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Review

Chromatographic methods of analysis of antibiotics in milk

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Abstract

The widespread use of antibiotics in dairy cattle management may result in the presence of antibiotic residues in milk. While rapid screening tests are commonly used to detect the presence of antibiotics in milk, more accurate chromatographic methods are required by government regulatory agencies to identify and confirm the identity and quantity of antibiotic present. This paper reviews recent developments in the chromatographic determination of antibiotic residues in milk.

Keywords: Reviews; Milk; Food analysis; Antibiotics; Aminoglycosides; Lactams; Tetracyclines; Macrolides; Aminocyclitols; Fluoroquinolones

Contents

1. Introduction

Antibiotics are widely used in dairy cattle management for the treatment of disease and as dietary supplements. They may be administered orally as feed additives or directly by injection. The use of antibiotics may result in drug residues being present in the milk, especially if they are not used according to label directions. The presence of antibiotic residues in milk may cause allergic reactions in sensitive individuals, interfere with starter cultures for cheese and other dairy products, or indicate that the milk may have been obtained from an animal with a serious infection [1]. There are concerns that the widespread usage of antibiotics may be responsible for the promotion of resistant strains of bacteria [2,3]. Recently the US Food and Drug Administration (FDA) placed severe restrictions on the use of two new classes of antibacterial drugs, the fluoro-

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quinolones and the glycopeptides [4], because of concerns about drug resistant bacteria.

Immunological or microbial inhibition screening tests are commonly used to determine if antibiotic residues are present in milk. Some drawbacks of screening tests are: they cannot identify which antibiotics are present in the milk, the presence of high somatic cell counts may result in false positives [5,6], and they may detect antibiotic residues at levels far below the officially mandated safe levels, resulting in the unnecessary destruction of the milk. Therefore, sensitive and specific analytical techniques for the identification and quantitation of antibiotic residues in milk are required. Liquid chromatography (LC) and gas chromatography (GC) are the techniques most commonly used for this purpose.

A clean-up is required to remove the large number of matrix coextractants prior to the chromatographic determination of antibiotics in milk. Since the antibiotics are typically polar compounds, extraction into nonpolar organic solvents may not be feasible. Most methods for the determination of antibiotics in milk entail precipitation of the milk proteins and extraction into polar organic solvents. The various extraction/deproteination methods used for the extraction of antibiotics from milk and tissues have been reviewed by Moats [7].

2. Aminoglycosides

Aminoglycoside antibiotics have an aminoglycoside moiety linked via a glycosidic bond to an aminocyclitol (an amino substituted hydroxycyclohexane) ring moiety. The aminoglycoside antibiotics most commonly used in food producing animals are gentamicin, neomycin, dihydrostreptomycin and streptomycin. There is concern about residues of aminoglycoside antibiotics in food since they have been found to cause damage to the kidneys and to cranial nerves, resulting in hearing loss [8,9]. The FDA has set tolerances of 125 ng/ml for dihydrostreptomycin and 150 ng/ml for neomycin in milk [10] and a level of concern of 30 ng/ml for gentamicin in milk [11].

The aminoglycosides are extremely polar, hydrophilic, compounds that do not contain analytically useful UV absorbing chromophores. These very polar compounds have little tendency to be retained on nonpolar reversed-phase LC columns. Two approaches have been used to facilitate the LC of these polar compounds. Pre-column derivatization with *ortho*-phthalaldehyde (OPA) to form a nonpolar, fluorescent compound prior to determination by reversed-phase LC has been used for neomycin [12] and gentamicin [13]. Ion-pair reversed-phase LC separation followed by in-line post-column derivatization forming a fluorophore has been used for the determination of neomycin [14], gentamicin [15], streptomycin and dihydrostreptomycin [16].

Salisbury [17] and Shaikh and Moats [18] reviewed chromatographic methods for the determination of aminoglycosides in food products. Since these reports provide a detailed review of methods for these compounds in milk, additional developments since then will be reviewed here.

Kwok et al. [19] reported a method using electrospray LC–MS–MS for the confirmation of residues of the aminoglycosides streptomycin, dihydrostreptomycin, neomycin, gentamicin and the aminocyclitols apramycin and spectinomycin in milk and in kidney tissue. The method entails precipitation of the milk proteins with hydrochloric acid, followed by a heptafluorobutyric acid ion-pairing reversed-phase solid-phase extraction (SPE) clean-up. Chromatographic determination was on a Zorbax RxC_8 column with an ion-pair gradient acetonitrile–water mobile phase. Recoveries (0.05–10 μg/ml) ranged from 60–80%.

Hormazábal and Yndestad [20] extracted dihydrostreptomycin from milk treated with trichloroacetic acid. An ion-pairing clean-up of the supernatant was on a C_{18} SPE column. The LC conditions employed were as follows: Supelcosil ABZ; acetonitrile–methanol–water ion-pair mobile phase adjusted to pH 3.2 and containing ninhydrin. Post-column reaction with NaOH resulted in a fluorescent derivative. The limit of quantitation was 25 ng/ml.

Kijak et al. [21] extracted gentamicin from milk treated with trichloroacetic acid. Clean-up of the supernatant was on a C_{18} SPE column. Ion-pair LC on a Spherisorb ODS-2 column with OPA post-column derivatization and fluorescence detection was used. The average recovery of fortified gentamicin residues (15–60 ng/ml) was 80%.

Märtlbauer et al. [22] demonstrated the use of immunoaffinity chromatography for the clean-up and concentration of streptomycin and dihydrostreptomycin residues from milk. Two types of Sepharose were coupled to monoclonal antibodies for streptomycin. Milk samples were defatted by centrifugation, diluted with phosphate-buffered saline (PBS) and eluted through the column. The column was washed with PBS and the antibiotics were eluted with glycine–HCl buffer. The columns bound 80% and 89% of 100 ng/ml streptomycin and dihydrostreptomycin residues in milk, respectively.

Fennell et al. [23] extracted gentamicin directly from milk using a hydrophobic ion-exchange (C_{18}COOH copolymer) SPE column. Gentamicin was eluted from the column with buffer and derivatized with OPA in the presence of mercaptoacetic acid (MAA). LC analysis was on a Zorbax SB-C_{18} using an ion-pair mobile phase and UV detection at 330 nm. They found that the OPA–MAA derivative was much more stable than the fluorescent OPA–mercaptoethanol derivative previously reported [13]. The limit of detection was 400 ng/ml.

Clark et al. [24] used GC for the determination of gentamicin and neomycin in milk. The sample preparation included an acid extraction and clean-up on a weak cation-exchange column. A two-step derivatization with *N*-trimethylsilylimidazole and *N*-heptafluorobutyrylimidazole [25] was followed by GC determination with electron-capture detection.

Schenck [26] extracted streptomycin and dihydrostreptomycin from milk treated with trichloroacetic acid. An ion-pairing clean-up of the supernatant was on a C_8 SPE column. The LC conditions employed were as follows: Spherisorb ODS-2 column; acetonitrile–water mobile phase containing octanesulfonate adjusted to pH 3.0 with phosphate buffer containing 1,2-naphthoquinone-4-sulfonate (NQS) or ninhydrin. Post-column reaction with NaOH resulted in a fluorescent derivative. Derivatization with NQS resulted in fewer interfering matrix peaks than derivatization with ninhydrin.

3. β-Lactams

The β-lactam antibiotics, which include the penicillins and cephalosporins, are widely used in veterinary medicine. There are concerns about β-lactam residues in food because of the potential for allergic reactions in certain individuals [18,27,28].

Regulatory requirements for β-lactam residues in milk in the USA are fairly stringent. Testing of all milk for β-lactam residues has become mandatory. Tolerances have been set at 5 ng/ml for penicillin G, 10 ng/ml for amoxicillin, ampicillin and cloxacillin and 20 ng/ml for cepaphirin [29]. Rapid screening tests are used to determine whether to accept or reject tanker loads of milk. As discussed in Section 1, there is a need for sensitive confirmatory tests that can be used to assess accuracy of the screening tests.

LC is the most commonly used methodology for the determination of β-lactams in milk, although GC [30] and gel electrophoresis [31] methods have been reported. The penicillins do not have any strong UV chromophores. They will absorb at 210 nm, but an extensive sample clean-up is required prior to the LC analysis to remove interfering milk matrix components which display a strong absorbance at this wavelength. The use of two-dimensional LC, which entails using one LC system for a sample extract clean-up and a second LC system for determination, has been reported for a number of β-lactams [32–36]. Alternatively, derivatizations to produce fluorophores [37,38] or UV chromophores that absorb at longer wavelengths [39,40] have been reported for certain β-lactams. The cephalosporins cepaphirin and ceftiofur, which display a strong UV absorbance at 290 nm, require a less extensive clean-up [41,42].

Boison [43] and Shaikh and Moats [18] reviewed chromatographic methods for the determination of β-lactam antibiotic residues in food products. Since these reports provide a detailed review of methods for β-lactams in milk, additional developments since then will be reviewed here.

Sørensen et al. [40] presented an LC method using pre-column derivatization, for the simultaneous determination of amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin and penicillin G in milk. This method is a modification of the method for penicillin G in milk developed by Boison et al. [39]. Milk was defatted by centrifugation and deproteinated with acid and sodium tungstate. Clean-up of the supernatant was on a C_{18} SPE column. This was followed by reaction of the amphoteric β-lactams amoxicillin and ampicillin with benzoic anhydride, a liquid–liquid

partition clean-up and derivatization with 1,2,4-triazole mercuric chloride producing UV chromophores that absorb at longer wavelengths. Chromatographic determination was on a Novapak C_{18}. The limits of detection for the six β-lactams ranged from 1.3–2.7 ng/ml.

Carson et al. [44] evaluated four published methods for the determination of β-lactams in milk in order to determine their suitability for regulatory purposes. The methods evaluated were a two-dimensional LC assay [36], a GC method employing a diazomethane derivatization [30], an LC method using a pre-column derivatization with mercury chloride and UV detection at 325 nm [39] and an LC receptogram assay [45]. Data were presented on the accuracy and precision of each procedure. A useful comparison of the advantages and limitations of each procedure was included.

Ang and Luo [37] reported a rapid method for the determination of ampicillin in milk. Milk was deproteinated with acetonitrile and trichloroacetic acid (TCA). After centrifugation, the supernatant was heated with formaldehyde and TCA forming a fluorescent ampicillin derivative. Determination was by LC using a Prodigy ODS-3 column with an isocratic acetonitrile–buffer mobile phase and fluorescence detection. The limits of detection for ampicillin in various types of milk ranged from 0.31–0.51 ng/ml. Amoxicillin could not be determined by this method because of matrix interferences. Luo et al. [38] modified the method so that both ampicillin and amoxicillin could be determined. Milk was deproteinated with sodium tungstate and sulfuric acid. After centrifugation, the drug residues in the supernatant were concentrated using a C_{18} SPE column. Ampicillin and amoxicillin were reacted with salicylaldehyde to form fluorescent derivatives. Determination was by LC using a Prodigy ODS-3 column with an isocratic acetonitrile–buffer mobile phase and fluorescence detection. The limits of detection were 1.1 and 1.0 ng/ml, for amoxicillin and ampicillin, respectively.

Ang et al. [46] compared the LC methods of Ang and Luo [37] and Luo et al. [38] with a microbial inhibition (MI) method (*Bacillus stearothermophilus*) for the determination of incurred amoxicillin and ampicillin residues in milk. No significant differences were found between the LC and MI assay methods for residue levels within the reliable range of the MI method. The LC method was found to be more sensitive than the MI method for residues at <10 ng/ml.

Hornish et al. [47] reported a method for the confirmation of ceftiofur residues in milk. The method utilized a two-dimensional analysis that included electrospray ionization and collision induced dissociation LC–MS–MS detection. The method was shown to have a limit of confirmation for parent ceftiofur residue of 50 ng/ml.

McNeilly et al. [42] developed a procedure for the determination of ceftiofur in milk. Milk proteins were precipitated by addition of ammonium acetate. Clean-up of the supernatant was on a C_{18} SPE column. Determination was on a Supelcosil LC-18-DB LC column using an isocratic acetonitrile–buffer mobile phase and 293 nm UV detection. The method was validated at levels of 25–100 ng/ml for ceftiofur. The limit of quantitation was 7 ng/ml.

Schermerhorn et al. [41] reported an LC method for the determination of cepaphirin and ceftiofur in milk. Milk was deproteinated with acetonitrile. Clean-up of the supernatant was on a C_{18} SPE column. Determination was by ion-pairing LC with UV detection at 290 nm. The method was validated at levels of 10–40 ng/ml for cepaphirin and 25–100 ng/ml for ceftiofur.

Moats and Harik-Khan [36] reported a multiresidue LC method for the determination of amoxicillin, ampicillin, ceftiofur, cepaphirin, cloxacillin, penicillin G and penicillin V in milk. Milk was deproteinated with tetraethyl ammonium chloride and acetonitrile. The filtrate was concentrated and loaded onto a Supelcosil LC-18 LC column. The β-lactams were eluted from the LC column with an acetonitrile–phosphate buffer gradient. Seven fractions were collected, each corresponding to the retention time of a standard. Each fraction was analyzed by LC using various isocratic mobile phases with Supelcosil LC-18 or LC-18-DB or polymeric PLRP-S LC columns. UV detection was at 210 or 290 nm. The limits of quantitation ranged from 2–5 ng/ml.

Harik-Khan and Moats [48,49] interfaced the above LC procedure with rapid screening kits. Milk samples fortified with amoxicillin, ampicillin, ceftiofur, cepaphirin, cloxacillin and penicillin G were

extracted and deproteinated and subjected to a gradient LC clean-up and fractionation. Each fraction was divided into equal parts, with one part being analyzed by four different test kits and the other being analyzed by isocratic LC. The four test kits evaluated were all found to be useful for screening the fractions for the presence of β-lactams. The authors proposed a protocol in which fractions would be collected from a gradient LC and evaluated by test kit. Those fractions testing positive for the presence of β-lactams would be subjected to further LC analysis.

Cutting et al. [31] used gel electrophoresis coupled with bioautography for the detection and identification of five β-lactam residues in milk. The method used a 2% agarose gel with an overlay of indicator agar seeded with *Bacillus stearothermophilus*. The method detected and separated β-lactam residues in milk at the following levels of detection: penicillin G (5 ng/ml), cepaphirin (20 ng/ml), ceftiofur (50 ng/ml), ampicillin (20 ng/ml), amoxicillin (30 ng/ml) and cloxacillin (30 ng/ml).

Tarbin et al. [50] developed a procedure for penicillin G in milk. Milk was defatted by centrifugation and deproteinated using sulphuric acid/sodium tungstate. Clean-up was by C_{18} SPE followed by fractionation on a Kromasil 5 C_8 LC column using a neutral pH acetonitrile–buffer mobile phase. Determination was on a Kromasil 5 C_8 LC column using a phosphoric acid–acetonitrile gradient, with post-column derivatization with imidazole–mercury chloride reagent followed by UV absorbance detection at 320 nm.

Kirchmann et al. [51] used electrochemical detection for the determination of eight penicillins in milk. Milk was deproteinated with acetonitrile and filtered. The filtrate was diluted with acetate buffer, loaded onto a μBondapak C_{18} Radial Pak LC column and concentrated on the column. The β-lactams were eluted from the column with an acetonitrile gradient and detected using a pulsed amperometric detector. The limit of detection for penicillin G in milk was ca. 70 ng/ml.

Tyczkowska [52] used LC with UV and electrospray mass spectrometric detection for the determination of amoxicillin, ampicillin, ceftiofur, cepaphirin, cloxacillin and penicillin G in milk. Milk was diluted with 50% acetonitrile and passed through a 10 000 molecular mass cut-off filter. The β-lactams were separated from coextracted milk components by ion pair chromatography with an acetonitrile–buffer mobile phase on an Ultremex phenyl LC column. Quantitation was by UV with a detection limit of 100 ng/ml. Confirmation of the β-lactam antibiotics was by LC–electrospray MS.

Straub et al. [53] used LC combined with electrospray quadrupole MS for the detection of six β-lactams in milk. Milk was diluted with 50% acetonitrile and passed through a 10 000 molecular mass cut-off filter. The ultrafiltrate was injected into an LC system using a perfusive-particle column packed with derivatized porous polystyrene–divinylbenzene. Both conventional electrospray and ultrasonic nebulization were evaluated. The LC separation of the six β-lactams in the milk ultrafiltrate was accomplished in 5 min, with no interference from the milk matrix components. The limits of detection were 3–5 ng/ml for ceftiofur, cepaphirin, cloxacillin and penicillin G and 20–30 ng/ml for ampicillin and amoxicillin.

Zomer et al. [45] combined LC with the Charm II multiresidue radioimmunoassay to identify and quantify β-lactam and tetracycline residues in milk or tissue. Milk was deproteinated with McIlvaine–EDTA (sodium citrate/phosphate in 0.1 *M* EDTA) buffer. For β-lactam analysis, the extract was subjected to clean-up on a C_8 SPE column and injected onto a LiChrosorb RP-8 LC column with an isocratic methanol–phosphate buffer mobile phase. Fractions were collected according to the retention times of standards and analyzed by the Charm II.

4. Chloramphenicol, florfenicol and thiamphenicol

Chloramphenicol, florfenicol and thiamphenicol are broad spectrum antibiotics that are suitable for the treatment of a variety of infectious organisms. Chloramphenicol, which has been found to produce aplastic anemia in a small percentage of humans exposed to the drug, is not approved for use in food producing animals in the USA [54,55]. Florfenicol has been approved for the treatment of bovine respiratory disease in the USA; the FDA has set a level of concern of 10 ng/ml for florfenicol in milk [56].

Chloramphenicol, florfenicol and thiamphenicol have strong UV absorption and can be determined directly by LC. Chloramphenicol has a maximum absorption at 278 nm, while florfenicol and thiamphenicol have a stronger absorption at 225–230 nm [57]. Unlike many of the more polar antibiotics, these three compounds can be extracted from biological matrices with an organic solvent. A single shake out with ethyl acetate is sufficient for the quantitative extraction of chloramphenicol [58] and florfenicol [59] from milk.

A comprehensive review of methods for the determination of chloramphenicol, florfenicol and thiamphenicol residues in food, which included eight GC methods and six LC methods for chloramphenicol in milk, has been reported by Nagata [57]. Since this report provides a detailed review of methods, only additional developments since then will be reviewed here.

Pfenning et al. [60] developed a GC method for the determination of chloramphenicol, florfenicol and thiamphenicol in raw milk. The milk is extracted with acetonitrile. This is followed by a C_{18} SPE clean-up, derivatization with Sylon BFT and GC determination with electron-capture detection. Average recoveries ranged from 92–104% at levels ranging from 5–80 ng/ml.

LC has been used for the determination of florfenicol in milk [59]. Florfenicol was extracted from milk with ethyl acetate. Clean-up was with C_{18} and Florisil SPE columns. Determination was on a Supelcosil LC-18DB column, using an acetonitrile–water mobile phase, with 225 nm UV detection.

Kijak [61] presented a GC–MS method for the confirmation of chloramphenicol residues in bovine milk. *Meta*-nitrochloramphenicol was added as a surrogate standard. Chloramphenicol residues were extracted from the milk by mixing the milk with ethyl acetate using a diatomaceous earth SPE column clean-up. This was followed by a C_{18} SPE clean-up and derivatization with Sylon HTP. Chloramphenicol was determined using GC with a 30 m methylsilicone column and negative ion chemical ionization mass spectrometric detection. The method was validated at levels of 0.5–2.0 ng/ml.

Bayo et al. [62] used diphasic dialysis to extract chloramphenicol from milk. Ethyl acetate was added to a piece of hydrated dialysis tubing. The tubing was placed into a flask of milk and mixed with an orbital shaker for 5 h. The ethyl acetate in the dialysis tubing was dried with sodium sulfate and evaporated. Determination was by LC using a Novapak C_{18} column and UV detection. The limit of quantitation was 5 ng/ml.

Keeukens et al. [63] presented preliminary studies on the determination of chloramphenicol in milk by modifying a previously reported LC method for chloramphenicol in meat [64]. Extraction and clean-up of the milk was with an Extrelut diatomaceous earth SPE column and water–toluene partitioning. The limit of detection was 0.5 ng/ml.

Clark et al. [65] presented a GC method for the determination of trace levels of chloramphenicol in milk. Milk was partially defatted by centrifugation. This was followed by a C_{18} SPE clean-up and derivatization with Sylon-HTP. GC was with a 2 m×4 mm column packed with 3% dimethylsilicone (OV-101) on gas chrom Q and electron-capture detection. The method was validated at levels of 0.50–1.5 ng/ml chloramphenicol in milk.

5. Tetracyclines

Tetracycline antibiotics are widely used for the treatment of bovine mastitis and are added at subtherapeutic levels to cattle feeds for prophylaxis. The FDA has set levels of concern for residues of chlortetracycline, oxytetracycline and tetracycline in milk of 30, 30 and 80 ng/ml, respectively [66].

LC is the chromatographic approach most commonly used for the determination of tetracyclines in milk. The tetracyclines are amphoteric, forming crystalline salts with acids and bases. The UV spectra show a strong absorbance at 360 nm in acidic solution. The tetracyclines have a tendency to bind irreversibly to the silanol groups in silica-based LC stationary phases resulting in peak tailing. This problem has been overcome by adding oxalic acid to the mobile phase [67–69] and using polystyrene–divinylbenzene LC columns such as the Polymer Labs. PLRP-S [70–73]. Similarly, binding to silanols has resulted in low recoveries when C_{18} SPE columns are used for clean-up of milk extracts [74]. To avoid this problem, C_{18} SPE columns have been silylated [75] or pre-treated with EDTA [69]. Alter-

natively, metal chelate affinity columns [69,71,72,74], ultrafiltration [67] and extraction into organic solvent in the presence of ion-pairing agents [76] have been used for the clean-up of milk extracts containing tetracyclines.

Oka and Patterson [77], Shaikh and Moats [18] and Barker and Walker [78] reviewed chromatographic methods for the determination of tetracyclines in food products. Since these reports provide a detailed review of methods for tetracyclines in milk, additional developments since then will be reviewed here.

Anderson and co-workers [79,80] used both LC and radioimmunoassay methods to study the occurrence of oxytetracycline residues in milk resulting from different routes of administration. The milk was analyzed by both the Charm II radioimmunoassay test and by the LC method of White et al. [71]. Intravenous and intramuscular administration resulted in potentially violative levels of the drug in milk, while oral dosing even at 5× the label dose did not. The levels of oxytetracycline found by the Charm II tended to be higher than those obtained using the LC method.

Carson and Breslyn [72] reported on an interlaboratory collaborative study of the LC method of Carson [70]. Fortified control and coded blind samples containing chlortetracycline, demeclocycline, doxycycline, methacycline, minocycline, oxytetracycline and tetracycline were analyzed. Average recoveries reported by eight participating laboratories ranged between 60 and 110%. This method was adopted by AOAC International as an official method.

Carson et al. [81] reported on a 1991 study that involved an evaluation of the Charm II multiresidue radioimmunoassay method for the detection of oxytetracycline and chlortetracycline in milk. Milk was first analyzed by the metal chelate affinity column clean-up LC method of Carson [70]. The milk was sent to four laboratories for testing using the Charm II. They concluded that the Charm II marketed in 1991 detected tetracyclines in milk far below the FDA concern level of 30 ppb.

Carson [73] presented a method for the confirmation of chlortetracycline, demeclocycline, doxycycline, minocycline, oxytetracycline and tetracycline residues in milk by LC–particle beam MS. The metal chelate affinity column clean-up of Carson [70] was scaled up to accommodate the extraction of a larger volume of milk. The extracts were desalted and concentrated using a polymeric (ENVI-Chrom P) SPE column. The residues were chromatographed on a polymeric PLRP-S LC column using a methanol–5 m*M* oxalic acid mobile phase. The six tetracycline residues were identified in milk at a level of 30 ng/ml.

Zomer et al. [45] combined LC with the Charm II multiresidue radioimmunoassay to identify and quantify tetracycline and β-lactam residues in milk or tissue. Milk was deproteinated with McIlvaine–EDTA (sodium citrate–phosphate in 0.1 *M* EDTA) buffer. For the tetracycline analysis, the extract was subjected to clean-up on a C_{18} SPE column and injected onto a polymeric PLRP-S LC column with an ammonium oxalate–acetonitrile–methanol mobile phase. Fractions were collected according to the retention times of standards and analyzed by the Charm II.

Chen and Gu [75] used capillary electrophoresis for the simultaneous determination of chlortetracycline, doxycycline, oxytetracycline and tetracycline in bovine milk, serum and urine. The tetracyclines were extracted using metal-chelating affinity columns. Salts were removed from the column eluates by clean-up on a C_{18} SPE columns that had been pre-treated with dimethyldichlorosilane. Detection was by diode array detector at 370 nm. The limits of quantitation for the four tetracyclines studied in milk were less than 10 ng/ml.

Moats and Harik-Khan [82] modified a previously reported gradient LC method (White) to provide a more rapid isocratic LC determination of chlortetracycline, oxytetracycline and tetracycline in milk. Samples were extracted and deproteinated with HCl–acetonitrile and the filtrates were concentrated. Determination was on a polymeric PLRP-S LC column with an ion pairing mobile phase with UV detection at 380 nm. Limits of detection in milk were 2–4 ng/ml.

Podhorniak et al. [83] studied the stability of tetracyclines in milk under laboratory storage conditions. Raw milk samples fortified with 50 ng/ml chlortetracycline, demeclocycline, methacycline, minocycline, oxytetracycline and tetracycline were incubated at 4 or 25°C and analyzed using the LC

method of Carson [70]. No loss of tetracycline was observed after 48 h storage at 4°C or 24 h at 25°C. Slight losses were often noted after 72 h storage at 4°C and after 48 h at 25°C.

6. Macrolides

The macrolide antibiotics are most effective against gram-positive organisms and are used to treat a wide range of infections. There are relatively few reports of chromatographic methods for the determination of macrolides in milk. Horie [84] and Shaikh and Moats [18] reviewed chromatographic methods for the determination of macrolides in food products. Only additional developments since then will be reviewed here.

Pirlimycin–HCl has been approved by the FDA for the intramammary infusion treatment of clinical mastitis in lactating dairy cattle; a tolerance of 400 ng/ml pirlimycin in milk has been established [85]. Hornish et al. [86] reported an LC–MS method for both the assay and identification of pirlimycin residues in milk and liver. Milk was deproteinated with acidified acetonitrile. The supernatant was partitioned with organic solvent forcing pirlimycin into the aqueous phase. An additional clean-up of the extract was accomplished by extracting pirlimycin at basic pH into methylene chloride and a C_{18} SPE clean-up. Extracts were injected onto a CPS-Hypersil-2 endcapped cyano LC column. Detection of pirlimycin was by thermospray MS with selected ion monitoring. Heller [87] conducted an interlaboratory study of this method. Average recoveries obtained by three laboratories (200–800 ng/ml) ranged from 83–113%. This was the first method accepted by the FDA that used LC–MS for both determination and confirmation of a veterinary drug residue at regulatory tolerances.

Heller [88] reported an LC–UV method for the determination of pirlimycin in milk. Milk was subjected to the deproteination and liquid–liquid partition clean-up of Hornish et al. [86] described above. The pirlimycin was reacted with 9-fluorenylmethyl chloroformate (Fmoc). Determination was by LC on a Hypersil ODS column, using an acetonitrile–methanol–acetic acid mobile phase and 264 nm detection. Recoveries of fortified pirlimycin residues from milk (200–800 ng/ml) ranged from 87–91%.

Ngoh [89] reported an LC method for the determination of tilmicosin in milk. Samples were defatted by centrifugation. The skim milk was cleaned up on a C_{18} SPE column. Tilmicosin was determined by LC on an Apex phenyl column, using a ternary gradient and 280 nm UV detection. Recoveries of fortified tilmicosin residues in milk (50–200 ng/ml) ranged from 97–101%.

7. Aminocyclitols

The two aminocyclitol antibiotics commonly used in food producing animals are apramycin and spectinomycin. They are hydrophilic and do not contain usable UV chromophores. Neither of these two compounds is approved for use in lactating dairy cattle.

Kwok et al. [19] reported a method using electrospray LC–MS–MS for the confirmation of residues of apramycin and spectinomycin along with four aminoglycosides in milk and in kidney tissue. The method entails precipitation of the milk proteins with hydrochloric acid, followed by a heptafluorobutyric acid ion-pairing reversed-phase SPE clean-up. Chromatographic determination was on a Zorbax RxC_8 column with an ion-pair gradient acetonitrile–water mobile phase.

Schermerhorn et al. [90] used electrochemical detection for the LC determination of spectinomycin in milk. Milk was defatted and deproteinated and the supernatant was washed sequentially with dichloromethane, hexane and ethyl acetate. LC determination was on an Ultracarb ODS-2 column with an ion-pair acetonitrile–buffer mobile phase. Mean recoveries (100–400 ng/ml) ranged from 76–80%.

8. Fluorquinolones

Fluoroquinolones are a recently developed class of antibacterial drugs used for fighting infections in humans and animals. While these compounds are technically not antibiotics, since they were not derived from living organisms, they will be included

in this article because of concerns about residues of these antibacterial drugs in food.

There is concern among members of the human health community that the use of fluoroquinolones in food animals will increase the level of drug resistant pathogens than are infective to humans. Two fluoroquinolones, enrofloxacin and sarafloxacin, have been approved for use in chickens and turkeys in the USA. Under the law, veterinarians can prescribe extralabel uses of approved animal and human drugs in animals. The FDA has specifically prohibited the extralabel use of the fluoroquinolones in food producing animals [4].

Roybal et al. [91] have reported an LC method for the determination of residues of ciprofloxacin, difloxacin, enrofloxacin and sarafloxacin in milk. The method entails extraction of the milk with acidified ethanol, clean-up on a cation-exchange SPE column and LC analysis with fluorescence detection. An isocratic acetonitrile–2% acetic acid mobile phase with an Inertsil phenyl column was used. Average recoveries from fortified raw milk (5–100 ng/ml) were 70–90%.

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