Research Article

Detection of Veterinary Antimicrobial Residues in Milk through Near-Infrared Absorption Spectroscopy

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This study focuses on detection of antimicrobial residues in milk through Fourier transform near-infrared spectroscopy. Simulated and real samples were considered. The simulated ones take into account veterinary drugs added in milk samples in the following concentrations: enrofloxacin $100\,\mu g/L$, terramycin $100\,\mu g/L$, and penicillin $4\,\mu g/L$. The statistical tool used to discriminate the samples was the principal component analysis (PCA). Our results show that, with this experimental procedure, it is possible to discriminate different types of antimicrobials dissolved in milk. Moreover, the methodology was able to detect real sample milked on different days after the injection of ceftiofur hydrochloride which is in principle a zero grace period antimicrobial. The methodology proved to be fast and accurate within the maximum residue limits allowed by European Agency for Medicinal Products and Ministry of Agriculture Livestock and Food Supply from Brazil.

1. Introduction

According to the Food and Agriculture Organization of the United Nations, milk is one of the most consumed foods in the world. It not only has its importance in the nutritional level, but also plays an important role in the economy. Global consumption of milk and its derivatives exceeds 6 billion of consumers [1].

The milk contains protein, carbohydrates, lipids, minerals, and vitamins which accomplish important biochemical and nutritional functions, particularly to children and elderly people. The bovine milk contains about 87.1% of water, 4.0% of fat, 3.3% of protein, 4.6% of lactose, and 0.7% of ash. The basic protein content in milk is casein in 78.3%, whey protein 19%, and others totaling 2.7% [2, 3]. Concerning carbohydrates, lactose is the main one. Constituted by two monosaccharides, glucose and galactose, they carry nutritional important functions, such as providing 16.8 kJ/g of energy to people [4, 5]. Milk and dairy products are considered a good source of calcium due its high bioavailability. The latter can be understood as the fraction of ingested nutrient and food that is absorbed and used in physiological and normal metabolic functions and storage [6, 7]. Although the potentiality of milk as food is unquestionable, restrictions on its intake exist in people allergic to lactose and casein.

Adulterations in milk have been highly reported in developing countries, such as Pakistan, Brazil, India, and China [8]. Mostly the aim is to increase volume with the addition of water [9]. However, there are other problems, such as the contamination of milk by residues of veterinary drugs that may be present when the cow is milked in the grace period. The most common drugs are antimicrobials and anti-inflammatories. They are widely used in the treatment of dairy cattle diseases, such as mastitis, diarrhea, and lung diseases, also in prevention and control, or to increase the production and growth of animal [10–13]. A study performed by Van Boeckel et al. showed that, between

2000 and 2010, the consumption of antibiotics by the world population has increased by 36% and is related to the appearance of drug-resistant bacteria. From this total, 76% is mainly due to the countries that composes the BRICS (Brazil, Russia, India, China, and South Africa). Fraction of this increase is due to ingestion of animals or their food derivatives contaminated with antibiotics [14].

The overuse of drugs in dairy herd results in detectable traces in the milk. When their concentrations are over the maximum residue limit (MRL), they can cause health damage to the consumer ranging from allergic reactions to bacterial resistance [13] Contaminated milk is harmful to health and should be discarded [11, 15, 16]. In 2014, Van Boeckel et al. related the excessive use of antibiotics in animals to the appearance of super bacteria in humans. In order to monitor the milk content, the regulatory agencies use a variety of analytical methods to detect antibiotics traces in the milk, such as high-performance liquid chromatography (HPLC), gas chromatography coupled to mass spectroscopy (CG-MS), and antimicrobial detection kits. Nevertheless, there are drawbacks in their use such as sample preparation, qualified manpower, complex procedures, time consuming, and high cost. Moreover, normally the tests of antibiotics are specific to a class of antimicrobials.

The search for high sensitive techniques that allow the detection of residues of antibiotics in milk has been carried out for decades. In 1996, Verdon and Couedor used a reverse-phase HPLC technique to determine ampicillin residues which is able to detect 3μg/L and to quantify up to 10μg/L of the drug [17]. In 2002, Sivakesava and Irudayaraj carried out a study showing the feasibility of measuring tetracycline at μg/L levels with Fourier transform near-infrared (FT-NIR) spectroscopy and Fourier transform medium-infrared (FT-MIR) spectroscopy. Nevertheless, they reported high prediction errors and suggested that the methodology should be confirmed with naturally contaminated samples and other drug residues [18]. In 2003, Jankovská and Sustová used FT-NIR to analyse cow milks. However, the technique was used to describe physical-chemical characteristics of milk. In addition, partial least squares (PLS) regression was used to develop calibration models for the examined milk components. Through these results, they suggest that the NIR spectroscopy is applicable for a rapid analysis of milk composition [19]. In 2010, Brandão et al. studied fat concentration in milk samples by means of noninvasive techniques, FT-IR and FT-NIR absorption. They concluded that the wavelength of 2308 nm can be used to determine the fat concentration of milk without other components' influence [20]. In 2014, Zhang et al. [21] examined UHT and pasteurized milks to verify the presence of residues of tetracyclines, sulfonamides, sulfamethazine, and quinolone. The milk samples were collected in highly populated cities of China and analysed by the enzyme-linked immunosorbent assay (ELISA). No residue of veterinary drugs has exceeded MRL established by China, European Union, and CAC (Codex Alimentarius Commission). Nevertheless, because of the high number of residues present in milk, they recommended that the control mechanisms should be rigorously applied in order to keep

Table 1: Result of physical-chemical characterization of milk samples. Average values and their standard deviations.

Analysis	Values	Values reference[a,b]
Cryoscopy	(0.536 ± 0.001) °H	(−0.550 to −0.530) °H
Acidity	(17.3 ± 0.6) °Dornic	(14 to 18) °Dornic
Density	(1.031 ± 0.000) g/mL	(1.029 to 1.040) g/mL
pH to °C	(6.72 ± 0.01)	(6.60 to 6.80)
Fat	(3.65 ± 0.01) %	≥3.00
Protein	(3.14 ± 0.01) %	≥2.90
Lactose	(4.50 ± 0.01) %	≥4.30
Solids	(11.29 ± 0.01) %	≥8.40

[a]IN 62; [b]FAO/TCP/KEN/6611.

these residues at safe levels. In 2015, Moharana et al. [22] analysed the veterinary drug enrofloxacin in cow milk samples obtained from two cities of India. According to the authors, the enrofloxacin is the most rampantly used drug in veterinary practice. To analyse the samples, they used reverse-phase HPLC. With a limit of detection of 100μg/L, they have verified that 8% of the samples had values above the MRL. From the brief historical review described above, it is clear that there is a need for more in-depth studies exploring detection limits, embracing bigger classes of antibiotics, and analysing real samples.

This work deals with Fourier transform near-infrared (FT-NIR) spectroscopy associated with principal components analysis (PCA) to detect traces of veterinary antimicrobials (enrofloxacin, terramycin, penicillin, and ceftiofur hydrochloride) bellow the MRL allowed by the legislation of European Agency for Medicinal Products (EMEA) which is adopted in Brazil by Ministry of Agriculture Livestock and Food Supply (MAPA) [23]. The detection of the ceftiofur hydrochloride in milk was performed for two consecutive days after the drug has been administered to the animal.

2. Materials and Methods

The analyses were performed in the Process and Products Laboratory (LPP) and in the Materials Spectroscopy Laboratory (LEM), located in the Physics Department of Federal University of Juiz de Fora, Brazil.

2.1. Milk Samples. Some samples used in this work were raw milk from nonmedicated cows. They were collected at a farm located in the city of Rio Pomba, MG, Brazil, near the laboratory. After milking, the raw milk samples were immediately stored and kept refrigerated at 5°C until analysis which was performed after approximately one hour. The samples were submitted to chemical-physical analysis to verify their conformity with the established standards [24], that is, cryoscopy, density, pH, acidity (Dornic and Alizarol tests), fat, protein, lactose, and solids [25–27]. Each measurement was performed in triplicate. The results are shown in Table 1.

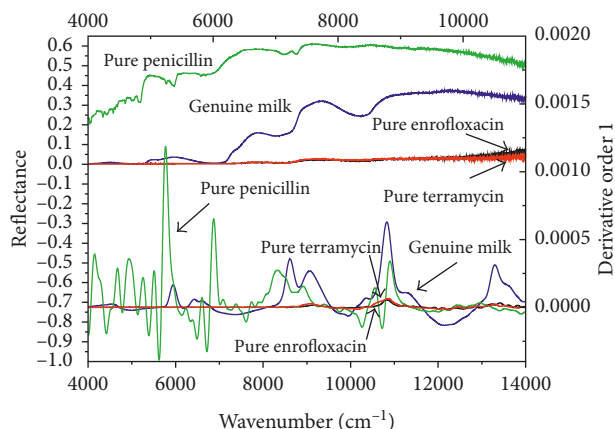*2.2. Contamination Simulation.* Initially, two portions were separated, one as a control sample and the other as

Figure 1: Reflectance spectra of pure samples of genuine milk and veterinary drugs with their respective first derivatives.

Figure 2: Reflectance spectra and their first derivatives of genuine and contaminated milk samples within the MRL.

a self-controlled one with a veterinary drug. The drugs used were the antimicrobials: enrofloxacin Baytril® injective 10% that has 10 g of enrofloxacin plus 100 ml of vehicle in its composition, terramycin/LA Zoetis/Pfizer® injective that has 20 g of oxytetracycline plus 100 g of vehicle in its composition, and reinforced pentabiotic Pfizer with penicillin.

The simulation was done according to the active principle of each drug and not in its volume. More explicitly, in the analysis with PCA, the discrimination of contaminated milk in μg/L is given through the active principle, MRL as provided by legislation, and not through the drug (excipients + active principle). For this purpose, each veterinary drug was first diluted in distilled water, and finally, part of this dilution was added in the genuine milk, in order to reach the concentration of the active principle in the milk. The values equivalent to the MRL allowed by EMEA and MAPA for the antimicrobials and its metabolites are as follows: 100 μg/L to enrofloxacin, 100 μg/L to terramycin, and 4 μg/L to penicillin [22, 28, 29]. This methodology was applied previously to the anti-inflammatory sodium diclofenac [30].

2.3. Real Contaminated Samples. First, the milk free from drugs was collected from a cow used as a control. The CeF-50 Ceftiofur Agner Union injective drug, which has 50 g of ceftiofur hydrochloride plus 1 mL of vehicle in its composition, was then administered to the cow. This drug does not have a grace period. Milk is then collected for two consecutive days. All the samples were immediately stored and kept refrigerated at 5°C until analysis which was performed after approximately one hour.

2.4. Analysis Using FT-NIR Method. Analyses of the samples were carried out with the Multi Purpose FT-NIR Analyser from Bruker operating in the reflectance mode in the range of 13.500 to 3.700 cm^{-1} wavenumbers with a Te-InGaAs detector and 4 cm^{-1} of resolution. The OPUS® software version 5.5 was used for data acquisition. The samples were placed in borosilicate cuvettes with 8 mm thickness. Each
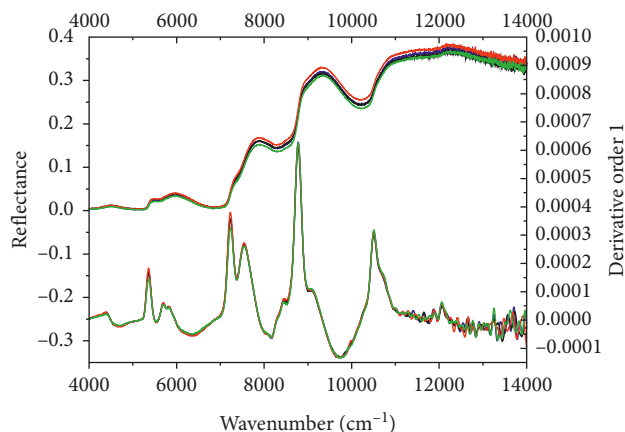
analysis was performed in triplicate with 32 scans for simulated and control samples.

2.5. Statistical Analysis. The reflectance spectra and their first derivatives were made and analysed with the software OriginPro®8 SR2 v.8.0891(B891). The eigenvalues were calculated with the software BioStat version 5.3. The principal component analysis was conducted with the software The Unscrambler® X version 10.3.

3. Results

Table 1 shows the average results obtained for the milk quality parameters of the samples and the reference value established by the Brazilian Normative Instruction 62 from MAPA and the FAO/TCP/KEN/6611 [24]. The results are in the range accepted by the legislation.

Figure 1 shows the reflectance spectra (top) and their first derivatives (bottom) of genuine milk and pure antimicrobials. From the figure, one can see that the spectra are very different from each other above all, in the first derivatives.

Figure 2 shows the reflectance spectra (top) and their first derivatives (bottom) of genuine and contaminated milk samples within the MRL. On the contrary of Figure 1, now the differences between the spectral profiles are not apparent due to the very low concentration of the antibiotics. Therefore, we have to rely on a statistical model. This will be accomplished by means of PCA to discriminate control and tampered milk samples.

The principal component of a set of data (dimensionality) is obtained by means of an analysis that consists in finding the eigenvalues of the covariance matrix [31]. Each eigenvector has a corresponding eigenvalue. The eigenvectors with higher eigenvalues are the principal components and are ordered from the higher to the smaller ones furnishing the components in significance degree [32]. Table 2 shows the explained and cumulative variances of the principal components of genuine and contaminated milk samples within the MRL.

TABLE 2: Explained and cumulative variances of genuine and contaminated milk samples within the MRL.

PC	Explained variance (%)	Cumulative variance (%)
PC1	99.9159	99.9159
PC2	0.0156	99.9315
PC3	0.0105	99.9420
PC4	0.0102	99.9522
PC5	0.0093	99.9615
PC6	0.0084	99.9699
PC7	0.0080	99.9779
PC8	0.0077	99.9857
PC9	0.0069	99.9926
PC10	0.0060	99.9986
PC11	0.0008	99.9993
PC12	0.0007	100.0000

FIGURE 4: Score plot of statistical analysis (PCA) for average values of samples: pure enrofloxacin (ENR), pure terramycin (TER), pure penicillin (PEN), genuine milk (GEN), milk-enrofloxacin and milk-terramycin in 100μg/L each (EN100 and TE100), and milk-penicillin in 4μg/L.

TABLE 3: Explained and cumulative variances of average values of samples: pure enrofloxacin, pure terramycin, pure penicillin, genuine milk, milk-enrofloxacin and milk-terramycin in 100μg/L each, and milk-penicillin in 4μg/L.

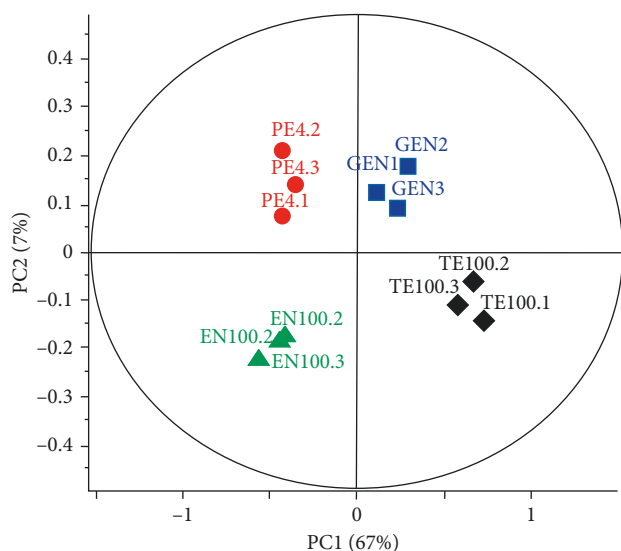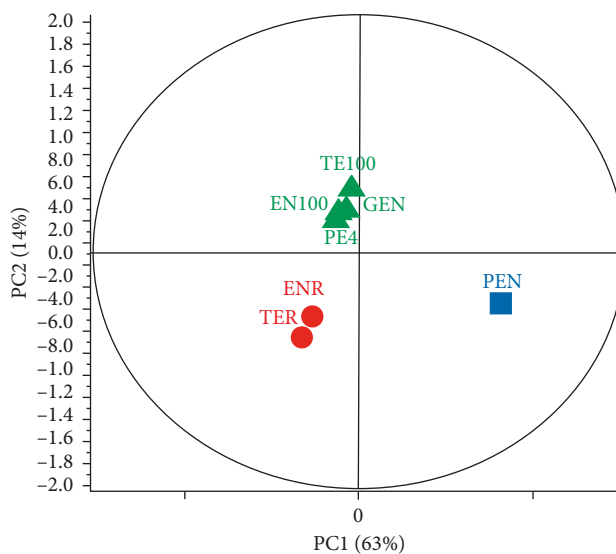| PC	Explained variance (%)	Cumulative variance (%)
PC1	81.9197	81.9197
PC2	13.6470	95.5667
PC3	3.6040	99.1707
PC4	0.8143	99.9850
PC5	0.0065	99.9915
PC6	0.0045	99.9660
PC7	0.0040	100.0000

FIGURE 3: Hotelling score plot of statistical analysis (PCA) showing clustering data for control milk samples GEN1, GEN2, and GEN3 (blue square); milk with 100μg/L of enrofloxacin, EN100.1, EN100.2, and EN100.3 (green triangles); 100μg/L of terramycin, TE100.1, TE100.2, and TE100.3 (black rhombuses); and milk with 4μg/L of penicillin, PE4.1, PE4.2, and PE4.3 (red circles).

Figure 3 shows the score plot with the clustering of milk samples: control (GEN1, GEN2, GEN3), contaminated with 100μg/L of enrofloxacin (EN100.1, EN100.2, EN100.3), contaminated with 100μg/L of terramycin (TE100.1, TE100.2, TE100.3), and contaminated with 4μg/L of penicillin (PE4.1, PE4.2, PE4.3).

From Figure 3, it is clear the formation of clusters resulting from the high degree of similarity between groups of samples. Four groups are present, one in each quadrant. Group 1 (squares) refers to genuine milk (control samples) and is located in the first quadrant. Group 2 is located in the second quadrant (circles). This cluster represents a group of contaminated milk samples with 4μg/L of penicillin. The third quadrant contains elements of group 3 which is related to contaminated milk samples with 100μg/L of enrofloxacin (triangles), while the fourth quadrant is occupied by group 4 (rhombuses) containing contaminated milk samples with 100μg/L of terramycin. Among the elements of the groups, none is far apart from each other which discard the presence of outliers. The PCA accurately discriminated the samples in groups despite the very low concentration of the antimicrobials. From PC1, one can observe that the penicillin and enrofloxacin have the same score in contraposition to terramycin and genuine milk. This trend may be depicted in the absorption spectra (Figure 2). Therefore, PC1 represents the degree of milk contamination with antibiotics. Note that the group of data containing genuine milk is close to the centre of the axis. The contaminated clusters have different distances and different positions from the centre. This is related to the fact that the concentrations of the drugs are different. For example, samples with 100μg/L of medication (groups 3 and 4) have similar position. From the above reasoning, we are led to infer that PC2 is related to the milk similitude.

All samples analysed until now were raw milk. Concerning pure antimicrobial data one may verify that the PCA
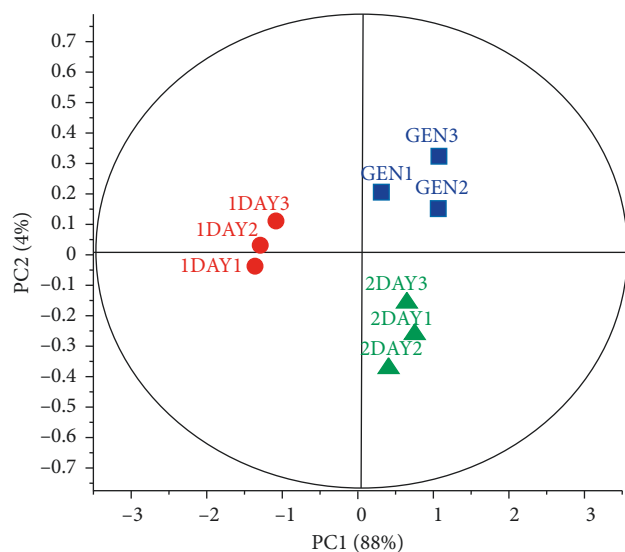
FIGURE 5: Score plot of statistical analysis (PCA) for samples of genuine milk (GEN) and milk collected for 2 days after the cow took the ceftiofur hydrochloride.

TABLE 4: Explained and cumulative variances of samples of genuine milk and milk collected for 2 days after the cow took the ceftiofur hydrochloride.

PC	Explained variance (%)	Cumulative variance (%)
PC1	99.7444	99.7444
PC2	0.1629	99.9073
PC3	0.0170	99.9243
PC4	0.0153	99.9538
PC5	0.0143	99.9538
PC6	0.0137	99.9675
PC7	0.0114	99.9790
PC8	0.0109	99.9898
PC9	0.0102	100.0000

is also able to discriminate between them. Figure 4 deals with such fact. It shows the PCA of average values of reflectance spectra of genuine milk, pure enrofloxacin, pure terramycin, and pure penicillin, and milk-enrofloxacin in 100 μg/L, milk-terramycin in 100 μg/L, and milk-penicillin in 4 μg/L, respectively. It can be seen that the milk-like samples (GEN, EN100, TE100, and PE4) are located very close in relation to PC1 which indicates the degree of similarity between them (cluster formation). For this reason, the samples of enrofloxacin antimicrobial (ENR), penicillin antimicrobial (PEN), and terramycin antimicrobial (TER) are so far apart from the group. Pure penicillin sample is detached from the other clusters because it was the only solid matrix (powder), while the others were liquid.

Table 3 shows the explained and cumulative variances for each of the principal components of the average values of pure enrofloxacin, pure terramycin, pure penicillin, genuine milk, milk-enrofloxacin and milk-terramycin in 100 μg/L each, and milk-penicillin in 4 μg/L.

Figure 5 shows the methodology applied in a real situation. It shows data of a control milk, without medication,

and contaminated milk in two consecutive days after the administration of the drug ceftiofur hydrochloride in a cow. Table 4 has the respective explained and cumulative variances from the data of Figure 5.

Cluster formation was observed for the genuine milks (GEN1, GEN 2, and GEN3) and one-day and two-day drug administered milk (1DAY and 2DAY). It turns out that the GEN and 2DAY groups are along the same PC1, showing the similarity between the groups. This is due to the metabolization of the antibiotic in the milked cow after two days of the drug administration. Reinforcement of this supposition is apparent when compared with one day milked sample with genuine milk. The PC2 is connected with time of milking as the spectroscopic measurements were performed after the last day of milking (2DAY).

4. Conclusions

This article dealt with the identification of the antimicrobials enrofloxacin, terramycin, penicillin, and ceftiofur in milk samples in the MRL permitted by regulatory agencies. The methodology developed is based on the combination of Fourier transform near-infrared (FT-NIR) spectroscopy jointly with principal component analysis (PCA). The experimental procedure was able to detect antibiotics traces in a fast and accurate way. The methodology was applied also to detect ceftiofur hydrochloride in milk of a cow where the drug was administered. Beyond trace detection, we are also able to follow the metabolization process in the animal. Our results clearly demonstrate the potentiality of the method for the development of a portable prototype.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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