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Detection of Penicillin Residues in Cow Milk Using High Performance Liquid Chromatography with UV-Diode Array Detection

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Abstract: Researchers describe the development of a Novel Method that is sensitive and reliable for detecting isoxazolyl penicillins (Cloxacillin (CLO) and Dicloxacillin (DCL)) in commercially available cow milk using High-Performance Liquid Chromatography with UV-Diode Array Detection (HPLC-DAD). CLO and DCL were extracted via deproteinization of milk samples, adjusting the pH to 4.5-5 using hydrochloric acid and then adding acetone/chloroform, followed by centrifugation and cleanup using a 0.45 μm nylon filter. CLO and DCL were separated using an Inertsil ODS-C_{18} column (4.6×250 mm×3.5 μm) via elution with a mobile phase consisting of 0.05 M phosphate buffer solution at pH 4.7 and acetonitrile at 22°C. Recovery of CLO and DCL was 96.47-106.94 and 97.76-104.86%, respectively. The limit of quantifications were 23.15 μg kg^{-1} for CLO and 2.28 μg kg^{-1} for DCL which are lower than the limits for residues in cow milk established by the European Union, indicating that the method is suitable for performing routine analysis. In order to determine this Novel Method's utility in a laboratory environment for analyzing real raw milk samples 100 raw milk samples were obtained from raw milk tanks belonging to milk producers. CLO and DCL were not detected in any of the samples. The present findings indicate that the Novel Detection Method is simple, rapid, sensitive and reliable, as compared to other detection methods and that as it uses fewer chemicals and does not require derivatization it could be considered the preferred method.

Key words: Cloxacillin, dicloxacillin, HPLC, milk, residue

INTRODUCTION

Monitoring of veterinary drug residues in food and foodstuffs is performed for ethical hygienic, technological and ecological purposes. Such residues may present pharmacological, toxicological, microbiological and immunopathological health risks for humans (Drackova *et al*., 2009; Kaya and Filazi, 2010; Filazi *et al*., 2005; Filazi and Yurdakok, 2010; Sanli *et al*., 1999; Sireli *et al*., 2006; Kurnaz and Filazi, 2011).

Veterinary drugs administered to lactating dairy cows may be detected in milk at various concentrations. The major source of milk contamination with antibiotics is intramammary administration followed by percutaneous, subcutaneous, intramuscular, intravenous and intrauterine administration (Jones and Seymour, 1988; Movassagh, 2011).

Worldwide, mastitis is the disease affecting dairy cows and as such, it is the most common reason antibiotics are administered to dairy cows. Bacteria from the genera *Staphylococcus* and *Streptococcus* are the most common causal agents of mastitis (Reybroeck *et al*., 2010). β-lactam antibiotics are the most commonly administered drugs for parenteral and intramammary treatment. The treatment of choice in many countries remains penicillin as minimum inhibitory concentration and minimum bactericidal concentration values for the most common mastitis pathogens are very low. If mastitis pathogens exhibit penicillin resistance, a combination of penicillins and clavulanic acid or isoxazolyl penicillins (cloxacillin and dicloxacillin) resistant to penicillinase are commonly used alternatives (Reybroeck *et al*., 2010; Mitchell *et al*., 1998).

As the elimination of all risks associated with drug residues and animal diseases in the human food supply is not possible, routine monitoring of the food supply is performed in an effort to protect that public health (Mitchell *et al*., 1998). In the European Union (EU), Maximum Residue Limits (MRLs) for veterinary drug residues in different animal tissues and milk were set by Council Regulation (EEC) 2377/90 and in many countries inhibitory substances are routinely monitored in farm milk samples. Residue levels below the MRL are considered safe. The EU Council Regulation set an MRL of 30 μg kg^{-1} for Cloxacillin (CLO) and Dicloxacillin (DCL) in cow milk (Filazi and Yurdakok, 2010). Additionally, EU Council Directive 96/23/EC concerns measures to monitor certain substances and residues in live animals and animal

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products and establishes that EU Member States must each draft a national residue monitoring plan for the groups of substances in Annex I. All national residue monitoring plans must comply with the sampling rules in Annex IV of the Directive. These same rules apply to Turkey, even though it is not an EU member.

In some countries that are concerned about food security very strict legislation of β-lactam antibiotic residues greatly increases the cost of transporting and incinerating cow milk and loss of the milk itself. The dairy industry is therefore interested in residue testing at the farm before milk is collected more of the responsibility for food safety to the farmer (Reybroeck *et al*., 2010). As such, sensitive, confirmatory tests for verifying farm-based rapid screening tests should also be used on the farm (Kaya and Filazi, 2010). Various techniques have been used to detect penicillins in animal-derived food products. High-Performance Liquid Chromatography (HPLC) is the prevalent method for the analysis of food matrices however, other non-chromatographic techniques have been proposed as well and gas chromatography and or gel electrophoresis are other possible used methods (Schenck and Callery, 1998). Numerous methods for analyzing penicillins have been reported, the majority of which can detect ≥1 compounds in a single matrix. Liquid chromatography has become the analytical method of choice for the detection and quantification of isoxazolyl penicillin residues in milk but these methods must become less complex more sensitive and more economical (Samanidou and Nisyriou, 2008).

To the best of the knowledge there are few data regarding the residue of isoxazolyl penicillins in milk as compared to other penicillins but these drugs are still widely used for the prevention and treatment of mastitis. As such, their residue may be detected in milk. For example in Germany β-lactams were detected in inhibitor-positive milk samples. Penicillin G was the predominant antibiotic detected (74.6%) during regulatory control, followed by ceftiofur (11%), ampicillin/amoxicillin (6.3%) and isoxazolyl penicillins (3.2%) (Kress *et al*., 2007).

The present study tested a Novel Method that is simple, sensitive and economical for detecting CLO and DCL in cow milk using reversed-phase High-Performance Liquid Chromatography with UV-Diode Array Detection (HPLC-DAD). The method was then used to analyze 100 raw milk samples.

MATERIALS AND METHODS

Reagents and standards: All reagents and solvents used were analytical or HPLC grade. CLO (97%, C27555) and DCL (98.2%, D 46182) were purchased as sodium salt from Sigma-Aldrich (St. Louis, MO). Acetone, acetonitrile, chloroform, hydrochloric acid and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Disposable syringe filters (Chromafil Xtra PVDF-45/25; pore size: 0.45 μm; membrane diameter: 25 mm) were purchased from Macherey-Nagel (Duren, Germany). Ultra-pure water was generated using a Millipore System (Millipore, Molfheim, France). Stock standard CLO and DCL solutions were prepared in ultra-pure water at a concentration of 1 mg mL^{-1} and then the solutions were stored at 4°C in the dark for up to 2 weeks. Working standard solutions at different concentrations were prepared daily via appropriate dilution in water.

Instrumentation: Chromatography was performed using a Shimadzu LC-20A System and SPD-M20A photodiode array UV-VIS spectrophotometric detector (Shimadzu, Kyoto, Japan). Separation and quantification was performed using an Inertsil ODS-C_{18} HPLC column (4.6×250 mm×3.5 μm) (GL Sciences, Inc., Torrance, CA, USA). The column temperature was maintained at 22°C and the injection volume was 20 μL with auto sampler (SIL-20ACHT prominence auto sampler, Shimadzu). HPLC gradient elution was performed using a mobile phase of 0.05 M phosphate buffer solution at pH 4.7 (A) and acetonitrile (B) at a flow rate of 1.2 mL min^{-1} (A:B = 75:25). Quantitative measurement of the peak areas was performed by selecting the appropriate detection wavelength for each compound, so as to achieve maximum sensitivity, therefore CLO and DCL were quantified at 215 nm.

Sample preparation: A 10 g sample of homogenized milk was placed into a 50 mL polypropylene centrifuge tube and adjusted to pH 4.5-5 using HCl and then acetone/chloroform solution (35/65 v/v) was added to 45 mL. The mixture was vigorously shaken for 2 min and then centrifuged at 2500 rpm for 10 min and allowed to stand until the complete separation of 2 phases. The organic phase was collected into a 100 mL flask. The procedure was repeated using an acetone/chloroform solution. The organic phases were combined and evaporated to dryness in a rotary evaporator. The residue was finally dissolved in 500 μL of water, filtered through disposable syringe filters and then transferred in vials for HPLC analysis.

Sample preparation for method validation: The selectivity of the method was estimated via preparation and analysis of 20 blank and spiked samples in the range of 1-16 mg kg^{-1} for CLO and 0.125-2 mg kg^{-1} for DCL. The probable interference from endogenous substances was

assessed by observing the chromatograms of the blank and spiked milk samples. The sensitivity of the method was assessed via Limit Of Detection (LOD) and Limit of Quantitation (LOQ). LOD was defined as the concentration at which the signal to noise ratio was 3:1 (measured from the injection of standard solutions containing isoxazolyl penicillins). LOQ was defined as the lowest concentration of analytes based on a signal-to-noise ratio of 10:1. A 6 point calibration curve was used to establish instrument response. Next, 15, 30, 45, 60 and 75 μg of DCL and 50, 75, 100, 125 and 250 μg of CLO were spiked to 1 kg of milk, respectively and standard addition samples were treated as described in the sample preparation section. For each point on the calibration plot, 10 injections were performed. The calibration curves were calculated using the following Linear Regression Method:

$$Y = aX+b$$

Where:

Y = Peak area of the analytes
X = Concentration of the analytes (μg kg^{-1})
a = Slope
b = Y-intercepts

To assess accuracy and precision 3 spiking levels were added to 1 g of samples (6 replicates for each level) as listed in Table 1. Fortified samples were treated as described in the sample preparation section. The recovery of isoxazolyl penicillins from milk at each concentration was calculated via comparison of the determined concentration of spiked samples with the theoretical concentrations.

Table 1: Limits Of Detection (LOD) and Limits Of Quantification (LOQ) of the Novel Ethod in bovine milk

Analyte	LOD (μg kg^{-1})	LOD (μg kg^{-1})
Cloxacillin	7.67	23.25
Dicloxacillin	0.75	2.28

Samples: Between December 2010 and November 2011 researchers collected 100 milk samples (25 samples were collected every 3 months) from various milk production facilities in Ankara, Turkey to assess seasonal variations. Researchers were careful to ensure that the milk samples analyzed were collected from different locations. Milk samples were refrigerated at 4°C until analysis which was performed within 24 h of collection.

RESULTS

According to the chromatographic conditions, the chromatogram of a solvent standard solution showed very good separation of isoxazolyl penicillins into a complete run of 60 min. Figure 1-3 show the chromatograms of the standard solution and of the blank milk and spiked milk samples (200 μg kg^{-1} of CLO and 80 μg kg^{-1} of DCL). Their peaks showed good resolution and no interference, indicating that the method described had high specificity and sensitivity. The absence of any interfering peaks for the analyte retention times during chromatographic runs shows that the sample cleanup procedure was suitable.

Retention times for spiked samples were 23 min for CLO and 46 min for DCL. LOD and LOQ for CLO were calculated as 7.67 and 23.25 μg kg^{-1}, respectively versus 0.75 and 2.28 μg kg^{-1}, respectively for DCL (Table 2). These levels were lower than the MRL set by the EU.

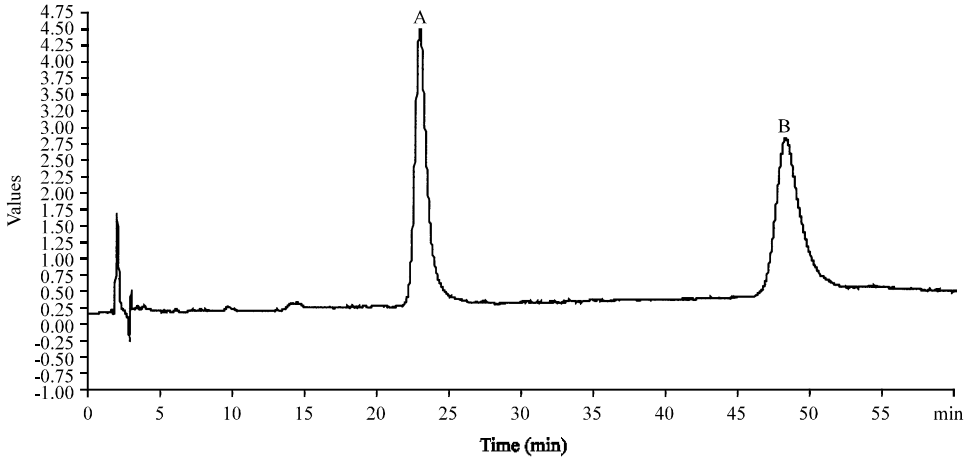

Fig. 1: HPLC-DAD chromatogram of standard solution containing (A) cloxacillin (16 mg L^{-1}) and (B) dicloxacillin (2 mg L^{-1})

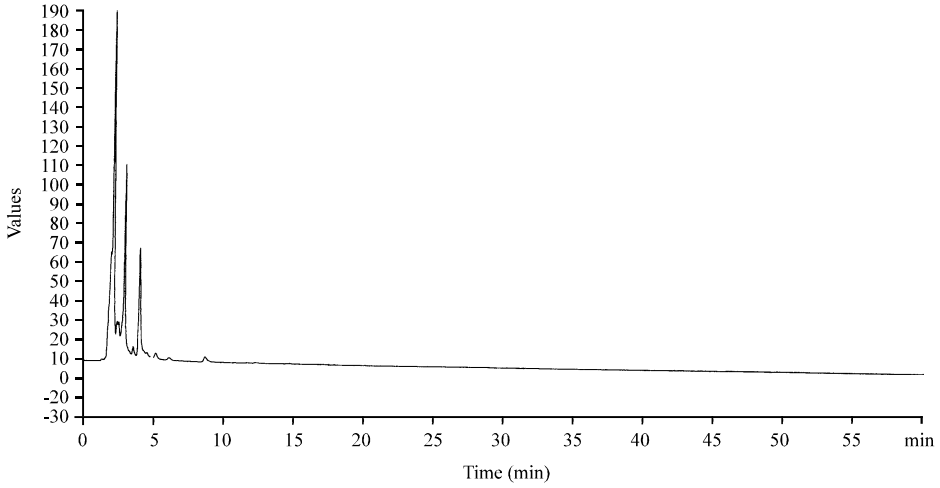

Fig. 2: HPLC-DAD chromatogram of blank milk sample

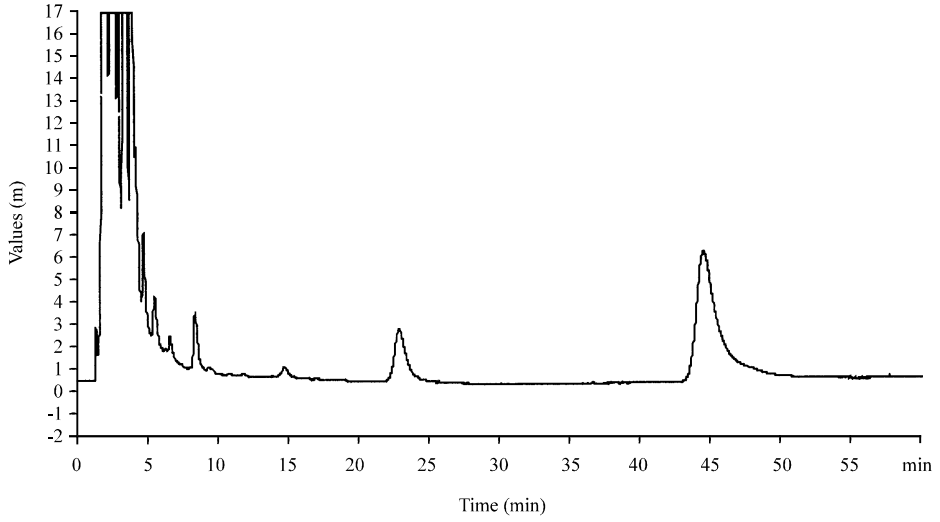

Fig. 3: HPLC-DAD chromatogram of blank milk sample spiked with (A) cloxacillin at 200 μg kg^{-1} and (B) dicloxacillin at 80 μg kg^{-1} (B)

Table 2: Intra and inte day, precision and accuracy of the novel method for the determination of cloxacillin and dicloxacillin in bovine milk samples

		Intra day (n = 6)		Inter day (n = 4 days and 3 determination)	
Analyte	Added (μg kg^{-1})	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Cloxacillin	50	100.98	2.82	102.86	3.32
	75	106.94	8.27	100.72	2.65
	100	101.75	4.69	96.47	4.14
Dicloxacillin	15	99.20	1.95	99.86	3.40
	30	100.36	1.38	97.76	1.30
	45	104.86	1.75	100.80	0.77

Mean recovery values in the present study were 96.47-106.94±0.77% (Relative Standard Deviation (RSD)) for CLO and 97.76-104.86±8.27% (RSD) for DCL (Table 1). Typical values for the regression parameters a, b and R^2 (determination coefficient) and the linear range of CLO and DCL are illustrated in Fig. 4 and 5. The equations were $y = 15.115x - 409.29$ and $R^2 = 0.9984$ for CLO and $y = 147.49x + 332.54$ and $R^2 = 1.0000$ for DCL. The linearity of each calibration curve was very good and similar correlation coefficients were obtained for CLO and DCL. The Novel Method described herein was used to analyze 100 raw cow milk samples. CLO and DCL were

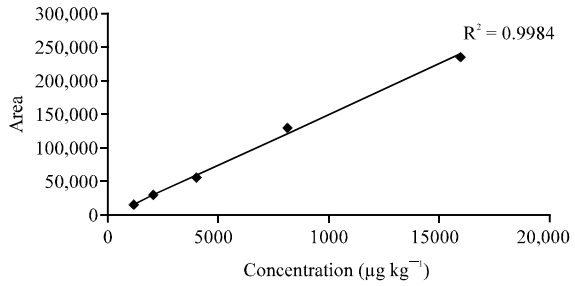

Fig. 4: Calibration curve for cloxacillin

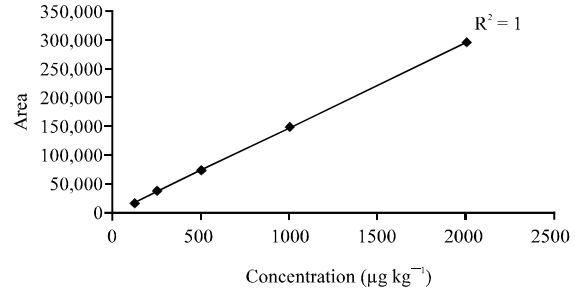

Fig. 5: Calibration curve for dicloxacillin

not detected in any of the samples which might have been because most of the samples were obtained from milk collection tanks and only 100 samples were analyzed.

DISCUSSION

Chromatographic analysis of chemical substances from a complex matrix such as milk is possible only following pretreatment of the sample which eliminates interference and ensures even analyte preconcentration and facilitates its subsequent analytical determination as indirectly the sensitivity and selectivity of the method is increased (Camara *et al*., 2013). Traditional extraction strategies for chemical substances in milk involve precipitation of proteins with organic solvents alone or in combination with strong inorganic or trichloroacetic acids as well as ultrafiltration with molecular mass cut off filters (Samanidou *et al*., 2007). Thus, isolation of isoxazolyl penicillins from such samples frequently involves protein precipitation using organic solvents prior to cleanup.

Liquid-liquid extraction is an all-purpose sample preparation technique recommended for many Standard Analytical Methods. Despite its widespread use, it is considered to be a time-consuming, tedious, multistage procedure. More importantly though use of large volumes of toxic organic solvents influences trace analysis, poses a health hazard to laboratory personnel and results in the production of hazardous laboratory waste thus, increasing the operational cost of waste treatment (Pena-Pereira *et al*., 2009; Psillakis and Kalogerakis, 2003). In the present study only acetone and chloroform were used for extraction and HCl was used for deproteinization followed by filtration through a hydrophilic membrane with a binding capacity for proteins of 20 µg for each 25 mm filter. Proteins are normally removed by addition of HCl and acetone, therefore the described method consumed little solvent.

Sorensen *et al*. (1997) reported that pH 4.6-4.8 was for the most suitable for separation of milk proteins. In addition, pH adjustment is very critical because penicillins are very sensitive to high proton concentrations in the present study milk pH was adjusted to 4.5-5 which was observed to be sufficient for good recovery of isoxazolyl penicillins in organic solvents.

US Food and Drug Administration (FDA) criteria for overall recovery for a determinative method in animal tissues are 80-110% with an RSD ≤10% for replicate analyses at concentrations ≥100 ppb and 60-110% with an RSD ≤20% at concentrations <100 ppb (FDA, 1994). Based on FDA criteria, the present findings are acceptable for detection of isoxazolyl penicillin residues in milk samples are better than those reported by Perez *et al*. (1997), Verdon and Couedor (1998), Takeba *et al*. (1998), Ibach and Petz (1998) and Marchetti *et al*. (2001) are similar to those reported by Sorensen *et al*. (1997), however Sorensen *et al*. (1997) used derivatization for extraction and reported that an analyst needed to prepare 8-10 milk samples for LC analysis on the same day without any automation. The present study did not use a derivatization agent and an analyst prepared 15-20 samples for analysis on the same day, therefore the novel method described herein is more advantageous than any another method that includes derivatization.

Mobile phase choice is another important step in residue analysis of food and foodstuffs. The pH value and compound of the mobile phase directly affect the separation process, therefore mobile phase selection must be based on technique, sample type and column (Alderete *et al*., 2004). Additionally, according to some studies, beta-lactam antibiotics have been separated using a reverse-phase column and acidic mobile phases (Camara *et al*., 2013; Samanidou *et al*., 2007). In the present study, 0.05 M phosphate buffer at pH 4.7:acetonitrile (75:25, v/v) was used as the mobile phase with a C18 column. Others reported that they used pH 5 acetonitrile:0.02 M potassium dihydrogen phosphate and pH 6.5 acetonitrile:methanol:0.01 mol L^{-1} phosphate buffer (37:5:58, v/v/v) (Perez *et al*., 1997). In the present study, all alternatives were evaluated and phosphate buffer and acetonitrile was observed to be the most appropriate option which might have been due the discrepancy pH and molarity of the mobile phase.

CONCLUSION

Researchers described a simple HPLC methodology for the quantification of CLO and DCL in cow milk using acetone, chloroform, HCl and a syringe filter for extraction. The use of a C18 column enables separation of antibiotics in 60 min without derivatization. No column blocking problems were noted after the analysis of antibiotics in milk. This Novel Method is sensitive enough for milk analysis with limits of quantification lower than the MRL (30 μg kg^{-1}) imposed by EU Legislation. Excellent levels of accuracy and precision were obtained for CLO and DCL. More importantly though, the method does not require use of toxic organic chemicals, offering protection to laboratory personnel and the environment and reducing the operational cost of waste treatment.

REFERENCES

Alderete, O., D.F. Gonzalez-Esquivel, L.M.D. Rivero and N.C. Torres, 2004. Liquid chromatographic assay for dicloxacillin in plasma. J. Chromatogr. B, 805: 353-356.

Camara, M., A. Gallego-Pico, R.M. Garcinuno, P. Fernandez-Hernando, J.S. Durand-Alegria and P.J. Sanchez, 2013. An HPLC-DAD method for the simultaneous determination of nine β-lactam antibiotics in ewe milk. Food Chem., 141: 829-834.

Drackova, M., P. Navratilova, L. Hadra, L. Vorlova and L. Hudcova, 2009. Determination residues of penicillin G and cloxacillin in raw cow milk using fourier transform near infrared spectroscopy. Acta Vet. Brno, 78: 685-690.

FDA, 1994. Guideline for approval of a method of analysis for residues. Guideline 3, Part 5, Center for Veterinary Medicine, Washington. DC., USA.

Filazi, A. and B. Yurdakok, 2010. Residue problems in milk after antibiotic treatment and tests used for detection of this problem. Turk. Klinikleri J. Vet. Sci., 1: 34-43.

Filazi, A., U.T. Sireli and O. Cadirci, 2005. Residues of gentamicin in eggs following medication of laying hens. Br. Poult. Sci., 46: 580-583.

Ibach, A. and M. Petz, 1998. HPLC-integrated solid-phase extraction with photochemical post-column derivatization for the determination of oxacillin, cloxacillin and dicloxacillin in raw milk. Zeitschrift Lebensmitteluntersuchung Forschung A, 207: 170-173.

Jones, G.M. and E.H. Seymour, 1988. Cowside antibiotic residue testing. J. Dairy Sci., 71: 1691-1699.

Kaya, S.E. and A. Filazi, 2010. Determination of antibiotic residues in milk samples. Kafkas Univ. Vet. Fak. Derg., 16: S31-S35.

Kress, C., C. Seidler, B. Kerp, E Schneider and E. Usleber, 2007. Experiences with an identification and quantification program for inhibitor-positive milk samples. Anal. Chim. Acta, 586: 275-279.

Kurnaz, E. and A. Filazi, 2011. Determination of metal levels in the muscle tissue and livers of chickens. Fresen. Environ. Bull., 20: 2896-2901.

Marchetti, M., I Schwaiger and E. Schmid, 2001. Determination of benzylpenicillin, oxacillin, cloxacillin and dicloxacillin in cows' milk by ion-pair high-performance liquid chromatography after precolumn derivatization. Fresenius J. Anal. Chem., 371: 64-67.

Mitchell, J.M., M.W. Griffiths, S.A. McEwen, W.B. McNab and A.E. Yee, 1998. Antimicrobial drug residues in milk and meat: Causes, concerns, prevalence, regulations, tests and test performance. J. Food Prot., 61: 742-756.

Movassagh, M.H., 2011. Detection of beta lactam antibiotics residues in iranian ultra high temperature milk by beta star test. Ann. Biol. Res., 2: 95-98.

Pena-Pereira, F., I Lavilla and C. Bendicho, 2009. Miniaturized preconcentration methods based on liquid-liquid extraction and their application in inorganic ultratrace analysis and speciation: A review. Spectrochim. Acta B: Atomic Spectrosc., 64: 1-15.

Perez, B., C. Prats, E. Castells and M. Arboix, 1997. Determination of cloxacillin in milk and blood of dairy cows by high-performance liquid chromatography. J. Chromatogr. B, 698: 155-160.

Psillakis, E. and N. Kalogerakis, 2003. Developments in liquid-phase microextraction. TrAC Trends Anal. Chem., 22: 565-574.

Reybroeck, W., S. Ooghe, H.F. De Brabander and E. Daeseleire, 2010. Validation of the β-s.t.a.r. 1+1 for rapid screening of residues of β-lactam antibiotics in milk. Food Addit. Contam. Part A Chem. Anal. Control Expo Risk Assess., 27: 1084-1095.

Samanidou, V. and S. Nisyriou, 2008. Multi-residue methods for confirmatory determination of antibiotics in milk. J. Separation Sci., 31: 2068-2090.

Samanidou, V.F., S.A. Nisyriou and I.N. Papadoyannis, 2007. Residue analysis of penicillins in food products of animal origin by high performance liquid chromatography. J. Liquid Chromatogr. Rel. Technol., 30: 1145-1204.

Sanli, Y.R.A., H. Yardimci, A. Bilgili, N. Kurtdede, A. Filazi and E. Yarsan, 1999. The effects of some antibacterial drugs on the immune system of poultry. Turk. J. Vet. Anim. Sci., 23: 547-555.

Schenck, F.J. and P.S. Callery, 1998. Chromatographic methods of analysis of antibiotics in milk. J. Chromatogr. A, 812: 99-109.

Sireli, U.T., A. Filazi and O. Cadirci, 2006. Effect of cooking and storage times on gentamicin residues in eggs. Ital. J. Food Sci., 18: 441-446.

Sorensen, L.K., B.M. Rasmussen, J.O. Boison and L. Keng, 1997. Simultaneous determination of six penicillins in cows' raw milk by a multiresidue high-performance liquid chromatographic method. J. Chromatogr. B: Biomed. Sci. Appl., 694: 383-391.

Takeba, K., K. Fujinuma, T. Miyazaki and H. Nakazawa, 1998. Simultaneous determination of β-lactam antibiotics in milk by ion-pair liquid chromatography. J. Chromatogr. A, 812: 205-211.

Verdon, E. and P. Couedor, 1998. Determination of isoxazolylpenicillins residues in milk by ion-pair reversed-phase high-performance liquid chromatography after precolumn derivatization. J. Chromatogr. B: Biomed. Sci. Appl., 705: 71-78.