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SIMULTANEOUS DETERMINATION OF LYMECYCLINE AND TETRACYCLINE RESIDUES IN BOVINE MILK FOLLOWED BY MATRIX SOLID-PHASE DISPERSION COUPLED TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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ABSTRACT

A simple, sensitive and inexpensive method was developed using matrix solid-phase dispersion (MSPD), together with high performance liquid chromatographic method for determination of antibiotic residues (Lymecycline and Tetracycline) in bovine milk. The evaluated parameters included the type and amount of sorbent (silica gel, C18 and alumina) and the nature of eluent (20% trichloro acetic acid , 0.01M citric acid, 0.01M disodium hydrogen phosphate, 0.01M EDTA and Methanol). The best results were obtained using 2mL of milk sample, 1.0 g of C18 as sorbent and 20mL of 20% trichloroacetic acid , 0.01M citric acid, 0.01M disodium hydrogen phosphate, 0.01M EDTA and Methanol (1:2:2:2:3, (v/v)).The method was validated using in milk samples spiked with antibiotics at different concentration levels (0.01 and 0.1 µg/mL). Average recoveries (using each concentration six replicates) ranged 88-96%, with relative standard deviations less than 2%, calibration solutions concentration in the range 0.01-2.0 µg/mL and limit of detection (LOD) and limit of quantification (LOQ) were 0.01 µg/mL and 0.03 µg/mL respectively.

Key words: matrix solid-phase dispersion, antibiotic residues, LOD and LOQ.

INTRODUCTION

Antibiotecs are in used in food-producing animals not only for treatment of disease, but also subtherapeutically to maintain health and promote growth[1-2]. The use of unauthorized antibiotics or the failure to follow label directions for approved antibiotics could result in unsafe antibiotic residues in food products. Therefore, monitoring antibiotic residues in food forms part of a general policy to prevent unapproved uses of antibiotics[3].

Traditionally most antibiotics have been determined by microbiological assay. However, it is very difficult to distinguish one antibiotic from another using microbiological method[5].

In modern farming practice, drugs are using in a large scale and are applied in animal husbandry for different reasons. They are used to prevent disease, cure animals, or as feed additive to promote growth. All drugs administered to milk-producing animals may lead to residues in the milk. In addition to the drug dosage, the levels of those residues depend on the animal products, which is called withdrawal period.

Milk is as ancient as mankind itself, as it is the substance created to feed the mammalian infant. All species of mammals, from man to whales, produce milk for this purpose. Many centuries ago, perhaps as early as 6000-8000 BC, ancient man learned to domesticate species of animals for the provision of milk to be consumed by them. These included cows, buffaloes, sheep, goats, and camels, all of which are still used in various parts of the world for the production of milk for human consumption.

The role of milk in the traditional diet has varied greatly in different regions of the world. The tropical countries have not been traditional milk consumers, whereas the more northern regions of the world, Europe (especially Scandinavia) and North America, have traditionally consumed far more milk and milk products in their diet. In tropical countries where high temperatures and lack of refrigeration had led to the inability to produce and store fresh milk, milk has traditionally been preserved through means other than refrigeration, including immediate consumption of warm milk after milking, by boiling milk, or by conversion into more stable products such as fermented milks.

Various methods have been described for the determination of antibiotics, using solid-phase extraction (SPE), solid-phase micro extraction (SPME), supercritical fluid extraction (SFE)

and matrix solid-phase dispersion (MSPD), However, none of the published researches to date have reported the simultaneous analysis of chemical classes such as Lymecycline and Tetracycline in milk.

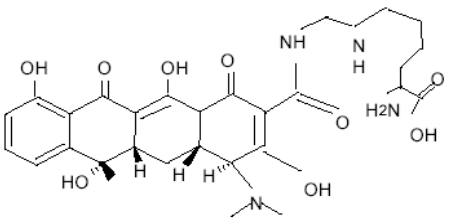
The matrix solid-phase dispersion (MSPD) technique was developed by Barker in 1989[4]. It has advantages over conventional techniques because it employs small amounts of sample and solvent, and the extraction procedure consists of only a few experimental steps. MSPD evolved from the solid-phase extraction (SPE) technique, modified for application to solid and semi-solid matrices. The MSPD procedure is based on the use of a sorbent, which acts as an abrasive in order to produce a modified "opening" of the solid matrix, facilitating the extraction process when using a suitable solvent for eluting the analytes. The use of MSPD for antibiotics recovery depends on the solubility of the antibiotics in the eluting solvent, as well as the interactions between the matrix components, sorbent and eluent.

Due to the lack of literature reports concerning the use of MSPD as an extraction technique for antibiotics belonging to different chemical classes from food products, this paper presents an MSPD method for determination of residue of antibiotics in bovine milk[6-10]. So, the present research considered two different chemical classes, namely Lymecycline and Tetracycline which analysis by high-performance liquid chromatography with ultraviolet detector (HPLC-UV).

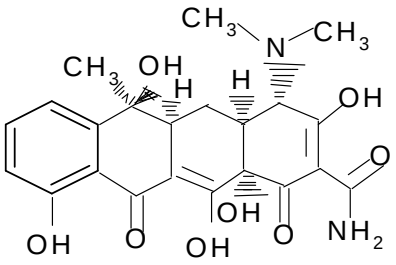
EXPERIMENTAL

Standards, Reagents and samples

Certificated analytical standards of Lymecycline (97.3%), and Tetracycline (98.2%) were obtained from Sigma Aldrich. Common names and structures of the antibiotics evaluated here are shown in (**Fig. 1)**. Acetonitrile was purchased from Rankem, New Delhi, Analytical grade solvents and chemicals trichloro acetic acid, 2-Methyl-2-propanol, methanol, disodium hydrogen phosphate, citric acid, tetra butyl ammonium hydrogen sulphate and EDTA, were supplied from Merck Limited, Mumbai, C18-bonded silica (50 µm) from phenomenex (Torrance, CA, USA), neutral alumina from Merck Limited, Mumbai, AR grade sodium sulphate from Merck Limited, Mumbai and Bovine milk is collected from local dairy form. They were brought to the laboratory and stored at refrigerator condition until they were processed in the laboratory.



Lymecycline



Tetracycline

Fig.1. Names and structures of two antibiotics evaluated

Standard stock solutions

The antibiotic standard stock solutions were individually prepared in acetonitrile at a concentration level 100 µg/mL and stored in a freezer at -18°C. The stock standard solutions were used for up to 3 months. Suitable concentrations of working standards were prepared from the stock solutions by dilution using acetonitrile, immediately prior to sample preparation.

Sample preparation

Representative 2 mL portions of bovine milk fortified with 100 µL of working standard solution. The mixture was then gently blended in the mortar for 30 min, to assess the homogeneity of the sample. The sample was allowed to stand at room temperature for one hour, before it was kept at refrigerator condition, until analysis.

Extraction procedure

2 mL of bovine milk sample was pipette out and homogenized with 1.0 g of C18 –bonded silica for 5 min. The homogenized sample was transferred to an MSPD column consisting of a 20mL capacity polyethylene syringe containing 1.0 g neutral alumina and 1.0 g of anhydrous sodium sulfate. The elution was performed under vacuum with and 20mL of 20% trichloroacetic acid , 0.01M citric acid, 0.01M disodium hydrogen phosphate, 0.01M EDTA and Methanol (1:2:2:2:3, (v/v)). The eluent was collected into a round bottom flask and evaporated to near dryness. Finally make up with 5mL of acetonitrile and analysed by HPLC-UV system.

Chromatographic separation parameters

The HPLC-UV system used, consisted Shimadzu high performance liquid chromatography

with LC- 20AT pump and SPD-20A interfaced with LC solution software, equipped with a reversed PLRP-S (250×4.6) mm and particle size 8µ, make: Varian Column temperature was maintained at 50°C. The injected sample volume was 20µL. 11.5:10:20:1:57.5 mixture of 2-Methyl-2-propanol,3.5% w/v di-potassium hydrogen phosphate,1.0% w/v Tetra butyl ammonium hydrogen sulphate,4.0% w/v di –sodium edetate and Milli-Q water was selected as mobile phase. The flow- rate used was kept at 1.5 ml/min. A detector wavelength was 254nm. The external standard method of Calibration was used for this analysis.

Method validation

Method validation ensures analysis credibility. In this study, the parameters accuracy, precision, linearity and limits of detection (LOD) and quantification (LOQ) were considered. The accuracy of the method was determined by recovery tests, using samples spiked at concentration levels of 0.03 and 0.3 mg/kg. Linearity was determined by different known concentrations (0.01, 0.05, 0.1, 0.5, 1.0 and 2.0 µg/ml) were prepared by diluting the stock solution. The limit of detection (LOD, µg/mL) was determined as the lowest concentration giving a response of 3 times the baseline noise defined from the analysis of control (untreated) sample. The limit of quantification (LOQ, µg/mL) was determined as the lowest concentration of a given antibiotics giving a response of 10 times the baseline noise.

RESULTS AND DISCUSSION

Specificity

The HPLC-UV analysis using the optimal extraction and detection conditions described shows the target analyte in the milk samples were free from the interference Representative chromatogram of obtained from standard, blank milk sample and LOQ level fortification in milk were given in **Figure 2-4.** The retention times of Lymecycline and Tetracycline were constant at 4.7±0.2, 5.3±0.2 min.

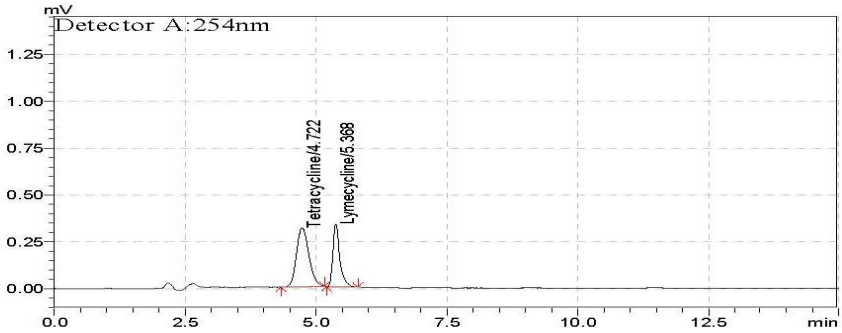


Figure2. A representative chromatogram obtained from Lymecycline and Tetracycline standard

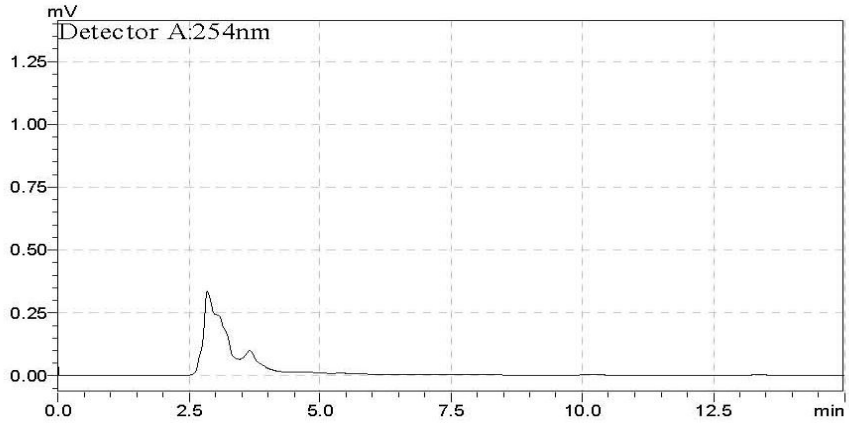


Figure 3. A representative chromatogram obtained from blank milk sample

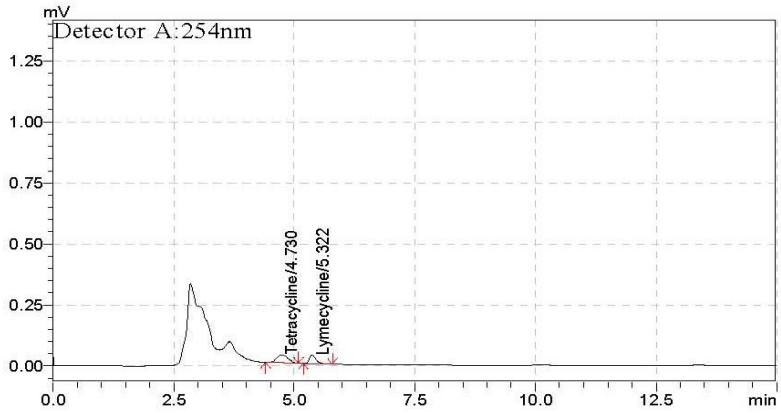


Fig.4. Representative Chromatogram at fortification level of(LOQ) 0.03µg/mL

Linearity

Different known concentrations of antibiotics (0.01, 0.05, 0.1, 0.5, 1.0, 2.0 µg/mL) were prepared in acetonitrile by diluting the stock solution. Each solution was prepared in triplicate. Injected the standard solutions and measured the peak area. A calibration curve has been plotted of concentration of the standards injected versus area observed and the linearity of method was evaluated by analyzing six solutions. The peak areas obtained from different concentrations of antibiotics were used to calculate linear regression equations. These were Y= 20956X+40.98 and Y=14961+27.55, with correlation coefficients of 0.9998 and 0.9999 for Lymecycline and Tetracycline respectively. A calibration curve showed in (**Fig. 5).**

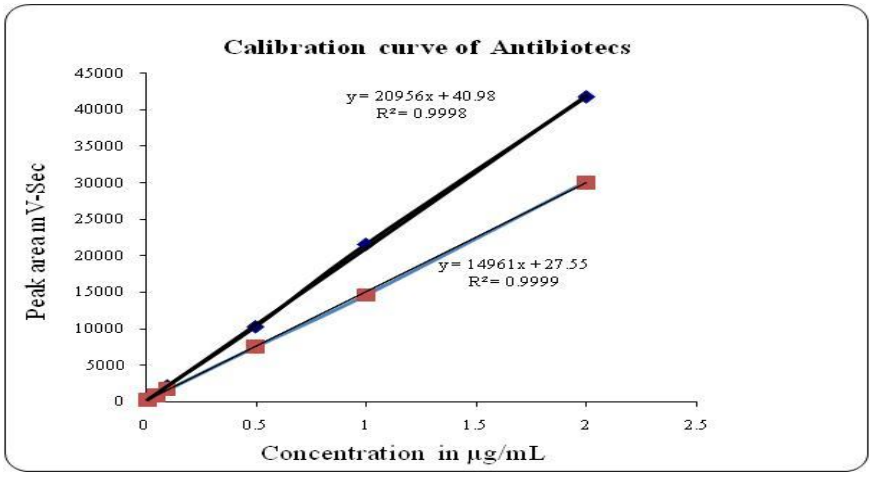


Fig.5. Representative Calibration curve of antibiotics.

Accuracy and Precision

Recovery studies were carried out at 0.03 and 0.3 µg/mL fortification levels for Lymecycline and Tetracycline in Bovine milk. The recovery data and relative standard deviation values obtained by this method are summarized in (**Table 1).**

These numbers were calculated from two (6) replicate analyses of given sample (Chlorothalonil and Biphenyl) made by a single analyst on one day. The repeatability of method satisfactory (RSDs<2 %).

Detection and Quantification Limits

The limit of quantification was determined to be 0.03 µg/mL. The quantitation limit was defined as the lowest fortification level evaluated at which acceptable average recoveries (90-98%, RSD<3%) were achieved. This quantitation limit also reflects the fortification level at which an analyte peak is consistently generated at approximately 10 times the baseline noise in the chromatogram. The limit of detection was determined to be 0.01 µg/mL at a level of approximately three times the back ground of control injection around the retention time of the peak of interest.

Storage Stability

A storage stability study was conducted at -20±1°C with bovine milk samples spiked with 0.1 µg/mL of Lymecycline and Tetracycline Samples were stored for a period of 30 days at this temperature. Analysed for the content of Lymecycline and Tetracycline before storing and at the end of storage period. The percentage dissipation observed for the above storage period was only less than 2% for Lymecycline and Tetracycline showing no significant loss of residues on storage. The results are presented in (**Table 2).**

Table 1. Recoveries of the antibiotics from fortified Bovine milk control sample (n=6)

Fortification Concentration in µg/mL	Replication	Recovery (%)	
		Lymecycline	Tetracycline
0.03	R1	86	85
	R2	84	86
	R3	88	87
	R4	88	86
	R5	85	83
	R6	86	83
	Mean	86	85
	RSD	1.86	1.97
0.3	R1	93	92
	R2	92	91
	R3	95	94
	R4	90	91
	R5	92	90
	R6	93	92
	Mean	93	92
	RSD	1.78	1.49

Table 2. Storage stability Details (n=6)

Fortified concentration in µg/mL	Storage Period in Days	Replication	Recovery in %	
			Lymecycline	Tetracycline
0.1	0	R1	92	91
		R2	93	93
		R3	95	94
		R4	92	91
		R5	93	90
		R6	94	93
		Mean	93	92
		RSD	1.25	1.68
	30	R1	90	88
		R2	89	89
		R3	91	90
		R4	92	91
		R5	89	91
		R6	89	90
		Mean	90	90
		RSD	1.41	1.30

CONCLUSIONS

This paper describes for the first time a fast, simple sensitive analytical method based on MSPD-HPLC-UV was developed and validated for the simultaneous determination of two antibiotic residues in bovine milk.

The MSPD extraction procedure of the described method is very simple and requires no sample preparation or pre-treatment, providing adequate clean-up of the matrix. Whole bovine milk extracts are very clean, with no interfering peaks at the retention time of the target compounds, indicating good selectivity of the proposed method.

The mobile phase 11.5:10:20:1:57.5 mixture of 2-Methyl-2-propanol,3.5% w/v di-potassium hydrogen phosphate,1.0% w/v Tetra butyl ammonium hydrogen sulphate,4.0% w/v di – sodium edetate and Milli-Q water yields good separation and resolution and the analysis time required for the chromatographic determination of the two antibiotics is very short (around 15 min for a chromatographic run).

Satisfactory validation parameters such as linearity, recovery, precision and very low limits were obtained and according to the SANCO guidelines[11]. For all of the antibiotics the sensitivity of the method was good enough to ensure reliable determination levels lower than the respective MRLs. Therefore, the proposed analytical procedure could satisfactorily be useful for regular monitoring of antibiotic residues on a large number of fruit samples.

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