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RESEARCH ARTICLE

An Easy Efficient Method of Veterinary Drug Residue Analysis in Raw Milk by RP-HPLC-UV with Application to Raw Milk

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Abstract: ***Background*:** This study reports an easy method of a veterinary drug investigation in raw milk, based on QuECHERS extraction followed by RP-HPLC-UV analysis. Use of this benchtop system was motivated by its availability and moderate cost relatively to other sophisticated methods such as LC-MS which are more efficient.

***Methods*:** This developed method has been optimized and then after validation according to EU legislation, it demonstrated good linearity with R^2>0.997, acceptable peak resolution within a short time (<9.5 min) and good recovery of the analyzed drugs (OXY, ALZ and IVR, respectively 87.08, 99.02 and 92.01 %). Additionally, we applied the method to the analysis of cow milk, collected in Nouakchott, capital of Mauritania.

***Results*:** The obtained results indicated a mixed level of drug use according to targeted molecules. In 42% of sampled farms, the anti-parasitics IVR and ALZ were detected whereas the antibiotic OXY was detected in 50%.

***Conclusion*:** This investigation shows that 17% of the sampled farms exceeded European standards for IVR drug.

Keyword: Veterinary drug residue, oxytetracycline, ivermectin, albendazole, QuECHERS method, sahel region.

1. INTRODUCTION

Populations in the Sahelian region, being essentially rural, rely on livestock for their subsistence. Also, this population is very keen to intensive daily consumption of raw milk [1]. In Mauritania, during last few decades, several milk producing companies, such as Tiviski, Toplait, Elwatania, Savaa, and SMPL society, emerged, leading to a phenomenal multiplication of small traditional milk producers where raw milk is sold out over main roads in Nouakchott, the capital of Mauritania. Moreover, it is remarkable that Mauritanian breeders, like their colleagues in the Sahel region, intensively use veterinary drugs to combat diseases affecting livestock and impeding milk production improvement [2-4]. Thus a relatively recent survey carried out in 2012 indicated that 44.4% of breeders make use of auto-medication for their animals and, from these ''automedicators'', 98 % use veterinary drugs such as oxytetracycline, ivermectin and albendazole [5]. Unfortunately, this uncontrolled use may originate toxic chemical residues in animal products [6, 7]. Consequently, it may affect, sometimes gravely, health security of foodstuffs causing perilous hazards for consumers, such as direct toxicity; allergy symptoms; carcinogenic effects and bacteriological resistance against human drugs [8-10]. Like other Sahelian countries, Mauritania has initiated a national comity of Codex Alimentaris and adopted national hygienic code [11]. This regional regulatory requirement, in addition to international regulations, such as European Union (EU) and U.S. Food and Drug Administration (FDA), shows the necessity for the elaboration of efficient analytical methods to analyze veterinary drugs residues in foodstuffs [12-18].

Several authors have been developing analytical methods using sophisticated equipment such as liquid chromatog-

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raphy coupled to mass spectrometry [19-33] in order to cope up with this issue. These methods are able to determine simultaneously up to hundred pesticides in complex foodstuff matrices. Our goal is to find an efficient sample benchtop method, being simple, fast and effective, able to simultaneously analyze veterinary drug residues in milk.

As indicated in the introduction, Mauritanian breeders, like other Sahelian, use intensively veterinary drugs to fight different diseases. The mostly used veterinary drugs are oxytetracycline; albendazole and ivermectin. That is, we have chosen these 3 veterinary drugs as the most hazardous in milk matrices from the Sahelian region.

2. MATERIALS AND METHODS

2.1. Chemical Products and Reagents

All the reagents [methanol and CH_3CN (HPLC-grade), anhydrous $MgSO_4$, formic acid (99.7 %), acetic acid (99 %), sodium acetate, disodium ethylenediamine tetraacetate dihydrated (Na_2EDTA, $2H_2O$) and Standard veterinary drugs (OXY, IVR and ALZ) were acquired from Sigma-Aldrich France. From these veterinary drug standards, a mother solution, 1000 µg/kg for each, was prepared, weighing precisely the necessary quantity of powdered standard and dissolving it in acetonitrile and 0.1% formic acid solution (10/90, v/v). 0.1 M Na_2EDTA was prepared by dissolving the necessary quantity of Na_2EDTA, $2H_2O$ in pure water solvent. The mixture was stocked in a dark place under -20°C.

2.2. Collection and Analysis of Raw Milk

100 mL milk samples of cow raw milk were collected opportunistically from 12 Nouakchott neighbouring farms, in August 2013 and September, 2017. They were transferred immediately to the laboratory and conserved in the refrigerator at -20°C and, later, analyzed by RP-HPLC-UV method 1.4 Equipment. The analysis was carried out using RP-HPLC-UV system, equipped with a reverse phase column (Luna, phenomenex) C18-type (150 mm × 4,6 mm, 5µm), Quaternary pump (DionexUltimat 3000) and a 1000 UV detector supplied by Thermo Fisher Scientific (Spectra SYSTEM UV). Also, the used centrifugator was from Thermo Fisher Scientific. Vortexing was carried out with the aid of Heidolph vortex (model Reax 2000).

2.3. Chromatographic Conditions

First, the C_{18}-type column was equilibrated with a mobile phase consisting of a mixture of aqueous formic acid 0.1% and acetonitrile. Before use, the mobile phase was degassed by applying a vacuum on the solvent tank approximately 15 min. The following chromatographic parameters were maintained: C_{18} column temperature at 25°C; flow rate 0.5 mL/min and 1 mL/min (Table **1**); wavelength at 254 nm and, finally, the injected volume was 50 µL. Calibration curve was obtained using standard solutions prepared in acetonitrile-water mixture 30:70v/v (water was slightly acidified) with the concentrations:0.1, 0.2, 0.5, 1, 2, 5, 7 and 10 µg/kg. Table **1** presents the optimized chromatographic program used to separate OXY, ALZ and IVR mixture.

2.4. Preparation of Analytical Sample

Extraction of veterinary drug residues from raw milk has been carried out according to a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure which necessitates a preliminary separation of the analysed molecules and other milk constituents, through several extraction and concentration steps [14]. A 5g sample of raw milk was transferred to a 50 mL centrifugation tube adding to it 5 mL of 1 % acetic acid solution (prepared in acetonitrile) and 5 mL of 0.1 M Na_2EDTA solution. After mixing and vortexing for 1 min, 2 g of anhydrous $MgSO_4$ and 0.5 g of CH_3COONa were added to this mixture. After homogenization and centrifugation for 5 min, a 2 mL of organic phase was taken off and filtrated through 0.20 µm. Finally, 50 µL from the filtered organic phase was injected into the RP-HPLC-UV system.

2.5. Procedure of the Method's Validation

The method was validated according to EU legislation 2002/657 / CE [14]. Therefore, several validation parameters, such as repeatability, linearity, recovery, precision, quantitation and detection limits were estimated using external calibration through 5 concentrations. A series of tests were conducted for 4 successive days with 3 measures per every day for blank sample (crude milk) doped with a mixture of 3 veterinary drugs concentrated at 10 µg/kg for each.

2.6. Statistical and Graphical Analyses

In order to assess method performance parameters, we have used triplicate samples for the evaluation of peak area, mean, standard deviation and correlation coefficient R^2. All of these parameters were calculated with the aid of the Microsoft Excel software (Microsoft® Office Excel 2010). Similarly, this software was used to construct graphics related to calibration, linearity and precision. *The software* ChemBio-Draw Ultra 12 was used to sketch chemical structures.

3. RESULTS AND DISCUSSION

Review of the published literature indicates that LC-MS (qMS, tofMS; tMS) is the most used technique [19-23,25-33]. We remark that RP-HPLC-UV is rarely used for the analysis of veterinary drugs in foodstuffs, even though some authors have used this system for analyzing pesticides in waters [34-37]. So, we chose to investigate an accessible technique like RP-HPLC-UV for analysing veterinary drugs

Table 1. Optimized RP-HPLC-UV chromatographic gradient.

RT (min)	0	4	5	6	7	11
Flow rate (µL/min)	500	500	500	1000	1000	500
Mobile phase (H_2O/CH_3CN)	70/30	70/30	35/65	10/90	10/90	70/30

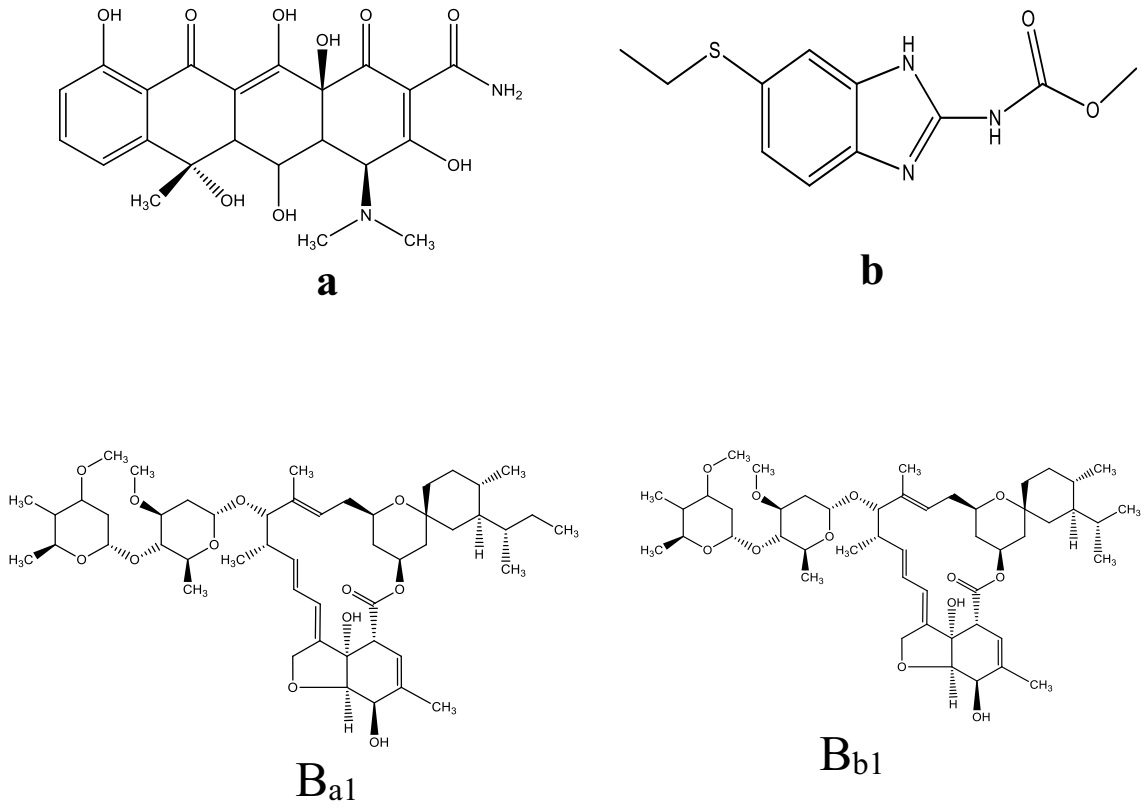

Fig. (1). Structures of analysed drugs (a: OXY; b: ALZ; c: (Ba1, Bb1): IVR).

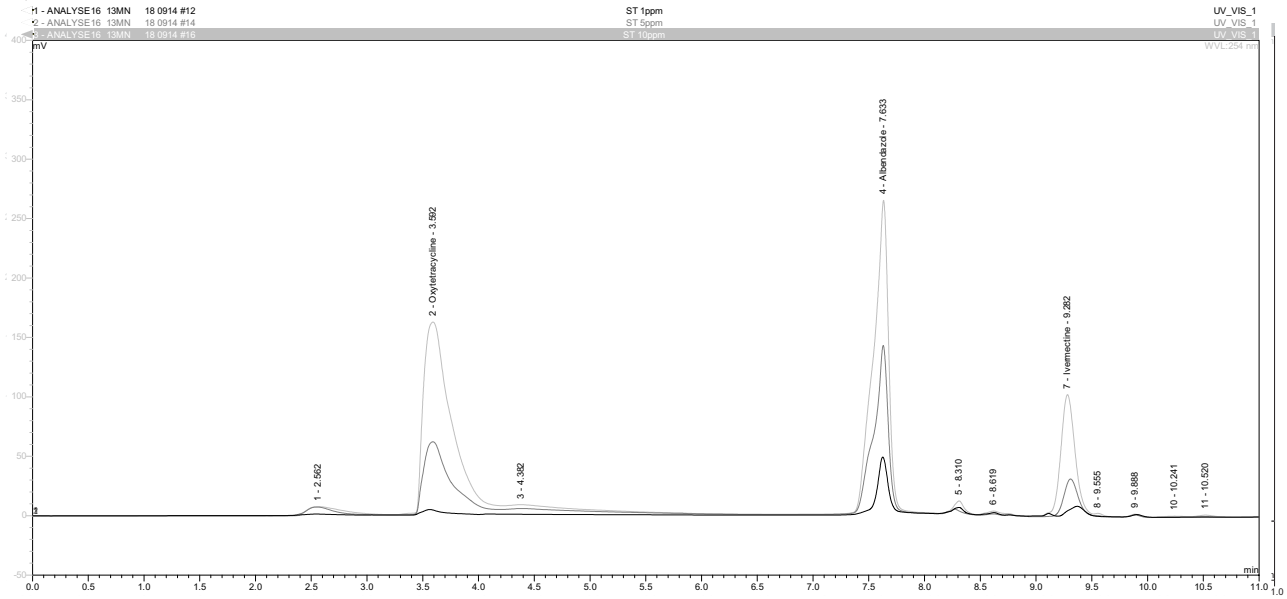

Fig. (2). Superimposed chromatograms of different concentrations (1, 5 and 10 µg/Kg).

Table 2. Validation parameters for 3 drugs, OXY; ALZand IVR.

Parameter / Drug	RT(min)	R^2	Recovery (%)	RSD (%)	LOD (µg/kg)	LOQ (µg/kg)	MRL* (µg/kg)
OXY	3.3	0.997	87.08	15.82	20	60	100
ALZ	7.5	0.998	92.02	5.30	5	15	100
IVR	9.3	0.997	99.01	10.35	0.7	2.0	10

*MRL values according to [15, 20].

in milk matrices. That is we developed a method based on RP-HPLC-UV analysis after extracting veterinary drugs with the aid of a modified QuECHERS procedure.

3.1. Validation of the Developed Method

Validation of this method was done according to the European Union legislation 2002/657/ CE which is based on linearity, precision and limit of detection [17].

3.1.1. Calibration Curve

The calibration curve was drawn for the concentration range 0.1-10 µg/kg. The peak area under wavelength 254 nm was recorded in the function of concentration for each of drug standards (OXY, ALZ and IVR). Fig. (**2**) presents superimposed chromatograms for different drugs concentrations showing good resolution of chromatographic peaks and excellent repeatability for different drug concentrations. Table **2** presents the main parameters of calibration for the 3 drugs. Analysis of these results allowed us to validate the method which showed good linearity of the peak area in the function of drug standard concentration ($R^2 \geq 0.997$), excellent repeatability of retention times and lower Limits of Quantitation (LOQ) which were smaller than MRL.

3.1.2. Precision

Relative Standard Deviation RSD is the absolute value expressed as the percentage of Standard Deviation relative to mean value (% RSD = (standard deviation) x 100/mean). This parameter, calculated from 3 measures, is used as a measure of both precision and repeatability of the developed method.

3.1.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Two closely related analytical parameters, the LOD and the LOQ, may be defined as the smallest content from which the analyst is detected or can be quantified, respectively [18]. These 2 parameters have been determined from several experimental assays and estimated with the following expressions: $\mathrm{LOD} = \frac{3.3 * Sy}{a}$, $\mathrm{LOQ} = \frac{10 * Sy}{a}$

(Sy = standard deviation of estimation and a = slope of regression line [13]).

3.1.4. Maximal Residue Limit

Maximal Residue Limit (MRL) is the maximal residue content of the veterinary drug in foodstuffs, commonly in µg/Kg, which cannot be surpassed. The concentration corresponding to MRL might not cause any scientifically proven health hazard for consumers [15, 20]. This threshold limit is fixed by Codex Alimentarius and other international organizations, such as EU and USA agencies [12, 13].

3.1.5. Optimization of Sample Preparation and Analytical Technique

3.1.5.1. Optimization of the Extraction Procedure

The extraction procedure of veterinary drugs multiresidues in milk may be very often the most sensitive part of the sample preparation, because of different properties of chemical substances which might be extracted simultaneously [21] as well as the extraordinarily rich milk matrices, being enriched in organics and minerals (water, mineral salts, proteins and vitamins) [21-23]. Thus we used sodium acetate to precipitate proteins, Na_2EDTA for complexing mineral salts and anhydrous magnesium sulfate for eliminating water. This preliminary preparation step, followed by successive extraction, allowed a significant enrichment in targeted molecules. Table **2** shows a weaker recovery of OXY relatively to IVR and ALZ. This may be attributed to the tendency of tetracycline to form chelate complexes with cations present in milk such as calcium and magnesium [19]. Fig. (**3**) presents a chromatogram of milk sample which was doped with 10 µg/kg of each of 3 targeted compounds OXY relatively to IVR and ALZ.

3.1.5.2. Optimized Wavelength

The efficiency of RP-HPLC-UV analysis depends closely on wavelength at which the analyte is detected. Therefore spectra of 3 drugs were recorded and λ_{max} (nm) was determined as for oxytetracycline (254, 270, 380); albendazole (230, 254, 300); ivermectin (254). These values were observed in conformity with the values reported in the literature. These values show that wavelength 254 nm is common to all 3 veterinary drugs where they absorb intensively. Therefore, we chose this wavelength as optimal.

3.1.5.3. Optimization of RP-HPLC-UV Parameters and Validation of the Method

Table **2** below presents the main validation parameters, such as relative standard deviation, limit of detection, limit of quantification, maximal limit of residue, linearity, retention time and interference ratio for all 3 targeted veterinary drugs.

As shown in Table **2**, recoveries for OXY; ALZ and IVR were 87.08, 99.02 and 92.01%, respectively. These recoveries are acceptable, being in the interval of 70-130%. LOQ values were lower than that of MRL demonstrating the applicability of our developed method for trace analysis. In addition, the short duration of analysis (≤9.5 min) highlights

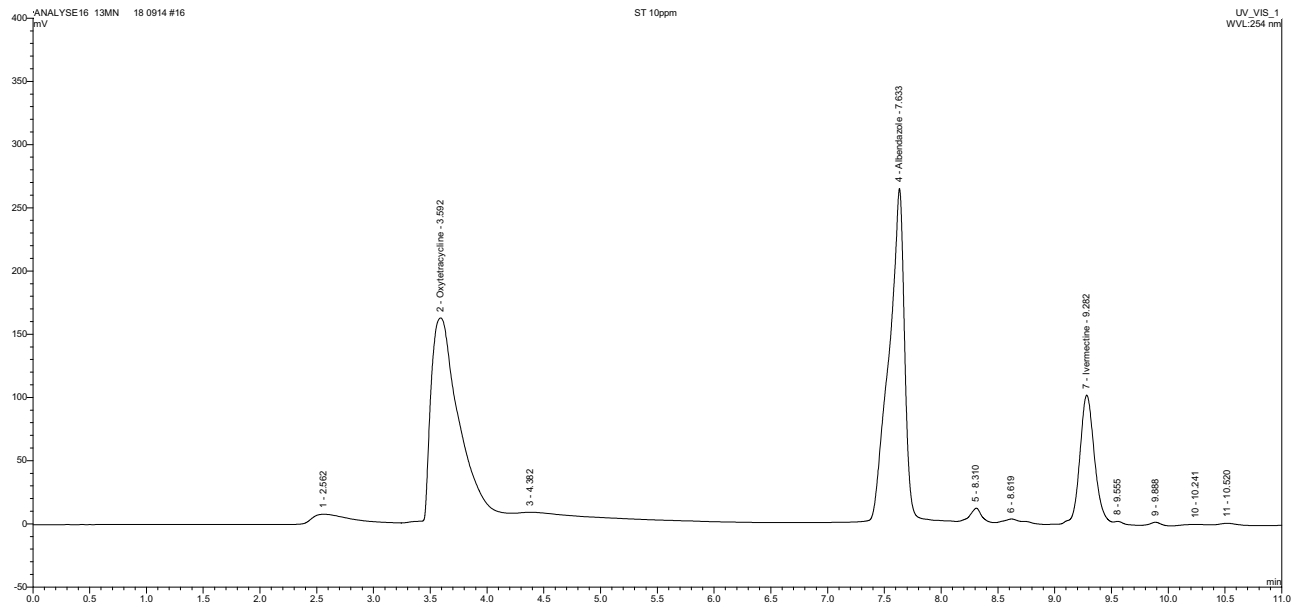

Fig. (3). Chromatogram of a milk sample doped with 10µg/Kg of 3 veterinary drugs.

the fact that this method is quite rapid for detection and quantitation of veterinary drugs in milk. However, we observed that the recovery of OXY was weaker, relatively to the other 2, ALZ and IVR as shown in Table **2**. This diminished recovery goes in favor of OXY complexation in the presence of mineral salts such as calcium and magnesium [19]. Repeatability, assessed by RSD, was 15.90; 5.30 and 10.35% for OXY; ALZ and IVR, respectively. Being comparable to the published data, these values are good for the two last veterinary drugs and substantial for OXY. However, the RSD value for OXY is comparable to that found by Aguilera-Luiz et al. and Zhan et al. in the same matrices [22, 29]. In addition, all these values were lower than 20% showing an acceptable level [14]. Correlation coefficients of linear regression from standard curves for OXY, ALZ and IVR showed good linearity (R^2 ≥0.99) over the concentration range 0.1-10 µg/kg. Additionally, these parameter values are comparable to the values reported in the literature [25-33].

3.2. Application of the Developed Method to Raw Milk Analysis

The collected samples were analyzed by extracted QuECHERS method and then by RP-HPLC-UV. Our preliminary survey shows that farmers use anti-parasitic IVR/ALZ against widespread diseases linked to parasites like internal/external worms; scabies and paramphistomum (locally known as 'Imendie'), whereas the molecule OXY is used as a general anti-microbial drug to alleviate wounds and common illnesses. The anti-parasitic residues IVR/ALZ may originate direct toxicity; allergy symptoms and carcinogenic effects whereas OXY molecule is linked to bacteriological resistance against human drugs [10]. It is very difficult to classify sampled farms according to fixed typologies like treatment history and the used drugs because breeders do not provide such information, fearing for official control and customers caprices. That is why we divided the sampled farms into 4 categories according to the presence or absence of detectable level of drugs: a batch with detectable ALZ residue; a batch with detectable IVR residue; a batch with detectable OXY residue and, finally, a batch with no detectable residue of the 3 drugs. Results presented in (Fig. **4**) show that OXY is the most frequently detected in samples with 50% detection frequency and a range of 25-50 µg/Kg for samples with detectable levels, whereas anti-parasites OXY, ALZ and IVR have a detection frequency of 17 and 33%, respectively [34-36]. Concentrations (µg/Kg) of samples with detectable level are as follows:

- IVR: 30; 25; 15; 15 for sample 3; 5; 6; 8 respectively

- ALZ: 60 and 30 for sample 1; 3 respectively

- OXY: 30; 30; 50; 35; 25 for samples 1; 2; 3; 7; 8 respectively.

We observed that in 33% of samples, there was no detectable level of the 3 veterinary drugs and that concentrations of 2 drugs OXY and ALZ were below the acceptable level (milk MRLs are 100 µg/Kg for both). Unfortunately, in 17% of sampled farmers, the concentration exceeded milk MRL value (20 µg/Kg for IVR).

Furthermore, it is known that MRL values for toxic residues in milk are established for a daily intake of 1.5L [17] which is pertinent to European daily consumption. However, this may be largely over-estimated for Sahelian consumption habits, particularly for women. Indeed it is known that Mauritanians, for example, have excessive consumption of locally produced raw milk [37, 38]. This study indicates a real hazard linked to uncontrolled farming habits of antibiotics use for Mauritanian population and generally for Sahelians. This preliminary investigation must be completed by further analysis with a more detailed and lasting monitoring of samples at known intervals from veterinary drugs treatment. Also, this developed method must be applied to other milk matrices like meat; eggs; vegetables and camel milk.

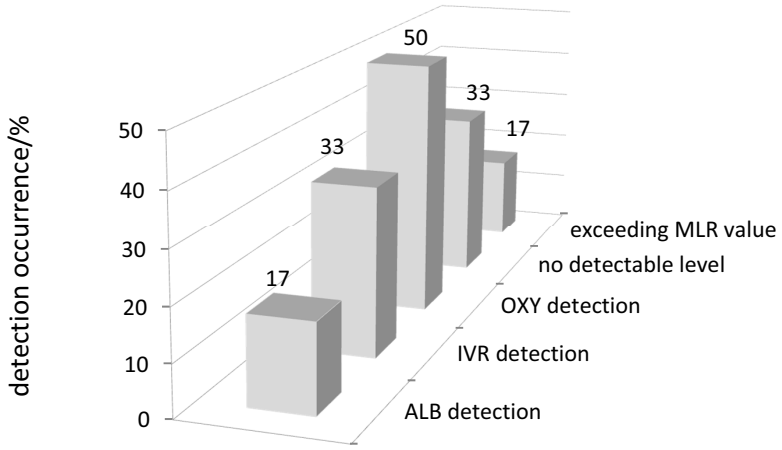

Fig. (4). Frequency of drugs detection in sampled farms.

3.3. Advantages of the Developed Method

Published methods use expensive sophisticated systems, such as LC-qMS; LC-TOFMS; LC-MS/MS; LC-orbitrapMS, for the analysis of veterinary drugs in foodstuffs [21-23, 25-33] which are more efficient. However, to the best of our knowledge, there is no developed method using LC coupled to UV detector for veterinary drugs analysis in foodstuffs. The main advantages of this method are cheapness and rapidity, being developed with available equipment, particularly in the Sahel region. The used wavelength, 254 nm is of low selectivity, giving potential perspective to extend the application to other drugs and other matrices. That is, the application of the developed method to crude milk analysis has offered promising results.

CONCLUSION

The developed method, based on QuECHERS extraction followed by RP-HPLC-UV analysis, has been validated according to EU legislation 2002/657 /CE. This validation was based on several parameters, namely repeatability, linearity, recovery, precision, limit of quantification and limit of detection. The performance of the developed method was demonstrated by linearity ($R^2 \geq 0.997$); rapidity ($\leq$ 9.5 min); good recovery (87-99%) and an acceptable LOQ < MRL. This method proved to be a simple, rapid and cheap analytical procedure for analysis of veterinary drug residues in milk matrices.

Application of this method to the analysis of 12 milk samples acquired from 12 Nouakchott neighboring farms indicated a mixed level of the targeted drugs. Although the detected residues were under MRL for OXY and ALZ, it was remarkable that in 17 % of the samples, concentrations of very toxic IVR significantly exceeded the MRL value (20µg/Kg). These results indicate the applicability of the developed method for analysis of veterinary drugs residues in milk matrices. However, it is clear that further analysis with a more detailed sampling at known intervals with monitoring of drugs use is needed. Also, it is very useful to make sensitization of milk chain production stakeholders for suitable hygienic practices.

As a perspective, we intend to further improve this technique and extend applicability scope to other matrices such as meat stuffs, varied milk; beverages and veterinary pharmaceuticals.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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