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Simultaneous Determination of Chloramphenicol and Aflatoxin M_1 Residues in Milk by Triple Quadrupole Liquid Chromatography−Tandem Mass Spectrometry

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ABSTRACT: A reliable, rapid, and sensitive liquid chromatography−tandem mass spectrometry (LC-MS/MS) method for simultaneous determination of chloramphenicol and aflatoxin M_1 in milk has been developed. This method includes simple extraction of sample with acetonitrile, separation on a MGIII-C_{18} column using 5 mM ammonium acetate aqueous solution/methanol (60:40, v/v) as mobile phase, and MS/MS detection using multiple reaction monitoring mode. The method was validated according to Commission Decision 2002/657/EC. The limits of detection (LODs) were 0.05 μg/kg for chloramphenicol and 0.005 μg/kg for aflatoxin M_1. The limits of quantification (LOQs) were 0.2 μg/kg for chloramphenicol and 0.02 μg/kg for aflatoxin M_1. The recovery values ranged from 88.8% to 100.6%, with relative standard deviation lower than 15% in all cases, when samples were fortified at three different concentrations. The decision limits (CCα) and detection capability (CCβ) of the method were also reported. This method has been successfully applied for simultaneous analysis of chloramphenicol and aflatoxin M_1 residues in milk from local supermarkets in China.

KEYWORDS: LC-MS/MS, chloramphenicol, aflatoxin M_1, residue, milk

INTRODUCTION

Milk and its products are basic foodstuffs and constitute an important source of nutrients in the daily diet of humans. With increasing concern about food safety, there has been a parallel increase in concern over the contaminants found in milk.

Chloramphenicol (CAP) is a broad-spectrum antibiotic widely used in veterinary practice as a cheap and effective drug. However, this drug has serious adverse effects such as dose-related reversible bone marrow depression and a severe nondose-related and irreversible aplastic anemia.[1] Even low doses of administered chloramphenicol may result in residues in edible tissues from treated food-producing animals. For these reasons, China, Japan, Canada, the United States, Australia, the European Union (EU), and some other countries have strictly banned the use of chloramphenicol in food-producing animals. The EU has set up the minimum required performance level (MRPL) at 0.3 μg/kg for chloramphenicol in milk.[2] Nevertheless, because of their easy access, low price, and steady antibacterial effectiveness, illegal use of chloramphenicol in livestock still exists and traces of this compound have been detected in various foods, including milk. It is necessary, therefore, to develop a highly sensitive method to control and monitor chloramphenicol residues.

Aflatoxin M_1 (AFM1) is another common contaminant that occurs in milk. It was first found in the milk of lactating animals that consume feedstuffs contaminated with aflatoxin B_1. Aflatoxin M_1 is the hydroxylated metabolite of aflatoxin B_1. It has been reported that approximately 0.3% to 6.2% of the aflatoxin B_1 initially present in animal foodstuff appears as aflatoxin M_1 in milk, and a linear relationship has been found between intake of aflatoxin B_1 in contaminated feed and the aflatoxin M_1 content of milk in cows.[3] Aflatoxin M_1 has comparable liver toxicity and cytotoxicity and can reduce the immunity of infants.[4] The International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) categorized aflatoxin M_1 as a group 1 human carcinogen.[5] Because of its serious health concerns, many countries have set maximum limits for aflatoxin M_1. The European Community prescribes that the maximum level of aflatoxin M_1 in liquid milk should not exceed 0.05 μg/kg, while in infant formula this level cannot be greater than 0.025 μg/kg.[6] Such low limits also require highly sensitive methods for detection and quantification of aflatoxin M_1 in milk.

A number of analytical methods have been reported for the detection of chloramphenicol or aflatoxin M_1 in milk and other products. In these cases, enzyme-linked immunosorbent assay (ELISA),[7−9] high-performance liquid chromatography (HPLC),[10−13] and gas chromatography with mass spectrometric detection (GC-MS) [14] are widely used. ELISA is often used for routine screening on account of its advantages such as rapidity, simplicity, and cost-effectiveness, but it is not fully reliable because of cross-reaction interference, especially when residues are at trace level.[15] The methods using GC-MS for the analysis of chloramphenicol can provide definitive qualitative and quantitative results, but it requires a derivatization step, which lengthens the analysis time and may compromise analyte recoveries. The HPLC method for analysis of aflatoxin M_1 needs a cleanup process with immunoaffinity columns before detection. Such sample preparation is multistage, expensive, and time-consuming.

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An LC-MS/MS method has been shown to offer significant advantages over other techniques, as it provides good sensitivity and confirmation of analytes.[16] Several papers describing liquid chromatography−tandem mass spectrometry with electrospray ionization methods for determining chloramphenicol or aflatoxin M_1 in different kinds of foodstuff have been published.[17−22] In previous studies, time- and labor-intensive sample preparation steps were needed. To our knowledge, methods for simultaneous determination of the two most concerned residues, chloramphenicol and aflatoxin M_1, in milk have not been published so far. In this study, a simple method comprising a single sample extraction with acetonitrile, but without an additional cleanup step, and an LC-ESI-MS/MS determination was developed for simultaneous analysis of chloramphenicol and aflatoxin M_1 in milk with low detection limits that can meet the proposed limit set by countries all over the world. The purpose of the present work is to report the performance characteristics of this procedure and provide an alternative for the simultaneous determination of chloramphenicol and aflatoxin M_1 in milk.

MATERIALS AND METHODS

Reagents and Solutions. Analytical standard chloramphenicol (99%) was obtained from Sigma-Aldrich (St.Louis, MO). Methanol and acetonitrile were of HPLC grade from Alfa Aesar (Ward Hill, MA). Ammonium acetate was of analytical grade from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q Plus system from Millipore (Brussels, Belgium).

A standard solution of aflatoxin M_1 (10 μg/mL in acetonitrile) was purchased from Supelco (Bellefonte, PA). It was stored at −20 °C and kept in the dark at room temperature before use. Working standard solution was made by diluting the purchased stock solution in methanol. It was stable for at least 5 days when stored at 4 °C.

A chloramphenicol standard stock solution of 1 mg/mL was prepared by dissolving 50 mg of chloramphenicol in 50 mL of methanol and stored at −20 °C for 1 year. An intermediate standard solution of 10 μg/mL was obtained by diluting stock solution 100 times with methanol. A chloramphenicol working solution of 100 ng/mL was made by diluting the intermediate standard solution with water:methanol (60:40, v/v). The working solution stored at 4 °C was stable for 1 month.

All of the working solutions were used to prepare calibration curves in matrix and to spike samples for recovery experiments and limit of detection. The structures of chloramphenicol and aflatoxin M_1 are shown in Figure 1.

Milk Samples. Liquid milk and milk powder samples were purchased from local supermarkets in Beijing. Different types of milk sample (whole milk, low fat milk, and skimmed milk) from different brands (both large and small manufacturers) were selected, and they were kept at 4 °C before analysis.

Sample Preparation. For powdered milk sample, an aliquot of 5 g powder was weighed into a 50 mL colorimetric tube. After 15 mL of deionized water was added, prewarmed in the water bath to 50 °C, the mixture was vortexed for 2 min then diluted to the 50 mL mark with acetonitrile. In the case of the liquid milk sample, an aliquot of 20 g of milk was weighed into a 50 mL colorimetric tube, and it was also diluted to 50 mL with acetonitrile. Extraction was performed by mixing the sample with a vortex mixer for 1 min and ultrasonicating the mixture for 15 min. An ultrasonic bath (Kunshan Ultrasonic Instruments Co. Ltd., Jiangsu, China) with ultrasonic power of 200 W and a frequency of 40 kHz was used. Then the sample was filtered through Whatman No.4 filter paper. A volume of 25 mL of the filtrate was concentrated to 2 mL with a rotary evaporator at 45 °C under vacuum. It was brought to a volume of 5 mL with water and then filtered through 0.20 μm syringe filter (Whatman, Dassel, Germany) prior to LC-MS/MS analysis. The extraction efficiency was evaluated by comparing the peak areas for the same compound in samples spiked before and after extraction step.

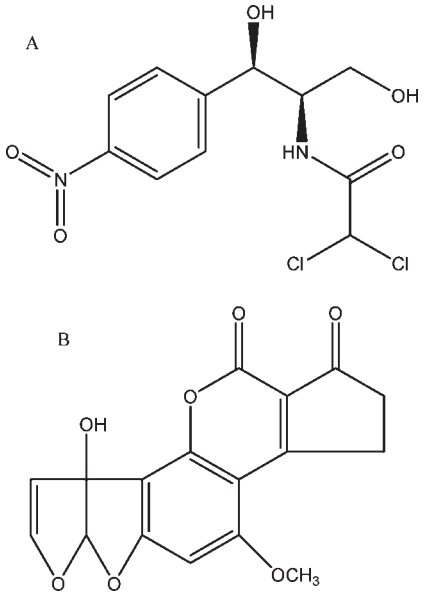

Figure 1. Chemical structures of chloramphenicol (A) and aflatoxin M_1 (B).

LC-MS/MS Analysis. Analysis was performed with an Agilent 1200 Series rapid resolution liquid chromatograph (Palo Alto, CA) coupled to an Agilent 6410 triple quadrupole mass spectrometer with an electrospray ionization (ESI) source. The column oven temperature was set at 30 °C. The injection volume was 50 μL. Separation was achieved using a Shiseido chloramphenicol cell pak MGIII-C_{18} (150 mm ×2.1 mm i.d., 1.8 μm) column (Shiseido, Tokyo, Japan). The isocratic mobile phases were 5 mM ammonium acetate aqueous solution/methanol (60:40, v/v), and the flow rate was 0.25 mL/min. A divert valve was utilized to help remove any matrix impurities from entering the MS/MS. The LC flow was diverted away from the mass spectrometer for the first 6 min. The MS was on from 6 to 12 min and the LC flow diverted again. In order to achieve maximum sensitivity for the analytes, the analysis of chloramphenicol was performed in negative ionization mode whereas aflatoxin M_1 was analyzed in positive ionization mode, so the mass spectrometer was operated in negative mode for the first 8.7 min and then operated in positive mode. Nitrogen was used as the nebulizer gas and the drying gas. Parameters for the ESI-MS were nebulizer gas pressure 40 psi, drying temperature 340 °C, and drying gas flow of 8 L/min. Cone voltage and collision energies were optimized for each analyte during infusion of the pure standard, and the most abundant fragment ion was chosen for the selected reaction monitoring. Quantitative analysis was carried out using multiple reaction monitoring (MRM) mode. The MRM parameters for the optimal yield of product ions were defined in individual time windows for each analyte as they eluted from the LC column.

Calibration Curves and Matrix Effects. Matrix matched calibration curves were prepared at six concentration levels from 0 to 10 μg/kg for chloramphenicol and from 0 to 2.5 μg/kg for aflatoxin M_1 by spiking the extracts obtained from blank milk samples with appropriate volumes of working standard solution. A corresponding calibration curve made from pure standard solution with the same amounts of chloramphenicol and aflatoxin M_1 was prepared by diluting the appropriate volume of the working standard solution with the chromatographic mobile phase. Matrix effects were assessed by comparing the ion

intensity of sample extracts with the analytes of interest added post-extraction with pure solutions prepared in mobile phase containing equivalent amounts of the analytes. The difference in response between the postextraction sample and the pure solution divided by the pure solution response determines the degree of matrix effect occurring in the analytes in question under chromatographic conditions.[23]

Method Validation. The method was validated according to the criteria of Commission Decision 2002/657/EC.[24] According to these criteria, method validation parameters include linearity, specificity, accuracy, precision (repeatability and within-laboratory reproducibility), and analytical limits (decision limits CCα and detection capability CCβ). Specificity was evaluated by analyzing 20 different blank samples (including liquid milk and powdered milk) in order to investigate possible interferents. Linearity was evaluated with a calibration curve. Method accuracy was evaluated by performing recovery studies using blank milk samples. According to the 2002/657/EC Decision, recovery experiments were conducted at three specified fortified levels. For chloramphenicol, the spiking levels were 1 × MRPL, 1.5 × MRPL, and 2 × MRPL, and for aflatoxin M_1, the spiking levels were 0.5 × permitted limit (PL), 1 × PL, and 1.5 × PL. Six replicates were obtained for each concentration. The accuracy was calculated as the relation between the measured chloramphenicol and aflatoxin M_1 content in fortified blank samples and the fortification level. Repeatability (within- and between-day) was calculated from the analysis of six aliquots of a blank liquid milk sample, fortified with chloramphenicol at 0.3, 0.45, and 0.6 μg/kg (which corresponded to 1, 1.5, and 2 times the MRPL) and with aflatoxin M_1 at 0.025, 0.05, and 0.075 μg/kg (which corresponded to 0.5, 1, and 1.5 times the permitted limit) and performed by the same operator on three separate occasions in a 2 week period (thus a total of 18 experiments for each concentration level). Within-laboratory reproducibility was calculated by the same principle, but analyses were performed by two different operators on one occasion in a 2 week period (thus a total of 12 experiments for each fortification level). CCα was calculated from the within-laboratory reproducibility data of blank milk samples fortified at three levels. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the decision limit. CCβ was calculated from the corresponding concentration at the value of the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility.

RESULTS AND DISCUSSION

Sample Preparation. In residue-analysis methods, the critical step is the extraction and cleanup procedure, especially when the concentration of the analytes is at μg/kg levels. For this reason, extraction conditions have to be carefully selected to achieve the highest recovery while eliminating most of the interfering matrix components. The nature of the solvent is significant because it determines the extent of analyte extraction from and interferent retention in the food matrix. Acetonitrile, acetone, and ethyl acetate were used to optimize the extraction efficiency. Acetonitrile was considered the best organic solvent because of the acceptable recoveries in studies and because it gave us the cleanest extracts since milk proteins were easily precipitated in it. As an alternative to homogenization, ultrasound-assisted extraction was another widely used extraction method. Results showed that extraction efficiency by ultrasonication was 90.3% while it was only 75.6% for shaking. The use of ultrasonication is easy, and many samples could be treated at the same time. For these reasons, we preferred ultrasonication as the sample preparation procedure.

Optimization of LC-MS/MS. Determination of the optimal MRM transitions for chloramphenicol and aflatoxin M_1 was carried out using single-MS full scan mode followed by product ion scan mode through direct injection of individual standards at 100 ng/mL and 20 ng/mL, respectively. ESI in both positive and negative ion mode were conducted. It was found that chloramphenicol exhibited higher precursor ion signal intensities in negative ion mode while aflatoxin M_1 exhibited higher precursor ion signal intensities in positive ion mode. The dominant precursor ion of chloramphenicol obtained from ESI was m/z 321 $[M - H]^-$. This precursor ion was also measured by Vivekanandan et al.[25] in their study on honey, and by Rodziewicz et al.[17] for determination of chloramphenicol in milk powder using LC-ESI-MS/MS. The dominant precursor ion of aflatoxin M_1 was m/z 329 $[M + H]^+$. Aflatoxin M_1 may also be determined in negative ion mode as shown by Chen et al.,[26] for determination of aflatoxin M_1 in milk and milk powder using LC-ESI-MS/MS. However, the positive ion mode was selected in our study because it gave sensitivity considerably higher than that from the negative ion mode.

The spectra of chloramphenicol and aflatoxin M_1 at different cone voltages were studied to select characteristic fragments of chloramphenicol and aflatoxin M_1. The most sensitive transitions obtained in negative ion mode for chloramphenicol were m/z 257 and 152, and the most sensitive transition ions in positive ion mode for aflatoxin M_1 were m/z 273.1 and 258.9. According to the Commission Decision 2002/657/EC criteria for prohibited substances, at least one precursor ion and two product ions are required to confirm the presence of the analyte.[24] In order to satisfy the qualitative system of identification points (IPs) for chloramphenicol, we selected m/z 321 as precursor ion, and production of m/z 152 and 257 as quantification ion and confirmation ion, respectively. In the case of aflatoxin M_1, we selected m/z 329 as precursor ion, and m/z 273.1 and 258.9 as product ions for quantification and confirmation. These ions represent the system of identification points for chloramphenicol and aflatoxin M_1 confirmation.

It is well-known that biological sample extracts usually contain high amounts of matrix coextractives. To prevent the electrospray source from contamination, a divert valve was used between the analytical column and the mass spectrometer which allowed the flow to pass through the mass spectrometer only during analyte elution. The use of a divert valve made it possible to analyze a high number of samples without having to clean the source of the mass spectrometer.

Matrix Effects. Many investigations into analytical troubleshooting encountered with LC-MS/MS detection have focused on the problems which arise due to matrix effects, and in particular ion suppression.[27] Matrix effects are the result of competition between nonvolatile matrix components and analyte ions for access to the droplet surface for transfer to the gas phase.[28] When ion suppression occurs, the sensitivity and lower limit of quantification of a method may be adversely affected.[23] Matrix effects were evaluated by a typical experimental system. Two sets of calibration lines, named "matrix-matched" and "solvent", respectively, were constructed. The actual experimental concentrations obtained from the calibration curves for the matrix-matched samples are compared to the theoretical values for the spiked solvent standards. Both experimental concentrations of chloramphenicol and aflatoxin M_1 in our procedures are significantly lower than the theoretical values as shown in Figure 2, which means that ion suppression occurs in our method.

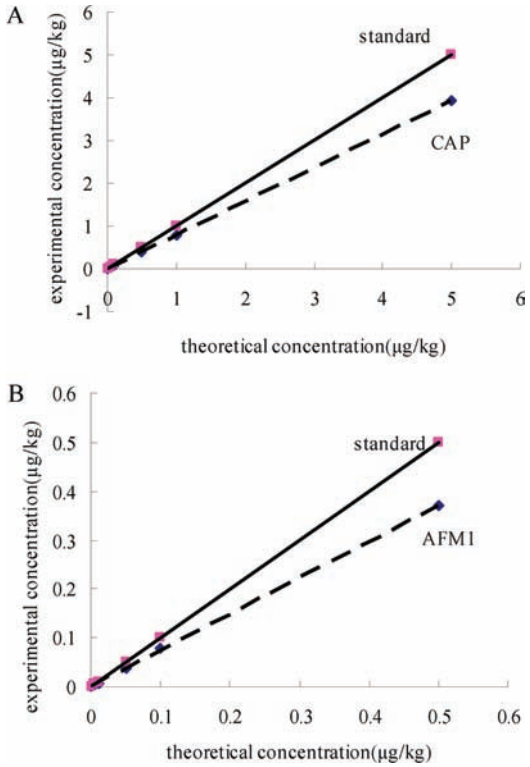

Figure 2. Ion suppression for chloramphenicol (A) and aflatoxin M_1 (B). Experimental values and theoretical values are plotted based on calibration curves of the matrix-matched extracted liquid milk samples and elution solvent samples, spiked with chloramphenicol (A) and aflatoxin M_1 (B).

Table 1. Results for Matrix Effects of Chloramphenicol and Aflatoxin M_1 for Liquid Whole Milk and Powder Milk

		matrix effect[a] (%) ± standard deviation	
analyte	level (μg/kg)	liquid milk	powder milk
chloramphenicol	0.05	−16.1 ± 2.0	−9.5 ± 1.6
	0.5	−18.1 ± 6.4	−9.2 ± 1.8
	5	−17.6 ± 5.2	−8.3 ± 0.8
aflatoxin M_1	0.005	−20.3 ± 9.5	−18.2 ± 4.3
	0.05	−21.2 ± 6.2	−17.6 ± 5.8
	0.5	−19.0 ± 5.0	−16.8 ± 3.6

[a] "−"represents a loss of the analyte signal (ion suppression), 0% represents no matrix effects, and "+"represents an enhancement of the analyte signal (ion enhancement).

Furthermore, data present in Table 1 show that the matrix effects of chloramphenicol ranged from −8.3% to −18.1% and −16.8% to −21.2% for aflatoxin M_1 in liquid milk and powder milk matrix, respectively. These results revealed that the determination of chloramphenicol and aflatoxin M_1 was affected by the interferences from real samples to some extent. Therefore, to provide reliable results, matrix-matched calibration curves were chosen throughout this study.

Method Validation. The specificity test was performed by preparation and analysis of 20 blank samples and spiked samples. No interference was observed at the retention time of the two analytes. Typical chromatograms of a blank liquid milk sample and liquid milk fortified with chloramphenicol at 0.1 μg/kg and aflatoxin M_1 at 0.01 μg/kg are illustrated in Figure 3. The chloramphenicol presented a retention time of 7.48 min and aflatoxin M_1 a retention time of 9.47 min, and there are no interfering compounds at these retention times. Linearity was evaluated by matrix-matched calibration curve. Analytical curves were linear in the range of 0.05 μg/kg to 10 μg/kg for chloramphenicol and 0.005 μg/kg to 2.5 μg/kg for aflatoxin M_1, respectively. Good linearity was obtained for each analyte with the corresponding correlation coefficient higher than 0.999. The precision of the developed analytical method were determined by calculating the within-day repeatability, between-day repeatability, and within-laboratory reproducibility for each analyte. Table 2 summarizes the overall performance data of the method, and satisfactory results were obtained in the study. Mean recoveries of chloramphenicol and aflatoxin M_1 ranged from 88.8% to 100.6%. The within-day and between-day precision, expressed as relative standard deviation (RSD), was below 7%, which reflects the robustness of the method. The within-laboratory reproducibility ranged from 6.6% to 14.7%, satisfying the criteria suggested by the EU Decision for the concentration level considered.

The sensitivity of an analytical method is generally established from the limits of detection (LODs) and the limits of quantification (LOQs). LODs and LOQs were based on the minimum amount of target analyte that produced a chromatogram peak with a signal-to-noise ratio of 3 and 10 times the background chromatographic noise, respectively. The LODs and LOQs for chloramphenicol in milk were 0.05 μg/kg and 0.2 μg/kg while those for aflatoxin M_1 were 0.005 μg/kg and 0.02 μg/kg. As there is no maximum permit level for chloramphenicol, it is more relevant to report the sensitivity of the method as CCα and CCβ. In the 2002/657/EC Decision, for a forbidden substance, the CCα is defined as "the limit at and above which it can be concluded with an error probability of 1% that a sample is noncompliant" and CCβ as "the smallest content of the substance that can be detected, identified and quantified in a sample with an error probability of 5%". The values CCα and CCβ were determined by the matrix calibration curve procedure according to ISO 11843.[29] Six curves obtained at four levels 0, 0.15, 0.3, and 0.45 μg/kg were used. The value CCα and CCβ for chloramphenicol in milk were 0.07 and 0.11 μg/kg, respectively. Not only the LODs and LOQs of the method but also CCα and CCβ of chloramphenicol in milk were all below the MRPL of 0.3 μg/kg for chloramphenicol and the maximum permitted level of 0.05 μg/kg for aflatoxin M_1, indicating that the proposed method is suitable for quantification of chloramphenicol and aflatoxin M_1 in milk.

Application of the Method. To evaluate the applicability of the method proposed, 50 samples were obtained from local supermarkets. Among the 50 samples, 4 samples were found containing chloramphenicol residues. The concentration of chloramphenicol detected was at levels up to 0.45 μg/kg which shows that chloramphenicol is currently illegally used in China. Among the 50 samples, 3 samples were found positive for aflatoxin M_1, with their concentrations levels were in the range of 0.01 to 0.25 μg/kg. Although aflatoxin M_1 content in most samples were below the maximum level established by EU, they can indicate that a risk of contamination of milk with aflatoxin M_1 exists. No sample was detected positive for both chloramphenicol and aflatoxin M_1.

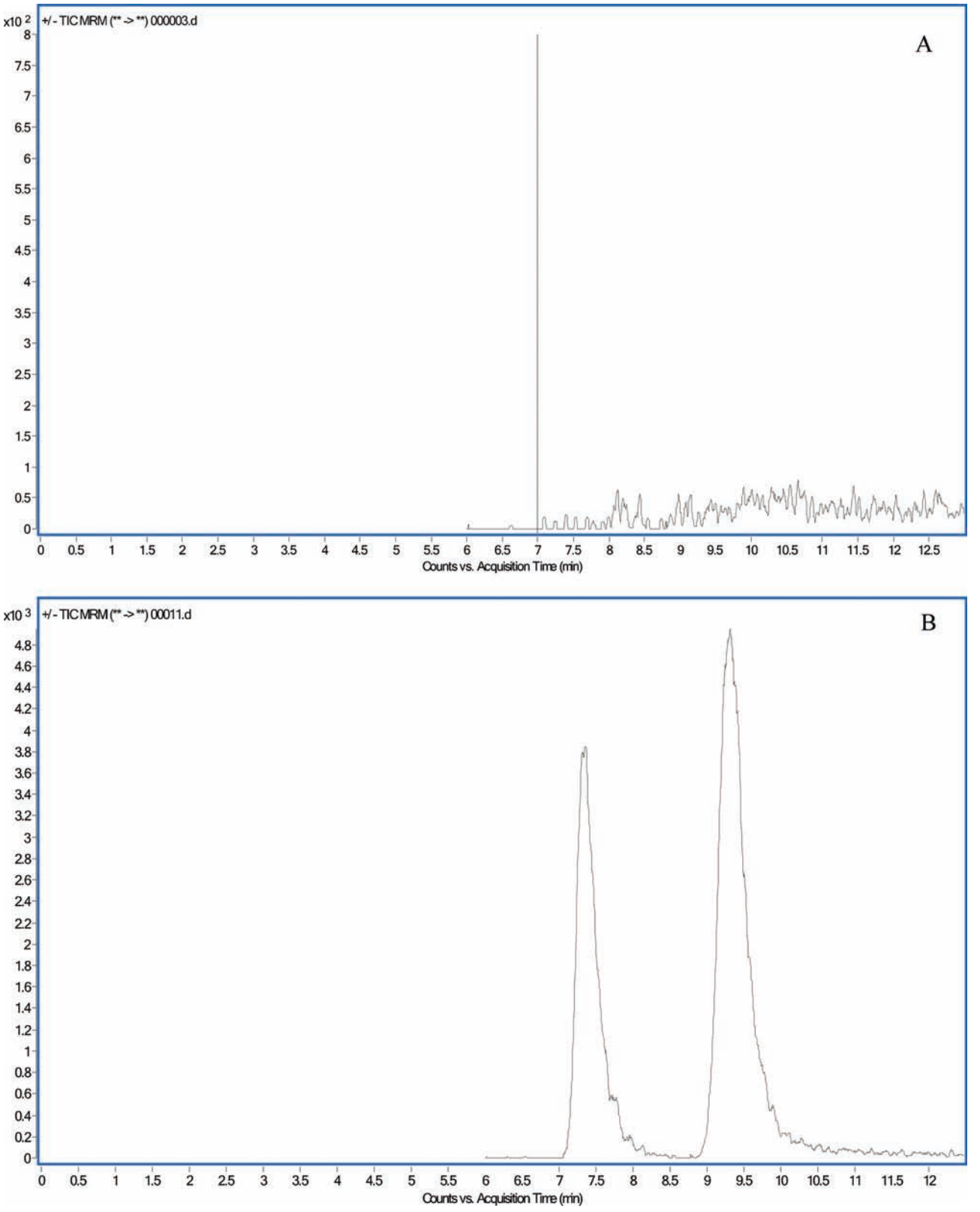

Figure 3. MRM chromatogram of blank liquid milk (A) and liquid milk fortified with chloramphenicol at 0.1 μg/kg and aflatoxin M_1 at 0.01 μg/kg (B).

In conclusion, the present method was successfully applied to simultaneous determination of chloramphenicol and aflatoxin M_1 in milk. In comparison to the previously reported methods, our method is sensitive, easily handled, and reliable. The extraction procedure involving only two steps: protein precipitation and ultrasonic extraction which is very effective, simple, and fast, with no further cleanup step. The LOQs of chloramphenicol and aflatoxin M1 were lower than the minimum

Table 2. Recovery Values (%) and Relative Standard Deviation (%) from Spiked Whole Milk Samples

		within-day precision[a]		between-day precision[b]		within-laboratory reproducibility[c]	
analyte	spike level (μg/kg)	mean recovery(%)	RSD (%)	mean recovery(%)	RSD (%)	mean recovery(%)	RSD (%)
chloramphenicol	0.3	99.4	3.4	95.6	4.6	94.2	9.8
	0.45	95.3	4.4	97.9	4.0	93.7	6.6
	0.6	100.6	3.9	99.1	4.2	90.8	7.1
aflatoxin M_1	0.025	91.7	6.2	90.3	6.0	88.8	14.7
	0.05	92.4	5.1	96.7	5.1	90.8	9.0
	0.075	97.4	5.6	93.6	5.9	94.9	7.6

[a] Number of replicates: 6. [b] Number of replicates: 6 × 3. [c] Number of replicates: 6 × 2.

required limit performance value for chloramphenicol (0.3 μg/kg) and maximum residue limits for aflatoxin M_1 in milk (0.05 μg/kg). The method was sufficiently efficient for routine quality control operations on milk products and suitable for residue confirmation analyses.

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