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Determination of Chloramphenicol Residues in Milk and Milk-products Using LC/MS/MS

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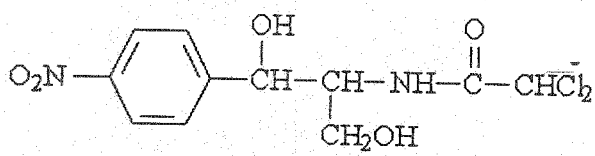
The aim of the present investigation was to demonstrate the presence of chloramphenicol residues in milk and milk products of dairy animals by performing liquid chromatographic mass spectrometric (LC/MS/MS) method for quantification. LC/MS/MS was used for quantitative detection of chloramphenicol in milk and milk products because it can determine very low concentration of antibiotics at about parts per million (ppm)/parts per billion (ppb) levels. Injection of 10% chloramphenicol prepared in sterile water and 2 mg/kg body weight was administered in livestock (food producing animal). Milk samples (with product prepared from it) were collected after 24 h of dermal administration of antibiotic, analyzed using LC/MS/MS and compared with commercially available milk samples for human consumption. It was found that considerable residue of the active substance is present in milk and milk products of the treated animal. Analysis in LC/MS/MS was conducted by extracting milk with ethyl acetate. Chloramphenicol was eluted from the C18 LC column at about 0.57 min using ammonium acetate-methanol as a mobile phase. The mass spectrometer was operated in the negative ion mode using selective reaction monitoring (SRM). The peak area was used for quantification. The milk fortified with chloramphenicol at 50, 100, 500 and 1000 ng/mL was used as standard.

Key Words: Chloramphenicol, Milk, Milk products, LC/MS/MS.

INTRODUCTION

Chloramphenicol (CAP) is a broad-spectrum antibiotic, having its wider activity against many gram-negative and gram-positive cocci and bacilli, including anaerobes. Being primarily bacteriostatic, CAP binds to the 50S subunit of ribosome and inhibits protein synthesis of many gram-negative and gram-positive bacteria. Miscellaneous toxic effects are due to the dichloride carbon alpha to the carbonyl group; this carbon readily undergoes substitution with nucleophiles such as those found on proteins (Fig. 1).



Structure of chloramphenicol: $C_{11}H_{12}N_2O_5Cl_2$
Fig. 1

Common antibiotics like CAP is widely used in veterinary medicine, due to their broad range of activity and low cost. The treatment of these animals, which are used for production of food, with CAP is prohibited in many countries including the European Union and the United States, as many of the side effects in humans have been found. The main potential human toxicity is depression of red blood cell production in bone marrow leading to dose independent fatal "aplastic anemia" and "leukemia", particularly in children.

In spite of its potential toxicity, CAP is sometimes used at therapeutic doses for treatment of serious infections in food producing animals. However, it has not been possible to identify a safe level of human exposure to CAP. Hence, it is important to identify CAP residues and monitor its exposure levels in different subjects, such as edible milk and milk products with Indian perspective.

Although the use of CAP in food producing animals and aquaculture is banned in many countries, use of CAP to treat animals in India remains a possibility due to its broad spectrum activity, ready availability and low cost.

Methods to detect CAP residues in biological matrices, especially in milk, include immunoassays[1] and mass spectrometry in combination with GC[2, 3] and HPLC[4, 5]. Immunological methods are suitable for screening purposes, whereas mass spectrometric methods are utilized for confirmation. Methods for the determination of CAP residues using GC/MS require additional chemistry steps, such as silylation of CAP, before the samples can be analyzed, while with the advent of liquid chromatography/mass spectrometry (LC/MS) and information dependent data acquisition function in mass spectrometry, it has become possible to quantify CAP without any derivatization and can be directly analyzed, minimizing possible derivatization problems, saving time and preventing compound losses.

EXPERIMENTAL

Ethyl acetate (HPLC grade), Methanol (HPLC grade), Millipore Milli-Q Plus ultra pure water, ammonium acetate and chloramphenicol sodium succinate.

Samples:

1. Loose milk sample obtained from a livestock which was administred with CAP at the dosage of 2 mg/kg **(Sample A).**
2. Sample extracted from yogurt prepared from sample A **(Sample B).**
3. Sample prepared from milk powder, commercially available **(Sample C).**
4. Branded milk sample **(Sample D).**

Equipment:

Perkin-Elmer Series 200 pump fitted with Perkin-Elmer Series 200 autosampler.

Analytical Column: Zorbax (50 mm length × 4.6 mm internal diameter) 5μ particle size.

Standards: Calibration and spike standards were prepared by dissolving 1 g of CAP powder with 10 mL sterile water (diluent).

The 100 mg/mL standard was used directly and diluted to yield five standards. Each of the five was again diluted 1 : 10 with unspiked milk sample matrix to yield CAP concentrations from 50 to 1000 ng/mL. These were used for the calibration curves.

Spiked samples: Spike recoveries were performed at CAP concentrations of 0.05 ppm, 0.1 ppm, 0.5 ppm, 1 ppm in the milk. Five recoveries at each level were run along with both samples A, B, C and D and a sample blank. The spike was added to the milk before extraction in step of the extraction-cleanup procedure.

LC conditions: Mobile phase: 0.01 M ammonium acetate: methanol in the volume ratio of 25 : 75

Mobile phase A	0.01 mM ammonium acetate in water		
Phase B	Methanol		
Gradient:	Minutes	% A	% B
	0	25	75
	5	25	75

Flow Rate: 0.5 mL/min; Column temperature: 40°C.

Autosampler conditions:

Auto-sampler	Injection volume: 25 µL (no waste injection)
	Syringe flush volume and wash volume: 5 mL
	Sample tray temperature: 10°C

Mass spectrometer conditions: Mode: Negative ion electro spray-with metal needle option. The metal needle was connected with a zero dead volume union to the fused silica capillary delivering the mobile phase to the ESI source. This eliminated the frequent need to reposition the capillary. Note that the parameters need to be optimized for each instrument. The following conditions were found to maximize response on our instrument (API-2000-Q TRAP) (Table-1).

TABLE-1

Precursor ions (m/z)	323.3	Entrance potential (EP)	–10
Product ions (m/z)	152.1	Declustring potential (DP)	–50
Ion spray voltage (IS)	–4.5 kV	Cell exit potential (CXP)	–7
Collision gas	Medium	Ion source gas 1	20
Capillary temperature	350°C	Ion source gas 2	50
Nebulizer gas (air)	80 arbitrary units	Interface heater	ON
Curtain gas (N_2)	20 psi	Collision entrance potential	–16.15 kV
Dwell time	500 ms/SRM transition		

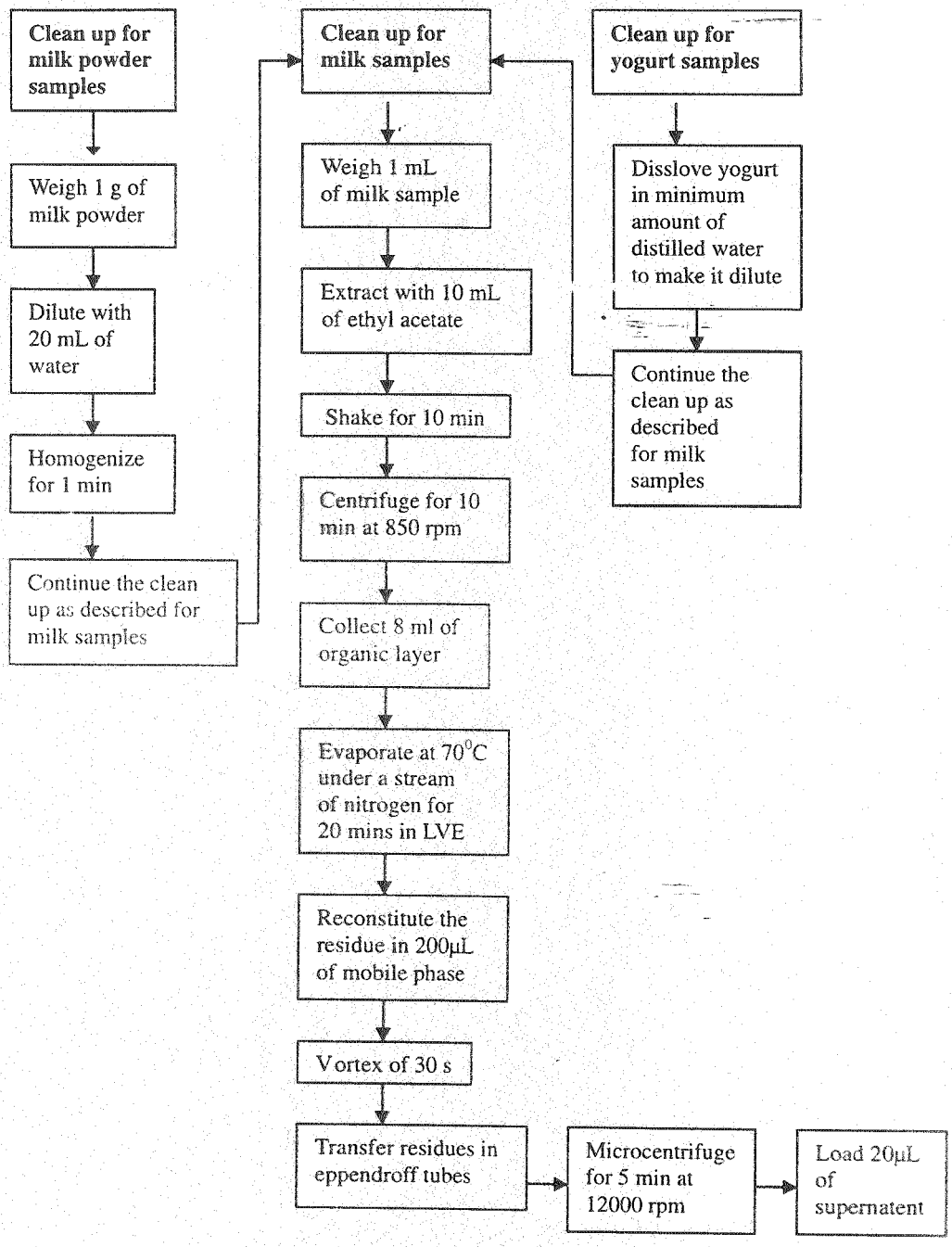
The collision energy (CE) was optimized for one of the SRM transitions which was monitored throughout the HPLC run. The collision energy for the SRM transition is listed below in Table-2.

TABLE-2

	Precursor ion (Q_1)	Precursor ion (Q_3)	Collision energy (CE)
m/z	323.3	152.1	–38

Under these conditions, the m/z 152.1 product ion was the base peak as depicted from chromatograms.

Method of sample preparation



RESULTS AND DISCUSSION

Blank milk samples were fortified with CAP in the concentration range of 0.05–1.0 ppm and the area and observed concentration were obtained. The area was found to be in the range of 49,000–9,70,000 and the observed concentration

was found to be in the range of 0.0524–1.005 ppm (as shown in chromatograms 1 to 4: STD chromatograms).

Subsequently four different milk samples were prepared for analysis as described in experimental part. All these samples were analyzed using LC/MS/MS and four chromatograms were obtained [chromatograms 5 to 8: chromatograms of samples A, B, C and D]. The area and the observed concentration obtained are given in Table-3.

TABLE-3

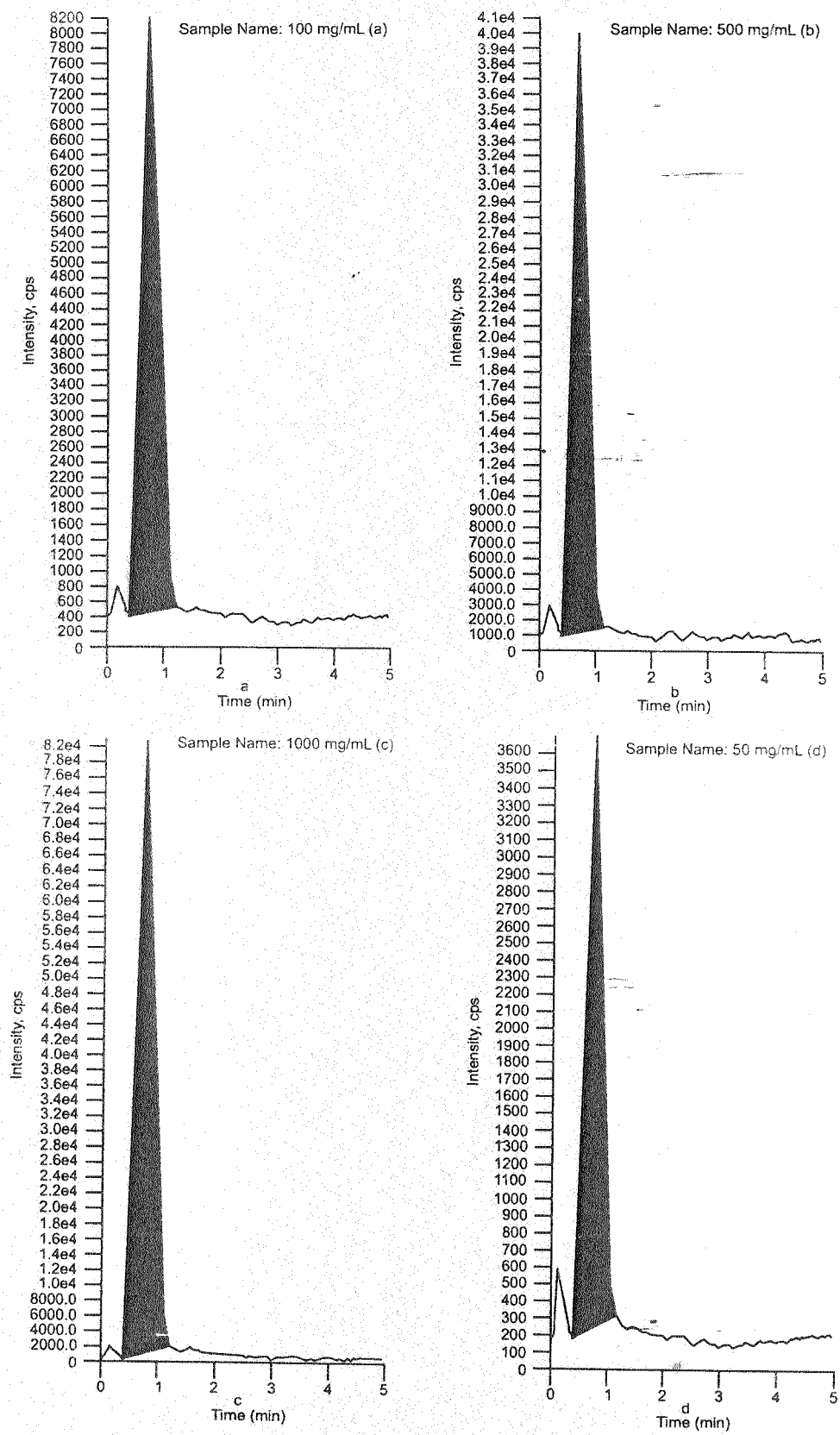
Samples	Area	Observed concentration (ng/mL)	Observed concentration (ppm)	Retention time (min)
Sample A	71784	73.42	0.07342	0.55
Sample B	10825	12.21	0.01221	0.62
Sample C	6396	7.77	0.00777	0.56
Sample D	5347	6.71	0.00671	0.56

The collision energy (CE) was calculated by selecting the characteristic product ions for CAP and using selective reaction monitoring transition. Table-2 represents the value of collision energy (CE) as –38.

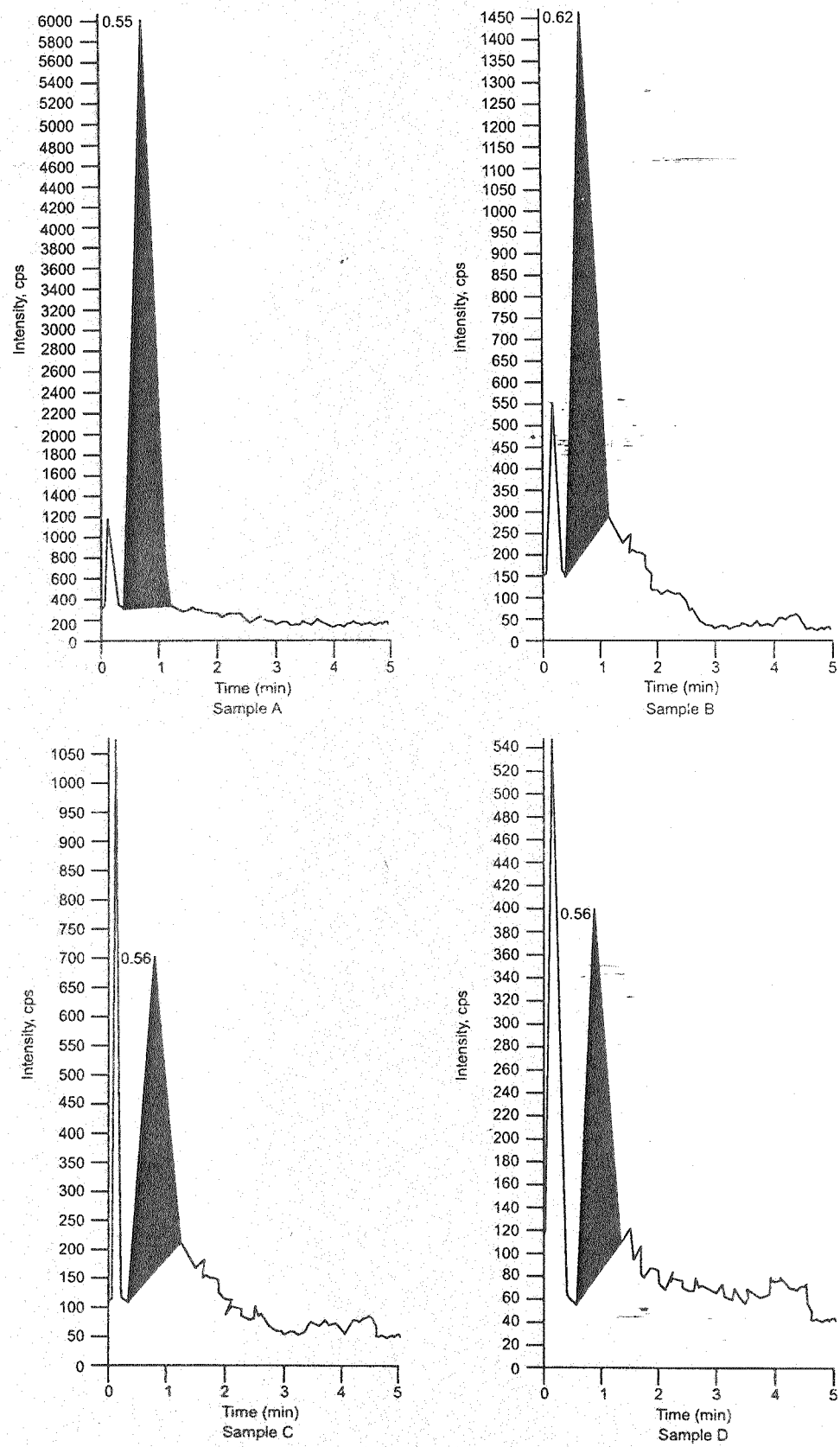
The performance criteria for mass spectrometric detection as a confirmatory method following either an on-line or an off-line chromatographic separation are measured as a system of identification points. For the confirmation of CAP a minimum of one identification point was essential. One identification point was obtained using LC/MS/MS with 1 precursor and 1 product ion and thus the performance criteria for the confirmation were fulfilled. Standard chromatograms showed 1 SRM in spiked milk samples. Hence the sample matrix peak detected in SRM chromatogram of the product ions m/z 152.1 was chosen for quantification purposes.

The chromatogram for milk samples C and D, *i.e.*, commercially available milk samples, showed the lowest and equal amounts of CAP which is 0.007 ppm whereas chromatogram for milk sample A, *i.e.*, loose milk sample collected after 24 h of dosage, shows maximum amount of CAP which is 0.0734 ppm. It is interesting to note that when the same loose milk sample was used to prepare yogurt the amount of CAP reduces by 1/6th of sample A. Sample B shows the presence of 0.0122 ppm CAP. The comparison of CAP concentration in samples A and B shows that CAP in sample B might have been utilized by the microbes present in the yogurt.

The recovery of the assay was tested using spiked blank milk samples from 0.05 ppm to 1 ppm CAP concentration range (n = 4). The calculated recovery was about 70% with a coefficient of variation 5%. The precision was determined using calibration curves, prepared with spiked blank milk samples from 0.05 to 1.0 ppm concentration range (n = 4).



Chromatograms-Standard (1–4)
Chromatogram (a) 0.1 ppm (b) 0.5 ppm (c) 1 ppm (d) 0.05 ppm



Chromatograms showing peaks for the presence of CAP in samples A, B, C and D

Table-4 shows that the coefficient of correlation of the calibration curves calculated for 1 SRM transitions was 0.99. The interferences in the chromatogram from different milk samples were investigated and eliminated using an appropriate HPLC column and using appropriate product ions. The product ions used are listed in Table-4.

TABLE-4

	Precursor ion (Q_1)	Precursor ion (Q_3)	Coefficient of correlation (r_2)
m/z	323.3	152.1	0.9993

For the chromatographic separation the deviations of the retention times between standard and samples were inside of 2.5%; the average retention time of the standard was 0.512 min and 0.558 min for milk samples. For the relative ion intensities of mass spectrometric detection of derivation of relative ion intensity in spiked milk samples *vs.* standard was lower than 10%.

TABLE-5

Sample No.	Actual concentration	Area	Observed concentration (ng/mL)	Observed concentration (ppm)	Retention time (min)
A	50 ng/mL level (0.05 ppm)	49000	52.39	0.5230	0.56
B	100 ng/mL level (0.1 ppm)	98100	103.16	0.1030	0.55
C	500 ng/mL level (0.5 ppm)	472000	489.76	0.4898	0.52
D	1000 ng/mL (1.0 ppm)	970000	1004.68	1.0047	0.51

Conclusion

Appreciable amounts of CAP are administered to control diseases in dairy cattle. However, concerns have been expressed about the genotoxicity of antibiotic CAP and its metabolites. Its embryo and fetotoxicity, and the lack of a dose-response relationship for aplastic anemia in humans have been reported. Unfortunately this antibiotic is making its way into consumer milk supply. The presence of CAP residues in milk could cause allergic reactions, affect starter cultures or create an environment favourable for resistant bacteria. Therefore, most countries in the world do not permit the use of CAP for treatment of food producing animals in order to protect the health of consumers. Despite such restrictions, CAP has been consistently detected in food samples and its residual presence has raised safety issues for our health.

The present method for the detection of antibiotic (CAP) drug residues in milk is one of the most significant tests performed by our laboratory. Through the aid of this method of milk detection, the presence of CAP in milk can be confirmed and when the detection is found positive, such milk can be destroyed and should not be used for production of other milk products.

The method is authentic, requiring only small quantities of reagents and involves minimal manual work-up procedures. The presented method is able to detect and quantify CAP residues in milk and milk products in the lower ppb/ppm concentration ranges. Due to its excellent sensitivity along with its proven reliability and stability, the API 2000 system provides a dependable and consistent determination of CAP as demonstrated by the precision of the correlation coefficient (r_2) at > 0.99 for the single calibration curves and the 99% variability. Due to its high specificity, sensitivity and stability the LC/MS/MS assay of the API 2000 system for antibiotic detection fulfils the criteria of confirmed detection at lower concentration range.

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