Research Progress on Detection Techniques for Veterinary Drug Residues in Milk (Postprint)

Authors: Du Bingyao, Wang Jiaqi, Wenfang, Zheng Nan, Zhang Yangdong, Li Guodong, Guo Xiaodong

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Abstract

In recent years, frequent incidents of milk quality and safety issues have posed a serious threat to public health. Therefore, milk quality and safety has become a focus of international concern. The presence of veterinary drug residues in milk can cause adverse effects on consumers. This article provides a relatively comprehensive review of detection technologies for veterinary drug residues in milk, examining their principles, advantages, and disadvantages, and provides an outlook on the future development directions of these technologies.

Full Text

Advances in Detection Techniques of Veterinary Drug Residues in Milk

DU Bingyao[1,2,3], , WANG Jiaqi[1,3], , WEN Fang[1,3], *, ZHENG Nan[1,3], , ZHANG Yangdong[1,3], , LI Guodong[1,3], , GUO Xiaodong[1,3],

(1. Laboratory of Quality & Safety Risk Assessment for Dairy Products (Beijing), Ministry of Agriculture, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, China; 2. College of Animal Science, Xinjiang Agricultural University, Urumqi 830052, China; 3. Milk and Dairy Product Inspection Center (Beijing), Ministry of Agriculture, Beijing 100193, China; 4. State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, China)

Abstract: In recent years, frequent milk quality and safety incidents have posed serious threats to public health, making milk quality and safety a focus of international concern. Veterinary drug residues in milk can adversely affect consumers. This paper provides a comprehensive review of detection techniques for veterinary drug residues in milk, covering their principles, advantages, and disadvantages, and offers perspectives on future development trends in this field.

Keywords: veterinary drug residues; milk; microbiological detection; instrumental analysis; immunoassays; aptasensors

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In animal production, veterinary drugs are commonly used for disease treatment and performance enhancement. However, increased usage leads to drug residues in animal-derived foods such as milk, muscle, and liver, compromising food quality and safety while causing adverse health effects in consumers, including allergic reactions, antimicrobial resistance, and toxic reactions [1-2]. Due to the widespread application of veterinary drugs, residues are prevalent in animal-derived foods, prompting countries worldwide to establish maximum residue limits (MRLs) to protect consumer health.

Given the hazards posed by veterinary drug residues, accurate detection is essential [3-7]. With technological advances, various techniques have been widely applied for residue detection. However, several challenges complicate the detection and quantification of veterinary drug residues in animal-derived foods: simultaneous determination of drugs with different properties is complex; residue concentrations may be very low; and the natural matrix is highly complex. Therefore, appropriate analytical methods are required for detecting veterinary drug residues in animal-derived foods.

Milk is an important source of protein in human diets, characterized by high protein and fat content, but its complex matrix can bind analytes and interfere with extract leachates [8]. From an analytical perspective, even small amounts of fat can degrade analytical columns and contaminate detection systems. Consequently, effective separation of proteins and fats during sample pretreatment is crucial for accurate quantitative detection of trace-level veterinary drug residues in milk. The main veterinary drug residues in milk fall into four categories: sulfonamides, tetracyclines, macrolides, and aminoglycosides. In response to this issue, both China and the European Union have established MRLs for veterinary drugs in milk, as shown in . Government agencies and dairy enterprises attach great importance to accurate detection of veterinary drug residues in milk, necessitating the establishment of rapid, simple, economical, and efficient methods to ensure consumer safety.

China has established several national standards for detecting veterinary drug residues in milk: 1) HPLC method for quinolone residues [11]; 2) LC-MS/MS method for penicillin antibiotics in animal-derived foods [12]; 3) LC-MS/MS method for sulfonamides in animal-derived foods [13]; 4) LC-MS/MS and HPLC methods for tetracycline residues in animal-derived foods [14]; and 5) LC-MS/MS method for nitrofuran residues in milk and milk powder [15]. Although numerous, these standard methods require professional operators and expensive instrumentation. In recent years, several high-tech detection methods have been developed and widely applied for veterinary drug residue detection in milk. This paper reviews several relevant techniques for veterinary drug residue detection.

1 Microbiological Detection Method

Microbiological detection methods are based on the inhibitory effects of veterinary drugs on microorganisms, utilizing the suppression of microbial physiological functions and metabolism to determine residue levels, with results measured by inhibition zone diameter. These methods are simple, rapid, low-cost, and require neither chemical processes nor complex instrumentation, enabling broad-spectrum antimicrobial drug detection. However, they are labor-intensive, provide only qualitative measurements, and suffer from limited sensitivity and specificity that require further improvement [16-17].

Appicciafuoco et al. [18] reported a bio-optical method based on growth inhibition assays using *E. coli* for detecting quinolone residues in milk. Blank and spiked milk samples were inoculated with 10,000 CFU/mL *E. coli*; quinolone presence inhibited bacterial growth. The method achieved detection limits of 100 g/L for ciprofloxacin and enrofloxacin but could not detect low-concentration residues, indicating a need for improved sensitivity. Stead et al. [19] utilized the Delvotest method to detect ten veterinary drug residues in milk, including penicillin, ampicillin, amoxicillin, cephalosporins, ceftiofur, cloxacillin, sulfadiazine, oxytetracycline, neomycin, and erythromycin, with results meeting EU MRL requirements. The Delvotest scanning system effectively distinguished matrix effects, and the method was also applicable for detecting milk fat content and somatic cell counts.

Chloramphenicol is an inexpensive, broad-spectrum, and highly effective veterinary drug widely used in clinical treatment and disease prevention. However, it can cause adverse effects in humans, such as aplastic anemia. Samsonova et al. [20] developed methods for detecting chloramphenicol, thiamphenicol, and florfenicol residues in various food matrices (milk, meat, eggs), screening microbiological inhibition, antibody-based immunoassays, and biosensor methods. They discussed and compared the advantages and disadvantages of these approaches while analyzing current status and future trends in veterinary residue detection.

Kumar et al. [21] employed microbiological detection to monitor veterinary drug residues in milk by examining the inhibition of spore germination in *Bacillus* species. At 64°C for 2-3 hours, spores were activated to germinate, with color change from purple to yellow serving as the detection criterion. Analysis of 228 samples revealed residue levels of 10.08% and a false-positive rate of 0.43%, demonstrating applicability for dairy enterprises, though the method only provides semi-quantitative detection with low sensitivity and poor selectivity. Many regulations prohibit veterinary drug use in feed to protect consumer and animal health. Bohn et al. [16] developed a simple, low-cost method for detecting feed residues, validating 14 representative drugs with detection limits of 10 g/L for ciprofloxacin and 10 mg/L for clopidol, though the method exhibited some false negatives requiring further improvement. Yamaki et al. [22] used microbiological detection to analyze veterinary drug residues in sheep milk from a Spanish farm,

finding a 2.6% positive rate, with approximately 25% being -lactams. Heating positive samples at 82°C for 10 minutes reduced the positive rate by 0.9%, with highest rates in September and October, though the method showed some false positives and required sensitivity improvement.

2 Immunoassay Method

Immunoassays are based on specific antibody-antigen binding reactions. Due to the high sensitivity and specificity of these interactions, and the clear functional relationship between antigen (or antibody) quantity and reaction intensity, these methods enable qualitative or quantitative sample analysis. A series of sensitive and efficient immunoassay methods based on this principle have been widely applied for detecting veterinary drug residues in milk [23].

Ceftiofur is a potent veterinary drug commonly used to treat bovine pneumonia during lactation, with improper use leading to milk residues. Stanker et al. [24] developed a monoclonal antibody-based competitive enzyme-linked immunosorbent assay (ELISA) for detecting ceftiofur and its metabolites in milk. The method is simple, requiring only sample dilution, with a detection limit of 1 g/L and recovery rates of 80.8%-116.8%. Zhi et al. [25] employed immunoassay for cephalosporin detection in milk, achieving recoveries of 74%-120% and detection limits of 1 g/L, enabling rapid and accurate analysis. Pennacchio et al. [26] developed a novel immunosensor for penicillin G detection in milk, conjugating penicillin G with bovine serum albumin (BSA) in carbonate buffer to form a penicillin G-BSA complex for immunizing rabbits to obtain IgG antibodies. The method directly detects penicillin G residues without interference, achieving a detection limit of 0.356 g/L.

Clinafloxacin is commonly used to treat animal diseases, but its residues in dairy products can harm human health by inducing bacterial resistance or allergic reactions. Chen et al. [27] established a fluorescence polarization immunoassay using a fluorescent tracer, synthesizing fluorescein isothiocyanate-labeled drugs and measuring fluorescence polarization values after competitive binding between different drug concentrations and the tracer for antibody binding. This enabled highly sensitive detection of clinafloxacin residues in dairy products, with recoveries of 86.8%-104.5%, relative standard deviations of 4.1%-7.2%, and a detection limit of 3.3 g/L for enrofloxacin. Gao et al. [28] synthesized monoclonal antibodies for tetracycline residue detection in milk, immunizing mice with doxycycline and chlortetracycline immunogens to produce monoclonal antibodies. By screening optimal antibody-antigen combinations, they developed an ELISA method capable of simultaneously detecting seven tetracyclines, with recoveries of 75.3%-106.8% and detection limits ranging from 1.5 to 6.9 g/L. However, the method may suffer from various interfering factors, potentially producing false positives and cross-reactivity without precise protocols and quality control standards, requiring further improvement.

3 Instrumental Analysis Method

Instrumental analysis methods determine veterinary drug content through specific properties or reactions of functional groups in drug molecules, such as gas chromatography and high-performance liquid chromatography (HPLC). HPLC coupled with UV or mass spectrometry detectors is widely used for veterinary drug residue analysis in milk. Pretreatment steps employ protein precipitation and extraction to isolate drugs from milk.

Samanidou et al. [29] used HPLC with diode array detection for tetracycline residues in milk, extracting analytes with oxalate buffer containing 20% trichloroacetic acid as a protein eluent via solid-phase extraction, achieving spiked recoveries of 93.8%-107.2% with relative standard deviations below 8.5%. Yu et al. [30] developed a simple, rapid, sensitive, and low-cost technique using C18 stir bar sorptive extraction combined with HPLC-MS/MS for six sulfonamides in milk. The C18 silica particle-coated stir bar was simple to prepare, exhibited good mechanical strength for over 20 reuses, and provided increased surface area beneficial for extraction. After optimizing extraction time, ionic strength, pH, and stirring speed, detection limits of 0.9-10.5 g/L were achieved, enabling application in milk and milk powder analysis.

Arroyo-Manzanare et al. [31] employed dispersive liquid-liquid microextraction and QuEChERS pretreatment methods for nine sulfonamides in milk. These approaches replaced solid-phase extraction, reducing organic solvent usage while being simple and feasible. Recoveries were 90.8%-104.7% for dispersive liquid-liquid microextraction and 83.6%-104.8% for QuEChERS, with detection limits of 1.21 and 2.73 g/L, respectively. -lactam residues in milk adversely affect human health. Cámara et al. [32] described a sensitive and reliable HPLC-UV diode array method for simultaneous multi- -lactam detection, optimizing chromatographic columns, mobile phases, temperature, and flow rates to achieve recoveries of 79%-96%, relative standard deviations of 0.5%-4.9%, and detection limits of 3.4-8.6 g/L, meeting national minimum residue limit requirements.

Adlnasab et al. [33] used dispersive liquid-liquid solid-phase extraction for -lactam residues in milk, achieving relative standard deviations of 4.3%-8.5% and detection limits of 50-500 g/L after optimization. Wang et al. [34] developed an air-assisted liquid-liquid microextraction pretreatment combined with HPLC for quinolone residues in milk powder and eggs, optimizing extraction solvent type, centrifugation time, and pH to achieve coefficients of variation below 8%, recoveries of 72%-115%, and detection limits as low as 5 g/L, offering a rapid, simple method with minimal organic solvent consumption and short centrifugation times.

Lombardo-Agüí et al. [35] used capillary liquid chromatography with laser-induced fluorescence detection for seven veterinary drugs in milk, comparing QuEChERS and molecularly imprinted polymer extraction. QuEChERS proved faster with higher recoveries and lower detection limits, achieving 0.4 g/kg for danofloxacin. Xu et al. [36] combined solid-phase extraction with

ultra-performance LC-MS/MS for tetracycline residues in milk, obtaining recoveries of 81.5%-101.4% and detection limits of 0.61-10.34 g/kg after optimization. Lv et al. [37] developed a rapid, sensitive, and specific method for tetracyclines in milk using molecularly imprinted solid-phase extraction with reversed-phase HPLC. They synthesized molecularly imprinted organic-inorganic hybrid composites using tetracyclines as templates, methacrylic acid as functional monomer, tetraethoxysilane as inorganic precursor, and 3-(trimethoxysilyl)propyl methacrylate as coupling agent, establishing a novel online solid-phase extraction HPLC method with recoveries of 85.3%-98.3% and detection limits of 0.76 g/kg.

Santos et al. [38] compared capillary electrophoresis with HPLC for six veterinary drugs in milk. HPLC could not distinguish amoxicillin, with detection limits of 0.48-1.09 mg/L and relative standard deviations below 5%. Capillary electrophoresis offered simplicity, short analysis time, low detection limits, and high sensitivity with wide measurement ranges, though requiring rigorous sample pretreatment and expensive equipment.

4 Aptamer Sensor Detection Method

The concept of nucleic acid aptamers was first proposed by Ellington et al. [39] and Tuerk et al. [40] in 1990. Aptamers serve as signal recognition units in detection systems, offering flexible structural design that changes or reconstructs upon target binding, making them excellent choices for signal transduction. They can detect various targets including veterinary drugs, proteins, cells, mycotoxins, and heavy metals [41-42]. With stable structures that can be synthesized in vitro without natural or physical constraints, aptamer-based detection has attracted widespread research attention as a novel rapid detection method for veterinary drugs in food [43].

Current HPLC methods are time-consuming, require professional technicians, and lack portability. Sun et al. [44] developed an aptasensor based on synergistic effects among graphene-gold nanoparticles, multi-walled carbon-cobalt phthalocyanine, and chitosan-gold nanoparticle composites. Electrochemical performance during electrode modification was characterized by cyclic voltammetry, and nanocomposite morphology was examined by scanning electron microscopy. Under optimal conditions, the aptasensor exhibited high sensitivity, specificity, and low detection limits, achieving 3.37 g/L for kanamycin in milk. Zhou et al. [45] established an electrochemical aptasensor for kanamycin detection, linking aptamers to gold electrode surfaces via thiol groups. Specific binding between kanamycin and its aptamer induced conformational changes, increasing surface coverage and hindering electron transfer, which was characterized by differential pulse voltammetry. The method showed good specificity and high sensitivity with a detection limit of 8.15 ng/L.

Guo et al. [46] modified electrode surfaces with tetracycline aptamers via gold nanoparticles, then added methylene blue. Upon tetracycline addition, com-

petitive binding occurred between tetracycline and methylene blue for the aptamer. Due to stronger tetracycline-aptamer affinity, methylene blue was released, causing current reduction that correlated with tetracycline concentration. The method exhibited good selectivity and high sensitivity for tetracycline detection in milk, with recoveries of 94%-108% and a detection limit of 1.86 ng/L. Zhi Wenting [47] developed a colorimetric aptasensor for tetracycline detection where aptamer binding caused gold nanoparticles to lose protection, and high-concentration sodium chloride solution reduced interparticle distance, inducing color changes. The method achieved a detection limit of 7.8 g/L. Xu et al. [48] constructed an aptasensor based on a signal amplification strategy using horseradish peroxidase and graphene oxide-polyaniline. The graphene oxide-polyaniline film was modified on the electrode, and both horseradish peroxidase-modified oxytetracycline aptamer and oxytetracycline were added. Oxytetracycline-aptamer binding reduced horseradish peroxidase on the electrode surface, decreasing electrochemical signals that correlated with oxytetracycline concentration. The method showed good selectivity and high sensitivity with a detection limit of 2.3×10 mg/L, though it suffered from poor reproducibility requiring further improvement.

With advancing science and technology, veterinary drug residue detection techniques continue to improve. In milk quality and safety analysis, the sensitivity of currently common methods—including microbiological detection, instrumental analysis, and immunoassay—has significantly increased, while aptamer sensor detection is gradually being refined. Compared with traditional methods, aptamer sensors offer numerous advantages including high sensitivity, strong specificity, small size, simple operation, and low cost, attracting widespread attention from researchers, though some issues remain to be addressed. In conclusion, we anticipate the emergence of more novel detection methods with high sensitivity, strong applicability, and simplicity for veterinary drug residue detection in milk.

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Note: Figure translations are in progress. See original paper for figures.