passed through the cartridge at a flow rate of 0.3 mL/min, the cartridge was equilibrated for 30 minutes to complete interaction and adsorption with the analytes. The loaded cartridges were washed in turn with 3 mL water and 3 mL methanol, and the eluate was discarded. The analytes in the cartridge were eluted with 3 mL methanol:acetic acid (30:70, v/v). The eluate was evaporated to dryness under a stream of nitrogen at 40°C. The obtained residue was redissolved in 1.0 mL of mobile phase for UPLC analysis.

2.5. UPLC analysis

After the UPLC column was conditioned with a mobile phase of 1mM ammonium acetate-acetic acid solution (pH 4.7) and acetonitrile (4:96, v/v), a 10-μL volume of sample solution was injected into the column at 35°C and eluted with the mobile phase at 0.4 mL/min. DCD, MEL, and CYR, were detected at 220.0 nm, 235.7 nm, and 239.2 nm, respectively. The relationship between the standard solution concentration in the range of 0.007–3.35 μg/mL for DCD, 0.0134–6.7 μg/mL for CYR, and 0.02–10 μg/mL for MEL (number of points, $n = 7$), and their peak areas were calculated by using weighted least-squares regression.

3. Results and Discussion

3.1. Optimization of ASE conditions

The effect of different conditions on extraction efficiency of ASE was investigated via recovery test. Three replicates of each extraction experiment were carried out. The selection of a suitable extraction solvent is the first challenge in ASE method development. Several solvents have been used in ASE for the preparation of food samples [21]. The polarity of the extraction solvent should closely match that of the target compounds. In this work, simultaneous extraction of DCD, CYR, and MEL from a pork sample were investigated using different solvents at 70°C and 10 MPa for 5-minute static times.

The data in Table 1 show that high recovery and precision were achieved by using methanol, acetonitrile, or aqueous trifluoroacetic acid (pH 3.0). The use of hot water as an extraction solvent has steadily become an efficient, low-cost, environmentally friendly method. The effect of aqueous trifluoroacetic acid with pH from 2.5 to 7.0 on the extraction was investigated. The recovery of the three analytes decreased with increased pH from 3.0 to 7.0. Satisfactory recovery was achieved with water at pH 3.0.

Temperature is one of the most important parameters for ASE. The effect of temperature from 40°C to 80°C on the recovery was investigated. The recovery of DCD, CYR, and MEL increased with increasing temperature from 40°C to 70°C due to the solubility of the target analytes to be increased, but when > 80°C, the recovery of DCD (decomposition temperature: 80°C) decreased to 77.2%, and > 85°C, the recovery of CYR decreased to 79.1%. This may be because an increase of the temperature has a large impact on the degradation rate of

Table 1 – Recovery of the three analytes using different solvents.

Analyte	Methanol		Acetonitrile		Aqueous trifluoroacetic acid (pH 3)	
	Recovery	RSD, $n = 3$	Recovery	RSD, $n = 3$	Recovery	RSD, $n = 3$
Dicyandiamide	98.7	4.3	98.1	2.7	99.3	2.1
Cyromazine	99.2	1.6	98.9	2.3	99.7.	2.0
Melamine	99.8	3.1	97.4	1.5	101	3.7

Data are presented as %.
RSD = relative standard deviation.

DCD and CYR. In this work, to ensure their stability in the extraction process, the temperature was set at 70°C.

The extraction process can be conducted in a static or dynamic mode. The static process can be repeated several times to obtain better extraction efficiency. In this work, the extraction efficiency was investigated using static times of 5 minutes, 10 minutes, and 15 minutes, and two cycles. The results showed that significant amounts of the analytes were found with 5 minutes of static time in the first extract. In order to evaluate the number of extraction cycles, an additional three consecutive extractions were made. The results indicated that two extraction cycles were adequate for obtaining extraction efficiency > 92%. Thus, 5 minute static times and two static cycles were used for further work.

Flush volume was also investigated to ensure that all analytes were eluted and closely related to the final volume. Different flush volumes were used to extract analytes. To minimize solvent and time, a flush volume of 40% (cell volume, 11 mL) was enough to extract the target analytes in the samples with obtained high-extraction efficiency.

3.2. *Optimization of clean-up conditions by MISPE*

To completely eliminate matrix interferences and concentrate the analytes, we investigated the effects of the MISPE washing and eluting conditions. The purpose of the wash step is to remove all interfering compounds from the complex matrix without eluting out the target analytes. Different volumes of water, methanol, and acetonitrile in the range of 1.0–6.0 mL were investigated. The interfering components were removed, and DCD, CYR, and MEL were fully retained when using 3 mL water and 3 mL methanol, therefore, this combination was chosen as the washing solvent. The nature of the elution solvent is also important, because the target analytes should be efficiently desorbed while other matrix components are retained in the cartridge. To achieve the best recovery, we evaluated a series of acidic elution solutions, including methanol–acetic acid (70:30, v/v), ethyl acetate–acetic acid (70:30, v/v), and acetonitrile–acetic acid (70:30, v/v). The best recoveries (74.6–100%) were obtained when using 3.0 mL methanol–acetic acid (70:30, v/v) as the eluting solution.

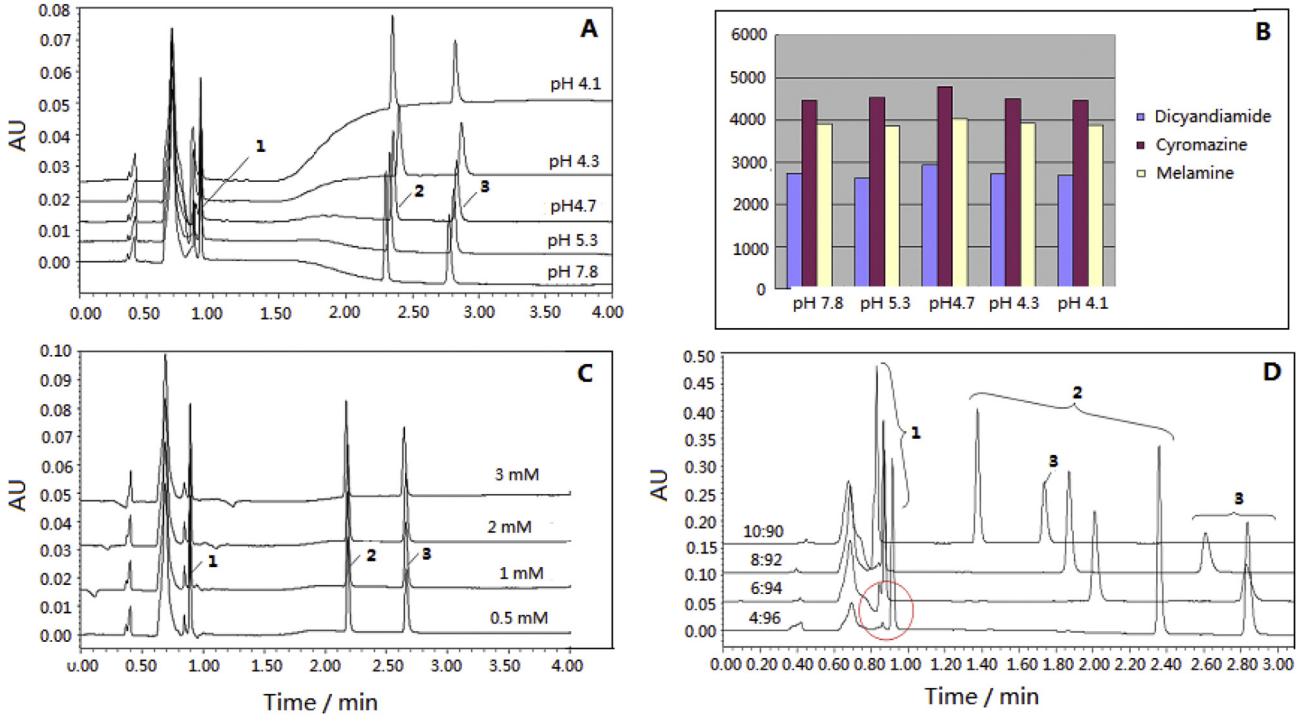

Figure 1 – (A) Chromatograms of the analytes under different pH conditions, (B) effect of different pH on the signal response of the analytes, (C) chromatograms of the analytes using different concentrations of ammonium acetate solution, and (D) chromatograms of the analytes using different proportions of ammonium acetate solution in the mobile phase 1—dicyandiamide, 2—cyromazine, 3—melamine.

3.3. Optimization of UPLC separation conditions

Because DCD, CYR, and MEL are small and highly polar molecules, it is difficult to achieve sufficient retention using traditional C18 columns. The separation condition of these analytes was investigated by using an Acquity UPLC BEH HILIC column (100 mm × 2.1 mm, 1.7 μm i.d.) and ammonium acetate–acetic acid solution–acetonitrile as mobile phases.

The pH of ammonium acetate solution influenced the stability and ionization of analytes. CYR could be hydrolyzed at pH ≤ 2 to melamine, and the dissociation constants pK_d are 5.0 for melamine [22], therefore, the pH of the ammonium acetate solution was adjusted to 4.1–5.3 with acetic acid. The effect of pH on the retention time and peak area of the analytes was investigated. The result showed no obvious change in retention time at a pH range of 4.1–7.8 (Figure 1A); however, the peak areas for DCD, CYR, and MEL increased with decreasing pH from 4.1 to 4.7, and when pH > 4.7, the peak areas decreased (Figure 1B). The best response for the three analytes was achieved at pH 4.7, which was used in subsequent work.

We also investigated the effect of the concentration of ammonium acetate solution on the retention time and peak area of the three analytes. Figure 1C shows no obvious effect of ammonium acetate concentration in the range of 0.5–3mM on retention times. Additionally, no effect of ammonium acetate concentration on peak areas was observed. Therefore, 0.5mM ammonium acetate solution was used in subsequent work. We then investigated the effects of different proportions of ammonium acetate solution in the mobile phase on the peak area and retention time of the three analytes in an isocratic elution program (Figure 1D). The results showed that the retention time increased with the decrease in volume ratio of ammonium acetate–acetic acid solution in mobile phases, especially for CYR. Using ammonium acetate–acetic acid solution–acetonitrile (4:96, v/v) as mobile phases achieved the baseline separation of the DCD peak from solvent peaks. Therefore, the isocratic elution program with 0.5mM ammonium acetate–acetic acid solution (pH 4.7)–acetonitrile (4:96, v/v) as a mobile phase was favorable for separation and detection.

3.4. Performance of the method

3.4.1. Specificity

Under the optimized conditions, chromatograms of real samples and spiked samples were obtained. Figure 2 shows the chromatograms of blank samples and spiked samples after the MISPE process.

After the MISPE process, DCD, CYR, and MEL were selectively extracted and no interferences from the matrices were observed. This demonstrated the high selectivity of the synthesized MIPs to DCD, CYR, and MEL. The retention time of the target analytes in the five matrices was accordant. No interfering peaks were observed at the retention times of any analyte. this indicated that this procedure had high selectivity and specificity.

3.4.2. Linearity and detection limit

Under the optimized conditions, we evaluated the linearity of calibration curves for the analysis of real samples with the

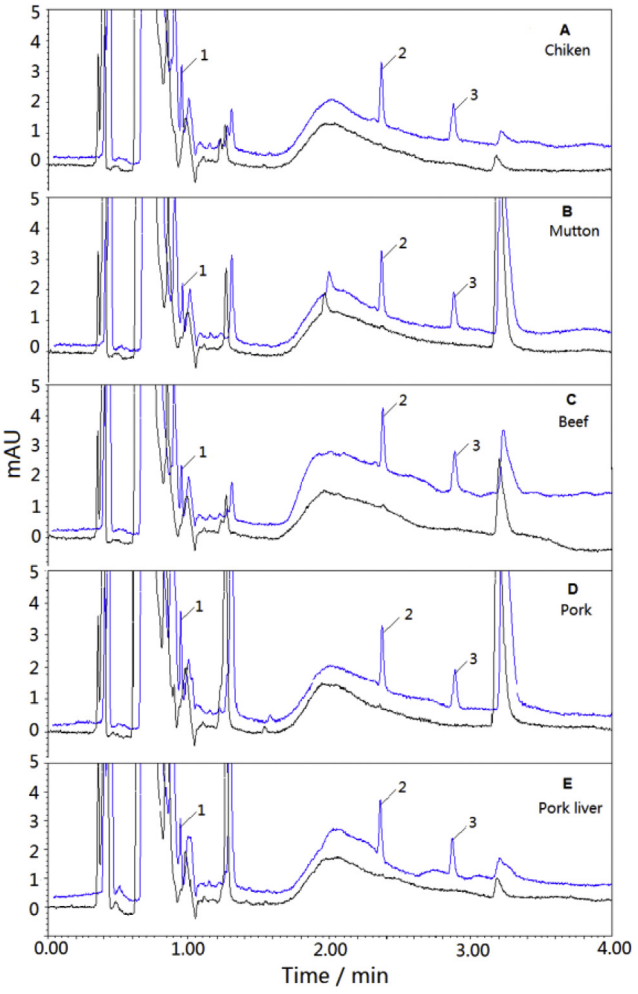

Figure 2 – Chromatograms of (A) blank chicken sample and spiked blank chicken sample, (B) blank mutton sample and spiked blank mutton sample, (C) blank beef sample and spiked blank beef sample, (D) blank pork sample and spiked blank pork sample, and (E) blank pig liver sample and spiked blank pig liver sample after the treatment by ASE–MISPE. Mobile phase—0.5mM ammonium acetate–acetic acid solution (pH 4.7): acetonitrile (4:96 v/v); spiked level: 1—dicyandiamide, 2.0 μg/kg; 2—cyromazine, 2.0 μg/kg; 3—melamine, 5.0 μg/kg. ASE-MISPE = accelerated solvent extraction molecularly imprinted polymer solid-phase extraction.

measured peak areas of the standards against their concentrations (number of points, $n = 7$). The linear regression equations are listed in Table 2 and show good linearity for each analyte, with correlation coefficients (r) of 0.9999.

The LOD was determined as the sample concentration that produced a peak height 3-fold higher than the level of the baseline noise, and the LOQ was calculated as the sample concentration that produced a peak height 10-fold higher than the signal-to-noise ratio. The LOD and LOQ values for the instrument (solution) are also given in Table 2. The instrument LOD was 0.002 μg/mL, 0.004 μg/mL, and 0.006 μg/mL and the

Table 2 – Linear regression equation, correlation coefficient (r), linearity range, LOD, and LOQ for the analytes.

Analyte	Linear regression equation	r	Linearity range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Dicyandiamide	A = 222377.0034C–2272.4688	0.9999	0.007–3.35	0.002	0.007
Cyromazine	A = 197932.6918C–5456.8541	0.9999	0.0134–6.7	0.004	0.013
Melamine	A = 117932.6247C–5011.1573	0.9999	0.02–10	0.006	0.02

LOD = limit of detection; LOQ = limit of quantification.

instrument LOQ was 0.007 μg/mL, 0.013 μg/mL, and 0.02 μg/mL for DCD, CYR, and MEL, respectively.

For 4-g samples and a 1 mL final solution, the method LOD was 0.5 μg/kg, 1.0 μg/kg, and 1.5 μg/kg for DCD, CYR, and MEL, respectively, and their method LOQ was 1.7 μg/kg, 3.2 μg/kg, and 5.0 μg/kg, respectively.

The LOQ values are at least three orders of magnitude smaller than the MRL [5,6]. Moreover, since the sample preparation procedure (AES-MISPE) has a higher enrichment factor, recovery yield, and is without matrix effects, the sensitivity of the method for DCD was higher than that observed using the LC [12] and LC-MS/MS methods [4,13–15,18,19]. Similarly, the sensitivity for CYR and MEL was higher than those observed using LC [20] and LC-MS/MS [4,18,20].

3.4.3. *Repeatability*

The precision of the method was investigated by analyzing the three analytes in a spiked blank sample. The intra-day precision of the method was expressed as the relative standard deviation (RSD) of nine determinations made for intra-day accuracy, and inter-day precision of the method was also expressed as RSD of three assays for each day within 3 days. The intra- and inter-day RSDs for spiked blank pork matrix at levels of 20 μg/kg were in the range of 1.7% and 3.1% for DCD, 2.9% and 6.3% for CYR, and 3.1% and 4.5% for MEL, respectively. It was shown that the repeatability and reproducibility of the method was satisfactory for residue determination of the studied analytes in animal-derived foods.

3.5. *Analysis of real samples*

The ASE–MISPE–UPLC method was used to analyze DCD, CYR, and MEL resides in five animal-derived foods. To investigate the effect of the sample matrix on accuracy, a recovery experiment was carried out in triplicate by spiking 10 μg/kg, 50 μg/kg, and 100 μg/kg of the analytes into animal-derived food samples. The results are given in Table 3 along with the RSD. The average recoveries of analytes from blank chicken, beef, mutton, pork, and pig liver samples spiked with the three levels varied from 91.2% to 107% with RSD of 1.7–8.3% for DCD, 89.0–104% with RSD of 2.1–6.1% for CYR, and 94.8–105% with RSD of 1.1–6.6% for MEL. It was indicated that the proposed MISPE–UPLC method had feasibility for the determination of these analytes in complex tissue samples.

4. Conclusion

This work provided optimized ASE conditions and MISPE clean-up procedures for animal tissue food sample preparation. Under the optimized conditions, DCD, CYR, and MEL were selectively concentrated and all matrix interferences were eliminated simultaneously, first achieving residue analysis of chicken, beef, mutton, pork, and pig liver samples. The proposed method had higher sensitivity than those previously reported [4,15–18,22], and are capable of effective and

Table 3 – Determination of the analytes in spiked tissue samples.

Matrix	Spiked (μg/kg)	Dicyandiamide			Cyromazine			Melamine		
		Found (μg/kg)	Recovery (%)	RSD[a] (%)	Found (μg/kg)	Recovery (%)	RSD[a] (%)	Found (μg/kg)	Recovery (%)	RSD[a] (%)
Chicken	10	9.9	99.1	6.5	9.9	99.0	4.6	9.9	99.0	6.2
	50	48.6	97.2	1.9	46.6	93.1	2.1	48.9	97.7	2.9
	100	96.5	96.5	2.7	98.2	98.2	4.9	96.0	96.0	2.0
Mutton	10	9.80	97.9	3.0	8.9	89.0	5.7	10.5	105	4.3
	50	47.4	94.7	4.9	45.1	90.2	4.1	49.6	99.2	4.7
	100	91.2	91.2	3.2	97.6	97.6	3.0	96.6	96.6	4.1
Beef	10	10.3	103	7.4	9.6	96.0	2.2	10.0	100	3.5
	50	47.9	95.7	3.7	45.5	91.0	4.7	49.1	98.1	1.1
	100	93.1	93.1	2.0	97.4	97.4	4.3	96.7	96.7	2.9
Pork	10	10.2	102	5.3	10.4	104	5.8	9.9	99.0	3.3
	50	49.1	98.1	3.9	45.0	90.0	2.8	47.4	94.8	2.7
	100	94.3	94.3	2.7	95.9	95.9	6.1	97.3	97.3	6.6
Pig liver	10	10.7	107	8.3	9.5	95.0	3.5	10.0	100	4.2
	50	49.5	99.0	2.9	49.7	99.3	5.8	49.5	98.9	2.6
	100	93.0	93.0	1.7	94.1	94.1	2.9	97.0	97.0	2.1

RSD = relative standard deviation.
[a] $n = 3$.

sensitive determination of DCD, CYR, and MEL at the μg/kg level in complex animal tissue samples.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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REFERENCES

[1] Yasuhara K, Shimo T, Mitsumori K, Onodera H, Kitaura K, Takahashi M. Lack of carcinogenicity of cyanoguanidine in F344 rats. Food Chem Toxicol 1997;35:475–80.
[2] Wang H, Dong YH, An Q. Research advance in biotoxicology and environmental behavior of cyromazine. Transaction of the CSAE 2008;24:246–9.
[3] Melnick RL, Boorman GA, Haseman JK, Montali RJ, Huff J. Urolithiasis and bladder carcinogenicity of melamine in rodents. Toxicol Appl Pharmacol 1984;72:292–303.
[4] MacMahon S, Begley TH, Diachenko GW, Stromgren SA. A liquid chromatography tandem mass spectrometry method for the detection of economically motivated adulteration in protein-containing foods. J Chromatogr A 2012;1220:101–7.
[5] The National Health and Family Planning of People's Republic of China. Chinese National Standard. GB 2763–2012: Maximum residue limits for pesticides in food. Chinese National Food Safety Standard. Beijing: China; 2012.
[6] World Health Organization. News release: International experts limit melamine levels in food. http://www.who.int/mediacentre/news/releases/2010/melamine_food_20100706/en/print.html. [Accessed 2 February 2015].
[7] Lucas GN. Dicyandiamide contamination of milk powders. Sri Lanka J Child Health 2013;42:63–4.
[8] Chu PWS, Chan KM, Cheung STC, Wong YC. Review of analytical techniques used in proficiency-testing programs for melamine in animal feed and milk. Tren Anal Chem 2010;29:1014–26.
[9] Sun HW, Wang LX, Liu N, Qiao FX, Liang SX. SPE then RP-LC for simultaneous analysis of cyromazine and its metabolite melamine in liquid milk and egg. Chromatographia 2009;70:1685–9.
[10] Sun FX, Ma W, Xu LG, Zhu YY, Liu LQ, Peng CF, Wang LB, Kuang H, Xu CL. Analytical methods and recent developments in the detection of melamine. Tren Anal Chem 2010;29:1239–49.
[11] Wu Y, Zhang Y. Analytical chemistry, toxicology, epidemiology, and health impact assessment of melamine in infant formula: recent progress and developments. Food Chem Toxicol 2013;56:325–35.
[12] Chen X, Chen W, Wang J, Huang L, Zhang D. Determination of dicyandiamide in dairy products by high performance liquidchromatography. Chin J Chromatogr 2013;31:875–7.
[13] Shen Y, Han C, Zhou X, Chen X, Huang F, Zhu Z. Microwave-assisted extraction and determination of dicyandiamide residue in infant formula samples by liquid chromatography-tandem mass spectrometry. J Dairy Sci 2013;96:6877–82.
[14] Inoue K, Sakamoto T, Min JZ, Todoroki K, Toyo'oka T. Determination of dicyandiamide in infant formula by stable isotope dilution hydrophilic interaction liquid chromatography with tandem mass spectrometry. Food Chem 2014;156:390–3.
[15] Wang JF, Cao YH, Zhang CM, Jia L, Fan XJ. Determination of dicyandiamide in infant milk powder by QuEChERs -UPLC-MS/MS. Chin J Anal Lab 2015;34:465.
[16] Chen XH, Zhou LX, Zhao YG, Pan SD, Jin MC. Application of nanoring amino-functionalized magnetic polymer dispersive micro-solid-phase extraction and ultra-fast liquid chromatography–tandem mass spectrometry in dicyandiamide residue analysis of powdered milk. Talanta 2014;19:187–92.
[17] Abernethy G, Higgs K. Rapid detection of economic adulterants in fresh milk by liquid chromatography–tandem mass spectrometry. J Chromatogr A 2013;1288:10–20.
[18] Yun H, Yan H, Zhang Z, Li J, Lu X, Liu X. Determination of dicyandiamide, melamine and cyanuric acid in milk and milk powder by ultra-performance liquid chromatography–tandem mass spectrometry. Chin J Chromatog 2013;31:404–9.
[19] Meng Z, Shi ZH, Liang SX, Dong XF, Lv YK, Sun HW. Rapid screening and quantification of cyromazine, melamine, ammelide, ammeline, cyanuric acid, and dicyandiamide in infant formula by ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry and triple quadrupole mass spectrometry. Food Control 2015;55:158–65.
[20] Yu H, Tao YF, Chen DM, Wang YL, Liu ZY, Pan YH, Huang LL, Peng DP, Dai MH, Liu ZL, Yuan ZH. Development of a high-performance liquid chromatography method and a liquid chromatography–tandem mass spectrometry method with pressurized liquid extraction for simultaneous quantification and confirmation of cyromazine, melamine, and its metabolites in foods of animal origin. Anal Chim Acta 2010;682:48–58.
[21] Sun HW, Ge XS, Lv YK, Wang AB. Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed. J Chromatogr A 2012;1237:1–23.
[22] Ge XS, Wu XQ, Wang JM, Liang SX, Sun HW. Highly sensitive determination of cyromazine, melamine, and their metabolites in milk by molecularly imprinted solid-phase extraction combined with ultra-performance liquid chromatography. J Dairy Sci 2015;98:2161–71.