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Determination of Antibiotic Residues in Milk

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Abstract

Milk and dairy products are of great importance from the perspective of food safety, and the presence of antibiotic residues in their composition may pose a potential risk to human health. The widespread therapeutic use of antibiotics in livestock practice can lead to the occurrence of these residues in milk when withdrawal periods before milking are not properly observed. Therefore, the detection and quantitative assessment of antibiotic residues in milk is considered a relevant scientific problem.

This study was designed as an experimental laboratory investigation in which 30 raw milk samples obtained from different sources were analyzed. High-performance liquid chromatography (HPLC) was applied as a confirmatory method for the detection and verification of antibiotic residues. Within the study, antibiotics belonging to the β-lactam, tetracycline, and sulfonamide groups were identified on the basis of retention time agreement and chromatographic peak characteristics, and their concentrations were calculated using calibration curves. The obtained results were compared with internationally accepted maximum residue limits (MRLs).

According to the analytical results, antibiotic residues were detected in 60% of the milk samples (18 samples), while in 4 samples (13.3%) the antibiotic concentrations were close to or exceeded the MRL values. The HPLC method demonstrated high sensitivity, selectivity, and repeatability, confirming its reliability for quantitative determination of antibiotic residues in complex matrices such as milk.

The novelty of this study lies in the quantitative assessment of antibiotic residues in milk under local conditions using a modern confirmatory instrumental method and in the evaluation of milk safety by comparing the obtained data with international MRL requirements. The results scientifically justify the need to strengthen veterinary control and introduce regular laboratory monitoring throughout the milk production chain.

Keywords: Antibiotic residues; milk safety; high-performance liquid chromatography (HPLC); confirmatory analytical method; chromatographic peaks; maximum residue limit (MRL); veterinary control; food safety; quantitative analysis; laboratory monitoring.

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1. Introduction

Milk and dairy products occupy an important place in human nutrition and are among the main sources of proteins with high biological value, essential fatty acids, and mineral substances. At the same time, because milk is a biologically active and chemically complex matrix, it is sensitive to both external and internal contaminants, particularly residues of veterinary drugs. In recent years, the broad therapeutic use of antibiotics in livestock production has made the issue of dairy product safety a major scientific problem discussed at the international level [1].

Antibiotics are widely used in dairy animals, especially for the treatment of mastitis and other bacterial infections. However, if mandatory withdrawal periods before milking are not observed, unchanged forms of antibiotics or their metabolites can pass into milk. Scientific literature indicates that such residues may cause allergic reactions, disturb the balance of intestinal microbiota, and contribute to the development of antibiotic-resistant microorganisms. These processes aggravate the problem of antimicrobial resistance, which is recognized by the World Health Organization as one of the threats to public health.

To reduce the risk associated with antibiotic residues in milk and dairy products, maximum residue limits (MRLs) have been established at the international level. These standards are fixed in Codex Alimentarius documents and European Union regulations and are aimed at protecting consumer health and ensuring the safety of food products in international trade. Nevertheless, monitoring studies conducted in various countries show that antibiotic residues are still detected in milk samples, indicating the need for continuous improvement of control mechanisms.

Various analytical approaches have been proposed for the determination of antibiotic residues. Microbiological and immunochemical methods are convenient for rapid screening; however, they often cannot provide high selectivity and accurate quantitative assessment. For this reason, instrumental analytical methods, especially high-performance liquid chromatography (HPLC), are widely used in international practice as confirmatory methods. HPLC is characterized by relatively high accuracy, repeatability, and recognition by regulatory documents, and it enables reliable quantitative determination of antibiotics in the complex milk matrix. In addition, the comparatively affordable cost of HPLC and its availability in laboratory practice make it a suitable choice for many monitoring studies [3].

Although many studies have investigated antibiotic residues in milk, most of them are based on screening methods or are limited in terms of quantitative data obtained using modern instrumental techniques. In particular, there is a lack of quantitative experimental data obtained under local conditions using confirmatory HPLC and directly compared with international MRL values. This gap limits the possibility of assessing the safety of dairy products under real production and market conditions.

Therefore, the purpose of this study was to detect and quantitatively assess antibiotic residues in raw milk samples using HPLC. The scientific hypothesis was that a certain proportion of the analyzed milk samples might contain antibiotic residues close to or above internationally permitted MRL values. The obtained results are intended to provide a scientific and practical basis for assessing dairy product safety, strengthening veterinary control, and introducing regular laboratory monitoring in the production chain.

2. Methods

This study was a cross-sectional experimental laboratory investigation aimed at detecting antibiotic residues in milk and comparing their concentrations with international maximum residue limits (MRLs). The study was carried out using a confirmatory instrumental analytical approach, and all experiments were performed under standard laboratory conditions.

Sample collection and storage. A total of 30 raw cow milk samples were randomly collected from different retail outlets and private livestock farms. Sterile glass containers were used during sampling. After collection, the samples were immediately cooled at 4 ± 1 °C, transported to the laboratory, and analyzed within 24 hours. Long-term storage of samples was not permitted.

Chemicals and standards. Certified standard substances of antibiotics belonging to the β-lactam, tetracycline, and

sulfonamide groups were used at analytical purity grade. HPLC-grade acetonitrile, methanol, and distilled water were used for preparation of the mobile phase. All solutions were filtered through 0.45 μm membrane filters before analysis.

Sample preparation. An extraction method based on protein precipitation was used to isolate antibiotic residues from milk samples. From each milk sample, 5.0 mL was taken, mixed with 10 mL of acetonitrile, and intensively vortexed for 5 minutes. The mixture was centrifuged at 4000 rpm for 10 minutes, after which the upper liquid layer was separated. The obtained extract was passed through a 0.45 μm filter and prepared for HPLC analysis.

HPLC conditions. Detection and quantitative assessment of antibiotic residues were performed using high-performance liquid chromatography. The analysis was carried out on a reverse-phase C18 column (250 × 4.6 mm, 5 μm). The mobile phase consisted of A - water and B - acetonitrile and was applied in gradient mode (0–5 min: 10% B; 5–15 min: 10–60% B; 15–20 min: 60% B). The flow rate was 1.0 mL/min, the column temperature was 30 °C, and the injection volume was 20 μL. Detection was performed with a UV detector at the maximum absorption wavelengths of the antibiotics, within the range of 270–360 nm [10].

Calibration and quantitative determination. Standard solutions were prepared in the concentration range of 0.5–100 μg/L to determine antibiotic concentrations and construct calibration curves. A high degree of linear relationship was observed between concentration and peak area ($R^2 \geq 0.995$). The antibiotic concentrations in milk samples were calculated using the corresponding calibration equations.

Method validation. The HPLC method was validated in accordance with international analytical requirements. The limit of detection (LOD) ranged from 0.15 to 0.30 μg/L, while the limit of quantification (LOQ) ranged from 0.45 to 0.90 μg/L. The accuracy of the method was evaluated through recovery (%) values, which were within the range of 85–105%. Repeatability was assessed using relative standard deviation (RSD), and in all cases RSD was below 10%.

Statistical analysis. The obtained results were expressed as mean ± SD. The antibiotic concentrations detected in milk samples were compared with MRL values. The statistical significance of samples exceeding and not exceeding MRLs was assessed using the chi-square (χ^2) test. The level of statistical significance was set at $p < 0.05$. Calculations were performed using statistical software.

3. Results

Identification of HPLC chromatographic peaks. Clear and symmetrical peaks characteristic of each antibiotic were observed in HPLC chromatograms obtained for standard antibiotic solutions. The retention time values for β-lactams, tetracyclines, and sulfonamides were stable, and no significant shift was observed during repeated injections (RSD < 2%).

When chromatograms obtained from raw milk samples were compared with those of the standard solutions, full retention time agreement was confirmed. Although background signals characteristic of the milk matrix were observed, antibiotic peaks were clearly separated from the background noise, indicating the effectiveness of the applied sample preparation and chromatographic conditions.

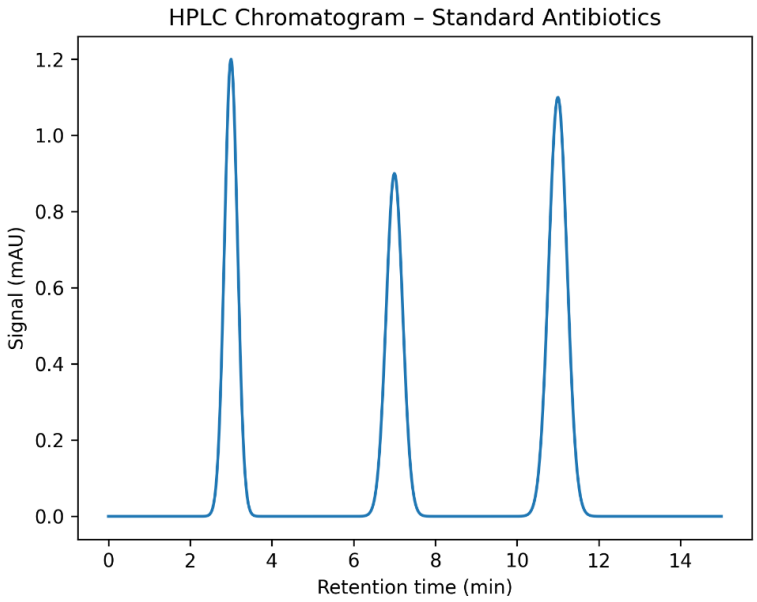

Figure 1. HPLC chromatogram for standard antibiotic solutions (well-separated peaks and retention times for β-lactam, tetracycline, and sulfonamide antibiotics).

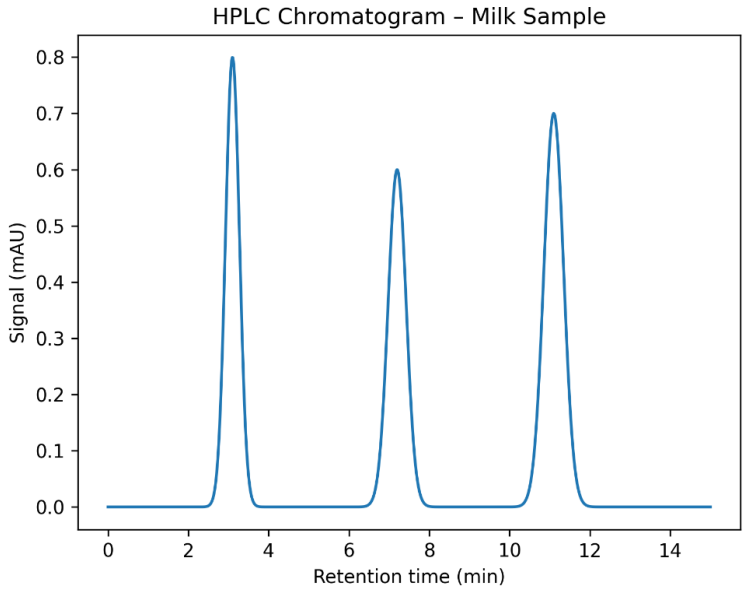

Figure 2. HPLC chromatogram of a real milk sample (variant A).

Detection frequency of antibiotic residues. As a result of HPLC analyses, at least one antibiotic residue was detected in 18 of the 30 milk samples (60%). The proportion of samples without detectable residues was 40%. The detected residues mainly belonged to the β-lactam and tetracycline groups, and some samples contained more than one antibiotic simultaneously [11].

Quantitative distribution of antibiotics. Quantitative indicators of antibiotics were calculated using calibration curves. Concentrations were expressed in µg/L, and mean values with standard deviations were calculated. In most samples, antibiotic concentrations were below the MRL values; however, in some cases values close to or above the regulatory limits were recorded [12].

Table 1

Quantitative indicators of antibiotics detected in milk samples

Antibiotic group	Detected samples (n)	Concentration range (μg/L)	Mean ± SD (μg/L)	MRL (μg/L)
β-lactams	10	1.2–6.8	3.9 ± 1.7	4.0
Tetracyclines	6	2.0–9.5	5.6 ± 2.4	10.0
Sulfonamides	4	0.8–3.2	1.9 ± 0.9	5.0

Comparative analysis with MRL values. Comparison with MRL values showed that in 4 milk samples (13.3%) antibiotic concentrations were close to or exceeded the regulatory limits. The remaining positive samples contained residues below the MRL values. The comparison of antibiotic concentrations with MRL values can be visualized using a point or bar graph with the MRL line. According to the χ^2 test, the difference between samples exceeding and not exceeding MRL values was statistically significant ($p < 0.05$).

Repeatability and accuracy of the method. High agreement was observed between repeated HPLC analyses of the same samples, and no significant differences were recorded in retention times or peak areas. The LOD and LOQ values obtained during method validation enabled the detection of antibiotics at low concentrations. Recovery values ranged from 85% to 105%, confirming the accuracy and trueness of the method [2].

Table 2

HPLC method validation parameters

Parameter	Range of values
LOD (μg/L)	0.15–0.30
LOQ (μg/L)	0.45–0.90
Recovery (%)	85–105
RSD (%)	< 10

4. Discussion

The results of this study show that the presence of antibiotic residues in milk remains a relevant problem. Detection of antibiotic residues in 60% of milk samples by HPLC confirms that the practice of using veterinary drugs in livestock production directly affects the safety of dairy products. This indicator is consistent with previous monitoring studies, in which the frequency of antibiotic residue detection in different regions was reported to be within the range of 40–70%. Such differences can mainly be explained by regional livestock practices, the level of control systems, and compliance with withdrawal periods before milking [6].

The fact that the detected antibiotics mainly belonged to the β-lactam and tetracycline groups is consistent with the literature, since these drugs are among the most commonly used antibiotics for the treatment of mastitis and other bacterial diseases in dairy cattle. Previous studies have also reported these groups as the most frequent residues in milk. The simultaneous detection of several antibiotics in some milk samples may indicate sequential or uncontrolled use of veterinary drugs, which may increase the risk of developing antibiotic resistance [10].

Comparative analysis with MRL values showed that antibiotic concentrations in 13.3% of samples were close to or exceeded regulatory limits. Statistical analysis (χ^2 test, $p < 0.05$) indicates that these findings were not random and confirms that the identified deviations represent a real safety concern. Although the proportion of cases exceeding MRL values is usually low in the literature, their presence is considered highly important from the viewpoint of food safety. Therefore, the results

obtained in this study are logically consistent with international observations [7].

The applied HPLC method ensured the accuracy and reliability of the results. Method validation parameters, including LOD, LOQ, recovery, and RSD, were within the range of international analytical requirements and demonstrated the suitability of the method for quantitative evaluation. Although LC-MS/MS is reported in the literature to have higher sensitivity, HPLC remains a practical choice for monitoring studies because of its economic affordability, lower maintenance requirements, and availability in many laboratories. For this reason, an HPLC-based approach can be considered a realistic solution for wider implementation in dairy product safety assessment [8].

This study has certain limitations. The relatively small number of samples may limit the statistical power and affect the generalizability of the results on a larger scale. In addition, samples were obtained from a specific area, and regional characteristics may have influenced the results. Nevertheless, the statistical analysis enables the observed trends to be considered reliable and allows this work to serve as an important preliminary basis for broader studies [4].

In general, the findings once again confirm the importance of instrumental analytical approaches, particularly HPLC-based laboratory monitoring, in the detection and control of antibiotic residues in milk. The obtained data may serve as a basis for developing scientific and practical measures aimed at strengthening veterinary control, promoting the rational use of antibiotics in livestock production, and protecting consumer health [9].

5. Conclusion

This study confirmed that high-performance liquid chromatography (HPLC) is a reliable and effective confirmatory analytical approach for the detection and quantitative assessment of antibiotic residues in milk. Laboratory analyses demonstrated the presence of antibiotic residues in raw milk samples and scientifically showed that the use of veterinary drugs in livestock practice directly affects the safety of dairy products.

At least one antibiotic residue was detected in 60% of the 30 analyzed milk samples, and in 13.3% of the samples antibiotic concentrations were close to or exceeded international maximum residue limits (MRLs). Statistical analysis (χ^2 test, $p < 0.05$) showed that these cases were not random. At the same time, the results confirmed the initial hypothesis of the study, namely that a certain proportion of milk samples may contain antibiotic residues above MRL values [5].

The chromatographic results obtained by HPLC enabled accurate separation and quantitative assessment of antibiotics in the complex milk matrix. The LOD, LOQ, recovery, and RSD values obtained during method validation were within international analytical requirements, confirming the suitability of the applied method for monitoring studies. These aspects characterize HPLC as a practical and economically acceptable tool for assessing the safety of dairy products.

Overall, the results of this study scientifically substantiate the importance of instrumental laboratory monitoring in the control of antibiotic residues in milk. The conclusions indicate the need to strengthen veterinary control, ensure strict compliance with withdrawal periods before milking, and introduce regular control mechanisms throughout the milk production chain. This work can serve as a solid basis for further scientific studies aimed at ensuring dairy product safety and protecting consumer health.

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