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Evaluating the Presence and Depletion of Residual Ivermectin in Milk Following Subcutaneous Injections on Lactating Cows

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ABSTRACT

Dairy farming industry in Kenya has experienced significant growth due to the rising consumption of milk and dairy products. Consequently, to improve output, veterinary drugs were administered prophylactically and/or therapeutically to dairy cattle. Commonly used drugs included beta-agonists, anthelmintics, antibiotics, and steroid hormones. Parasitic diseases more so tick infestations hindered production. Chemical methods were primarily used in tick management however, the technique proved expensive and developed resistance. Conversely, ivermectin effectively controlled ecto- and endoparasites in livestock, hence gaining popularity in tick management. Nonetheless, its residues accumulated in animal tissues, rendering it unacceptable on animals that produced milk for human use. Apparently, farmers in tick-infested areas in Nyandarua County, Kenya used ivermectin, but there lacked adequate evidence to show that it occurred in milk. Thus, this study examined the performance of Liquid Chromatography tandem mass spectrometry (LC-MS-MS) in order to analyse the presence and depletion pattern of ivermectin residues after it excreted in cow milk following subcutaneous treatment of dairy cows. Between day 0.5 and day 57 following treatment, 24 milk samples were purposefully collected and stored at -20°C.. The samples were prepared using QuEChERS, a solid-phase extraction technique. Further, positive Electrospray ionization (+ESI) LC-MS-MS qualitative analysis identified a precursor ion with the mass number of m/z 897.5 and corresponding product ions—m/z 897.5 > 329, m/z 897.5 > 240, and m/z 897.5 > 183—within a retention time of 8.782-8.858 (mean = 8.362±0.002) minutes. The solvent and matrix-matched calibration curves displayed satisfactory linearity with r=0.998 and 0.993 as their respective correlation coefficients. Additionally, the limit of detection was 2.5 ng/ml and limit of quantification reached 10 ng/ml. The analyte recovery ranged from 74.977% to 101.435%, within satisfactory limits. Hence, ivermectin residues were quantified in milk samples from the 0.5th to 17th day. The highest concentration (60.90±0.98 ng/ml) was observed on 2nd day. No traces of the residue were detected beyond day 18 and as a result, the study recommends a withdrawal period of 18days after subcutaneous ivermectin treatment. It is therefore important to perform further research on other dairy and animal products that people consume.

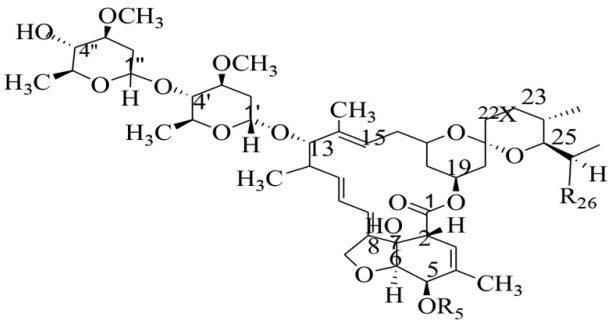
Keywords: avermectin, ivermectin, endectocides, residue, milk

INTRODUCTION

In Kenya, the milk production system operates on two scales: large-scale and small-scale, distinguished by operational size, management level, and input usage. However, small-scale farming has been the dominant force in the industry (Omore *et al.,* 2005). Dairy products have traditionally been considered crucial for a healthy diet among the population (Neumann *et al.*,

2002). Nevertheless, contamination can occur when anthropogenic contaminants are exposed to milk through a variety of sources, including contaminated hay, water, or grass. Hence, the potential for these compounds to bioaccumulate in the body has an adverse effect on the quality of milk produced (Raza & Kim, 2018). Additionally, veterinary drugs are regularly administered to female ruminants (such as cows, goats, and sheep) to enhance milk production and overall productivity. These drugs include pesticides for animal pest control, antibiotics and sulfonamides to cure bacterial related infections, anthelmintics for eliminating internal parasites, as well as beta-agonists and steroidal hormones used to stimulate growth (Lopes *et al.*, 2011; Stolker & Brinkman, 2005). More so, to combat parasitic infestation and ease the negative effects brought about by parasites, farmers primarily utilize veterinary drugs (Lopes *et al.,* 2015; Thornton, 2010). The most common and efficient methods for controlling ticks and other flying insects include the use of chemicals, such as acaricides. However, studies indicated that ticks are becoming resistant to different classes of acaricides which include; cypermethrin, chlorpyrifos and amitraz (Abbas *et al.*, 2014; George *et al.*, 2004). Similarly, their toxicity, high cost and proclivity to accumulate in animal-derived foods along with the environment as residues is a matter of great concern. Therefore, there is need to turn to other effective control techniques, such as vaccines (Rajput *et al.*, 2006) or plant-based repellents safe to the ecosystem (Wanzala *et al.*, 2018).

In veterinary medicine, macrocyclic lactones or macrolides (MLs) have been employed for their potent anthelmintic properties. This class of drugs comprises avermectins and milbemycins (Hennessy & Alvinerie, 2009). They have demonstrated effectiveness against various internal parasites, particularly nematodes at all life stages, and have proven effective against economically significant gastrointestinal and lungworm infections. Trials have also shown their efficacy against arthropod parasites that affect domestic animals, including biting flies, ticks, mites, and parasitic larvae (Campbell, 2012; Canga *et al.*, 2009; Campbell *et al.*, 1984; Chabala *et al.*, 1980). Importantly, these drugs have been successfully used in the treatment of human filarial worm infections (Campbell, 1989). The soil-dwelling bacterium *Streptomyces avermitilis* produces these macrolide endectocides as fermentation products. Among them, eight distinct avermectins exist, designated as A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, $B_{2a,}$ and B_{2b} with compound **1** serving as a representation of their general structures (Campbell, 2012).



Compound **1**

In relation to avermectin (1), the "A-series" molecules in subset 1 contain a methoxyl moiety at the C5, while the "B-series" molecules in subset 2 have an unmodified "hydroxyl group". All compounds in subset 1 exhibit an "olefinic bond between C22 and C23", whereas compounds in subset 2 have a "hydroxyl group" present at C23 due to double bond hydration. (Prichard *et al.*, 2012). The structural variations between the two subsets are illustrated in table 1, with reference to compound 1.

Table 1: Structural differences between avermectin subsets 1 and 2

Avermectin Subsets	R_2	R_{26}	C_{22}-X-C_{23}
A_{1a}	CH_3	C_2H_5	-CH=CH-
A_{1b}	CH_3	CH_3	-CH=CH-
B_{1a}	H	C_2H_5	-CH=CH-
B_{1b}	H	CH_3	-CH=CH-
A_{2a}	CH_3	C_2H_5	-CH_2-COH_2-
A_{2b}	CH_3	CH_3	-CH_2-COH_2-
B_{2a}	H	C_2H_5	-CH_2-COH_2-
B_{2b}	H	CH_3	-CH_2-COH_2-
Ivermectin	H	>80% C_2H_5 <20% CH_3	-CH_2-CH_2-

Hydrogenation of *cis* 22, 23-double bond in avermectin B1 results in the production of 5-O-dimethyl-22, 23-dihydro-avermectin B_{1a} and B_{1b} in an 80:20 ratio, which collectively formed ivermectin. This conversion is achieved using Wilkinson's catalyst; chlorotris (triphenyl-phosphine) rhodium (I), under varying H_2 pressure (1 to 150 bars) and temperatures (60 to 100°C) conditions (Campbell, 2012).Chemically, ivermectin constitute a combination of two structural isomers with varying degrees of toxicity and various modes of action. There two homologues, B_{1a} and B_{1b}, possessed distinguishing properties at the alkyl group at the R26 position, as indicated in compound 1 and table 1, and they were present in ratios of at least 80:20, respectively (Campbell, 1989). Furthermore, the B_{1a} and B_{1b} components had molecular weights of 875 and 860, respectively, with distinct formulae: $C_{48}H_{74}O_{14}$ and $C_{47}H_{72}O_{14}$ respectively (Campbell, 1992). The 22, 23-dihydro-avermectin B_1 was the 1st avermectin to be commercialised (Chabala *et al.*, 1980).

Ivermectin was available in subcutaneous and topical forms for animal use. However, there were concerns over its use since it was not specifically recommended for use on nursing animals whose milk was intended for human consumption, despite being affordable and effective against ticks (Sanderson *et al.*, 2007; Schenck & Lagman, 1999). Research have shown that misuse or failure to observe withdrawal periods before slaughtering or milking treated animals resulted in unwanted residues in food products (Teresiah *et al.*, 2016; Souza *et al.*, 2010). To ensure safe use of these drugs on nursing animals, maximum residue limits (MRLs), which are the safest concentrations permitted for human exposure, have been determined to assure consumers' safety (Whelan *et al.*, 2010). Consequently, international food safety bodies such as the Codex and European Union recommended MRLs in milk for various veterinary drugs, including ivermectin, and prohibited the use of those that persisted in foodstuff (Orwa *et al.*, 2017). Both bodies provided 0µg/kg MRL and a withdrawal period of 35days for injectable ivermectin (Kinsella *et al.*, 2009; Durden & Wotske, 2009). Primarily, Nyandarua County was a high milk producing area in relation to the high populations of dairy cows (Muia *et al.,* 2011). Moreover, tick infestation was a constraint to animal production (Maingi & Njoroge, 2010) and the information obtained from farmers through randomly distributed questionnaires made it clear that ivermectin injections were used to control tick populations. However, most brands were unlabelled and did not indicate withdrawal periods and the only information provided by most manufacturers was on milk withdrawal-time caution labelled as "not to be used within 2 months of calving". In this regard, there was inadequate information regarding occurrence of ivermectin residue in milk. In order to carry out qualitative and quantitative analysis on ivermectin molecule, the study optimized the performance of the solid phase extraction method that used QuEChERS and +ESI LC-MS/MS analysis technique. This made it possible for the study to assess if ivermectin residue was present and how it depleted in milk collected from lactating cows after ivermectin was administered subcutaneously. In view of this, the information generated can provide a withdrawal interval for milk after subcutaneous treatment. Besides, relevant bodies can advise farmers on the adoption of suitable tick control techniques and regulate the use of ivermectin as a matter of public health safety.

MATERIALS AND METHODS

Study design and area

A questionnaire was developed and administered randomly to different farmers to establish the mode of tick control in Nyandarua County. This is because Nyandarua County is a high- milk production zone in Kenya, with estimated annual amounts of approximately 40 million liters (Ministry of Livestock Development Department and Fisheries 2012). A farm was identified for sample collection in Matura, Magumu ward, South Kinangop sub-county, Nyandarua County, 0°49'43.4"S 36°35'13.3"E -0.828722, 36.587028, where cows were treated with the recommended dose of 0.2mg/kg of body weight subcutaneously.

Sample collection

The study applied purposive sampling technique. Over the course of nine weeks, 24 consecutive 250 ml milk samples were collected from a treated cow between 27th June 2019 and 23rd August 2019. The time interval was; after 12hours, 24hours, and on days 3, 4, 6, 9, 12, 15, 17, 19, 21, 22, 26, 29, 33, 36, 40, 43, 46, 47, 48, 50, 54 and 57. The samples immediately transported to Central Veterinary Laboratories, Kabete at room temperature after collection, and then refrigerated at -20^{o}C as described by Henshall, 2012 and Monardes *et al.*, 1996, where qualitative and quantitative studies were performed to detect and quantify ivermectin residues.

Standard, chemicals and reagents

Ivermectin standard (96.03%) (Norbrook Kenya Limited), deionized water (Merck KGaA), Acetonitrile (MeCN) (Fisher Scientific), methanol (HPLC grade) (Sigma-Aldrich), 98% Formic acid (analytical grade) (Sigma-Aldrich), Pre-packed 5g (4:1, w/w) portions of $NaSO_4$:NaCl respectively (Fisher Scientific), pre-weighed mixes of 100mg PSA sorbent (99% Sodium hydrogencitrate sesquihydrate- $C_6H_9NaO_8$) with 300mg anhydrous sodium sulphate ($NaSO_4$) (Sigma-Aldrich).

Sample preparation

In 50mL centrifuge tubes, 10g of milk samples were weighed for analysis. 10mL of MeCN was added into the weighed sample after which the tubes were covered and incubated for 10 minutes. The resultant mixture was then shaken gently by hand for a minute. Subsequently, 5g of 4:1, w/w $NaSO_4$ to NaCl was transferred into the solution and immediately vortexed for 1 minute to avert agglomeration during the hydration of $NaSO_4$. After that, the mixture was centrifuged for 5min at 4000 rpm to partition it (Dahiya *et al.*, 2010; Kinsella *et al.*, 2009). A 2mL aliquot of the supernatant obtained was drawn and put into a 15mL mini-centrifuge tube that contained a combination of 50mg 99% PSA sorbent and 150mg anhydrous $NaSO_4$ for sample clean up. The mixture was vortexed for 1 minute to homogenize the sample extract. At this point, the homogenate was centrifuged for 5 minutes at 4000 rpm and an aliquot of 500µL of

the supernatant was was taken, placed in an auto sampler vial, and then diluted with 500µL of LC water. In the end, however, a 10µL aliquot that had been diluted was transferred into the LC-MS/MS equipment. Sample preparation method was adopted from Whelan *et al.*, 2010, Dahiya *et al.*, 2010 and Kinsella *et al.*, 2009.

Preparation of ivermectin standard solutions

Ivermectin standard at a purity level of 96.03%, weighing approximately 0.9603 mg was dissolved in methanol and topped up to volume in a 100 mL volumetric flask. Further, 1mL of the prepared stock solution was transferred to a 100mL volumetric flask, which was then precisely filled to capacity using methanol. This generated a solution of 1µg/mL (1ppm) of ivermectin standard. Further calibration solutions of 1, 2.5, 5, 10, 25, 50, 75, and 100ng/mL were obtained by picking appropriate aliquots from the 1µg/mL standard solution and diluting them with methanol. As described by Whelan *et al.*, 2010, the solutions were kept at 2° to 8°C for use in the generation of solvent calibration curve.

Preparation of matrix-matched calibration standards

Ten (10) grams of blank or control milk portions were transferred into seven separate 50mL centrifuge tubes. The sample were prepared and ivermectin extracted according to standard protocols (Whelan *et al.*, 2010). A volume of 1 millilitre of the respective extracted sample was put into the auto sampler vials. The 1µg/mL stock solution of the standard was used to prepare matrix-matched calibration concentration of 1, 2.5, 5, 10, 25, 50, 75, and 100ng/mL. Hence, matrix-matched calibration curve was developed using matrix-matched calibration standards to demonstrate how the matrix affected the target analyte.

Preparation of mobile phases

The mobile phases constituted of two solutions, A and B, in a 5:95 ratio that were used in an isocratic flow. Solution A was constituted by transferring formic acid in 500mL of water, while solution B was made by adding formic acid in 500mL of methanol. Both solutions contained 0.1% formic acid. The solutions were then sonicated. The approach employed was the same as described by Dahiya *et al.*, 2010.

Preparation of spiked samples

Preparation of spiked samples was as described by Whelan *et al.*, 2010 and Kinsella *et al.*, 2009. Twelve blank liquid samples, each weighing 10g, were placed in 50mL centrifuge tubes. An analyte standard of 10ng/ml (10ppb) was added into the samples. After allowing the mixture to stand for 15 minutes, the sample extraction technique described earlier was carried out. An aliquot of 500µL was diluted with 500µL of LC water and a 10µL aliquot was introduced into the LC-MS/MS instrument for analysis.

Instrumentation and Apparatus

Instrumentation

An Agilent 1290 Infinity II LC system was used for LC-MS/MS analysis. The equipment comprised of an automated sampler, vacuum degasser, a column oven and a binary pump. The mass detector, Agilent 6460 triple quadrupole with an Electrospray Interface (ESI) acquired and process data, whereas analysis was done using Masshunter Workstation software (vB.08.00). In addition, separation and analysis were done using an Agilent ZOBRAX Eclipse Plus C18 column that had dimensions of 5µm, 2.1 x 100mm.

An analytical weighing balance with a readability range of 0.01mg to 180g was applied for measuring mass. A vortex (Digisystem) with a speed of 3000 rpm was utilised for mixing. A Powersonic 405 benchtop sonicator was used to sonicate the samples. Centrifugation was achieved using a Heraeus multifyge X1R high-speed chilled centrifuge.

Apparatus

Centrifuge tubes; non-reusable 50mL Fluorinated Ethylene Propylene (FEP) with screw caps, 2mL mini-centrifuge tubes, autosampler vials, volumetric flasks; 50 to 1000ml, micro-pipettes and disposable micro-pipette tips; 1µL to 1000µL, syringe filters; a diameter of 25mm and pore size of 0.2 microns.

Liquid Chromatography/ Mass Spectrometry (MS) conditions

Separation of components was performed using an Agilent ZOBRAX Eclipse Plus C18 column with dimensions of 5µm by 2.1 x 100mm. The mobile phase, however, was made up of two solutions (solution1 and 2). Solution1 contained 0.1% formic acid constituted in water, while solution 2 contained 0.1% formic acid diluted in methanol. The mobile phase ran in an isocratic mode, which had a flow ratio of 5:95. Meanwhile, the LC column underwent conditioning achieving an optimum temperature of 40°C. The ideal conditions indicated were achieved by introducing a 10.00µL aliquot of a 10µg/mL solution to the ESI interface.

Similarly, the MS was conditioned in the positive electrospray ionization mode using air pressure. More so, nebulization and desolvation processes carried out using nitrogen yielded satisfactory results. The mass spectra of

protonated molecules of ivermectin were obtained using a product ion scan method within the mass range of 100 to 20,000 Da. The following product ions: 897.5>329, 897.5>240, and 897.5>183 were scanned in Multiple Reaction Monitoring (MRM) mode for 50 milliseconds. The most abundant ion (897.5>183) was designated as the quantifier ion, while 897.5>329 and 897.5>240 were used as qualifier ions. The collision energy used was 68v whereas fragmentor voltage was 150v and capillary voltage of 4000v. Gas flow was maintained at 8 l/min and temperature of 325°C. The analysis used nebulizer pressure of 35 psi, sheath gas heater of 325°C and sheath gas flow of 12 l/min.

Optimization of LC-MS/MS for analysis of ivermectin molecule

The study adopted analysis method from Whelan *et al.*, 2010, Dahiya *et al.*, 2010 and Kinsella *et al.*, 2009. Hence, it was assessed for performance using different parameters described below to ensure qualitative and quantitative analysis of ivermectin molecule.

Selectivity and specificity

Selectivity was determined through analyzing eight (8) blank samples of milk that were spiked with ivermectin standard. The objective was to evaluate the effects of potentially endogenous substance interferences. The standard concentration of 1, 2.5, 5, 10, 25, 50, 75, and 100ppb were used to graph the calibration curve. Consistent with the acceptance criteria for the correlation coefficient (r^2) for standard calibration curves (Chhonker *et al.*, 2018; Kinsella *et al.*, 2009), the study recorded and employed a linearity for the analyte of 0.998, which was nearly a perfect linearity of 1. Further, the chromatograms from the blank and spiked samples were compared using the aforementioned method. Besides, by injecting the standard and looking for interference peaks throughout the prescribed retention period, the specificity of the assay was evaluated for the existence of chromatographic interferences that could be present in milk samples (Dahiya *et al.*, 2013; González & Herrador, 2007).

Precision

Repeatability and within-laboratory reproducibility was employed to evaluate precision on 12 milk samples to which 10 ng/ml of ivermectin standard were added. The acquired data was expressed as relative standard deviations (RSD), after which percentage RSD values for recoveries, concentrations and retention time were calculated. The acceptance criteria was set at a precision limit of ≤ 20 % of RSD (Chhonker *et al.*, 2018; Kinsella *et al.*, 2009). Furthermore, the acceptable RSD was determined using the Horwitz equation: RSD (%) = 2^ (1–0.5logC): C represents concentration present or added (Horwitz, 1982).

Recovery of the analyte

Efficiency of the extraction method was assessed using 12 blank milk aliquots spiked with 10ppb of ivermectin standard. The recovery for the analyte in the spiked samples was then determined for each spiked sample using the following formula in accordance with Kinsella *et al.,* 2009:

$$\text{Recovery (\%)} = \frac{\text{Analytical Result}}{\text{True Value}} \times 100 \qquad \text{Equation 1}$$

Linearity and matrix effect

Matrix-matched and solvent calibration curves were constructed using eight-point concentration levels ranging from 1, 2.5, 5, 10, 25, 50, 75, and 100 ng/ml. The calibrants were ran in triplicates, and the data were examined for linearity through linear regression analysis. The equation for the regression line was expressed as y= ax + b: x designates the concentration of the analyte, y represents the detector variable, whereas a and b indicate the slope and y-intercept of the fitted line, respectively. The matrix effect was established by comparing the two curves (Chhonker *et al.*, 2018; Zhou *et al.*, 2017). To minimize interfering matrices and reduce matrix effects, the study employed QuEChERS sample preparation and clean-up techniques (Kinsella *et al.*, 2009; Zhou *et al.*, 2017), along with a 1:1 dilution of extracted samples with LC-MS grade water and limiting injection volumes to 10 µL (Stahnke *et al.*, 2012; Lien *et al.*, 2009).

Estimation of Limit of Detection (LOD) and Limit of Quantification (LOQ)

Determination of LOD was guided by the highest mass transition relative to baseline noise given by the blank. The signal to noise (S/N) ratio used to calculate LOD was 3:1 whereas LOQ was calculated using an S/N ratio of 6:1 (González & Herrador, 2007). While applying a different approach, LOD was determined by the following equation: LOD = k * (sy/x/m), where *m* represented the slope, sy/x was the residual standard deviation of the regression curve, and the value for k was 3 (Miller & Miller, 1993).

Data analysis

All samples were analyzed in triplicates. The linearity of the matrix-matched calibration line that was used to quantify the samples was assessed using regression coefficient analysis. Additionally, the results were analysed using means, standard deviation as well as one and two-factor analysis of variance for hypothesis testing.

RESULTS AND DISCUSSION

Method performance

In the study, a precursor ion having mass-to-charge ratio (m/z) of 897.5 was detected using Selected Ion Monitoring (SIM) scanning mode at a retention time of 4.754 minutes. The system was switched to MRM mode upon which the same ion (m/z 897.5) was detected albeit with a slightly longer retention time of approximately 8 minutes. Since ivermectin has a molecular weight of 875 g/mol, the study alluded that the base peak for the detected mass spectrum resulted from chelation of ivermectin molecule with a sodium ion (Na^+): m/z 897.5 (M + Na^+). This is because; ivermectin is composed of two chemically modified avermectins, with molecular weights of 875 and 860. The major component is at least 80% 22, 23-dihydro-avermectin-$B1_a$ ($H2B1_a$), while the minor constituent is >20% 22, 23-dihydro-avermectin-$B1_b$ ($H2B1_b$) (Campbell, 1992; Fisher & Mrozik, 1992). Studies have shown that $H2B1_b$ metabolizes faster than the $H2B1_a$ homolog (González *et al.,* 2009). As a result, $H2B1_a$ is the primary metabolite found in body fluids and the most prevalent residue identified *in vivo* (Lobato *et al.,* 2006; Markus & Sherma, 1992). Further fragmentation of the precursor ion (m/z 897.5) resulted in the detection of three product ion transitions, eluting within retention times ranging from 8.782 to 8.858 minutes, as shown in figure 1.

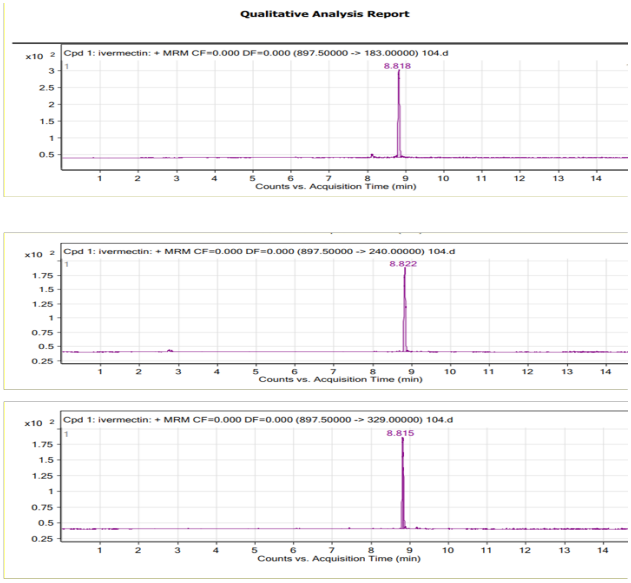


Figure 1: Mass spectrum of ivermectin fragment/product ions

The product ion transitions comprised of; **a** (897.5 > 183), **b** (897.5 >240), and **c** (897.5 >329) as illustrated in figure 2.

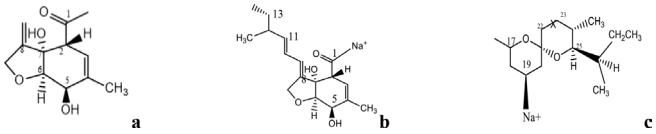


Figure 2: Ivermectin fragments

The secondary mass spectrum displayed in figures 1 and 2a showed a base peak m/z 183, which was identified as the benzofuran moiety present in ivermectin molecule. This moiety broke at C-1 and C-9 and chelated with Na^+ [160 + Na^+]. The reason associated with the breaking of bonds at these sites, rather than on the disaccharide moiety was the property of the disaccharide having a non-reducing end that can substitute *O*-atom linked to the anomeric carbon, and a reducing end linked to the rest of the ivermectin group. However, at the reducing end, the *O*-atom on the anomeric carbon cannot be substituted (Calvano *et al.*, 2017). Additionally, the fragment labelled as **b**, m/z 240 (figure 2), was identified as the benzofuran group broken from the main ivermectin molecule at C-1 and C-13, bonding with the disaccharide group and chelated with the sodium ion [217 + Na^+]. Furthermore, the fragment labelled as **c,** (figure 2), with a base peak at m/z 329, was identified as a spiroketal group of ivermectin molecule chelated with the sodium ion [306 + Na^+]. Moreover, the spiroketal group fragmented from the core structure at C-19, which was attaching to oxygen at C-1 and C-16. Hence, the study applied 240 and 329 m/z as qualifier ions for qualitative analysis. On the other hand, m/z183, the most abundant ion, was utilised as the quantifier ion for quantitative analysis. Throughout the study, the identification criteria for LC-MS/MS were verified by monitoring the relative retention times, which ranged from 8.782 to 8.858 (average = 8.362±0.002) minutes.

Likewise, the peaks obtained for 1ppb solvent and matrix-matched standards were well resolved indicating that there was no suppression or enhancement of signal in the matrix-matched curve. This demonstrated that the analytical performance of this method was unaffected by the matrix. Therefore, efficient ionization and separation of the analyte were achieved for both the solvent and matrix-matched chromatograms.

Selectivity and specificity

The analysis revealed that there were no endogenous contaminants in the chromatograms generated using the 10ppb of solvent and matrix-matched standards each. In addition, the study did not show any peaks that co-eluted or interfered with the identified analytes. These findings align with other the findings which indicated that "the high selectivity of MS in MRM mode enables clear distinction between peaks that elute closely based on

their distinct precursor and product ions" (Kinsella *et al.*, 2009). Thus, the method used demonstrated selectivity and specificity, ensuring that the quantitative results for ivermectin determination were not affected.

Precision

The repeatability of the procedure was demonstrated by the consistent in results provided by the data for the replicated twelve samples displayed in Table 2.

Table 2: Values for concentrations, recoveries and retention times

Samples (10ppb)	Conc.	% recovery	RT
Spike 1	9.412	94.12	8.362
Spike 2	8.613	86.131	8.365
Spike 3	8.528	85.267	8.363
Spike 4	9.306	93.06	8.363
Spike 5	9.014	90.136	8.362
Spike 6	10.112	101.12	8.363
Spike 7	10.144	101.435	8.365
Spike 8	7.794	77.939	8.367
Spike 9	8.988	89.884	8.363
Spike 10	7.498	74.997	8.366
Spike 11	7.783	77.828	8.363
Spike 12	10.101	101.01	8.341
Average	**8.941**	**89.409**	**8.362**

Consequently, the precisions for retention times, concentrations, and recoveries were calculated and determined to be 0.08, 10.419 and 10.419 respectively. All the parameters exhibited a precision of less than 20% indicating that, the values were within the acceptable tolerance specified in the European Commission's 2018 document criterion for precision acceptance. Additionally, at a 95% level of confidence, the calculated standard deviation for repeatability of concentration was 2.416. This value was deemed acceptable as it was lower than the theoretical value of 3% suggested by Horwitz, 1982. The results obtained are as indicated in table 3.

Table 3: Precision in percentage RSD for retention times, concentrations and recoveries

Variable	Mean ± SEM6	CV (% RSD)	SD	Sr
Retention time	8.362±0.002	0.08	4.59E-05	0.018
Concentrations	8.941±0.269	10.419	0.868	2.416
% Recovery	89.410±2.69	10.419	86.768	24.155
Theoretical RSD %	3			
Repeatability	2.810			
LOQ	Set at 10 ppb			
Matrix effect	-19.957 (no matrix effect)			

Recovery

The recoveries of ivermectin in milk samples ranged from 74.997 to 101.435 % (table 2), with a mean of 89.410±2.69 % (table 3). The variations below and above 100% in recoveries were attributed to repeatability within a run, which encompassed factors such as volumetric and gravimetric errors, heterogeneity of the test analyte and variations in the chemical treatment phases of the analysis (Thompson *et al.,* 2002). However, as noted by Becker *et al.*, (2004) both signal suppression and enhancement effects were commonly observed in complex biological matrices. Nonetheless, the results for this study met the criteria of the 70-120 % for method validation and analytical quality control processes for pesticide residue assessment in feed and food, which were recommended for implementation by January 1, 2020. Furthermore, the precision for recoveries was determined to be 10.419 % RSD thus falling below the required threshold of 20%.

Linearity and matrix effect

Both the matrix-matched and solvent calibration curves exhibited linearity within the range of 1–100 ng/mL, with correlation coefficients of 0.993 and 0.998, respectively. Similarly, it was suggested that matrix effect could be reduced or compensated for by optimizing sample preparation protocols. Moreover, reduction of the injection volume and sample diluting were employed to minimize the co-elution of components and mitigating matrix effects when the concentration for target analyte was high (Stahnke *et al.*, 2012). Similarly, the utilization of the matrix-matched calibration curve method during analysis helped alleviate matrix effect (Zhou *et al.*, 2017). This was due to the possibility that matrix effects could still be present regardless of LC-MS being a sensitive and selective analytical method, particularly when utilizing electrospray ionization (ESI) for analyzing complex matrices (Matuszewski *et al.*, 2003). Nevertheless, this limitation was successfully addressed by reducing injection quantities to 10µL and diluting the extracts with LC water in the ratio of 1:1.

LOD and LOQ

The LOD was 2.5 ng/mL, whilst 10 ng/ml was the lowest concentration that could be quantified. Comparable to this, when sample weight of 10g/10mL, a dilution factor of two, and a recovery with a precision of less than 20% were taken into consideration, the spike concentration of 10ng/mL was recognized as the LOQ. These findings aligned with the recommendations outlined in the document SANTE/12682/2019, EURL | Pesticide Residues | Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed (EURL).

Ivermectin excretion and depletion in raw milk samples from treated animals

It was evident that cows treated subcutaneously with ivermectin excreted the drug through their mammary glands, leading to contamination of the milk they produced. Ivermectin residues began to appear in milk samples 12hours after treatment with a mean value of 24.09±0.48ng/ml. Subsequently, the highest mean levels reaching 60.90±0.98ng/mL were observed, on day 2. Nevertheless, despite a weak signal response on day 17, levels were still detectable at 0.95ng/ml. No further detectable amounts of ivermectin were identified from day 18 onwards. The levels of the residue present in the sampled milk are presented in Table 4.

Table 4: Concentrations of ivermectin in milk from treated crossbred dairy cattle

Day	Concentration of Ivermectin (µg/ml)
0.5	24.09±0.48^{d}
1	43.39±1.39^{c}
2	60.90±0.98^{a}
4	49.18±1.57^{b}
6	24.42±1.11^{d}
7	23.95±1.29^{d}
9	19.37±1.63^{d}
12	4.23±0.33^{e}
15	1.47±0.53^{e}
17	0.95±0.11^{e}
18	0.00±0. 00^{e}

Means followed by different small superscript letters column-wise denotes significant difference (p<0.05)

However, the peaks corresponding to the weak signals on day 17 could still be identified based on their distinct precursor and product ions, (Kinsella *et al.* 2009). The optimal retention time was still present, and all of the ions remained easily visible. The data obtained, presented in table 4, allowed for the determination of the depletion pattern of ivermectin residue in milk after treatment as depicted in figure 4.

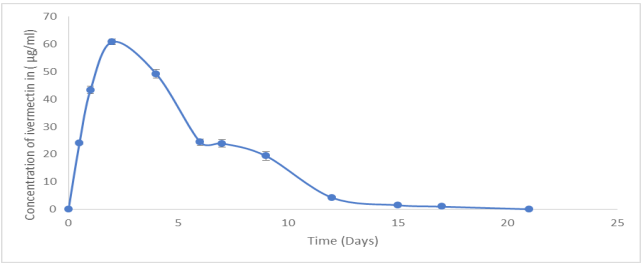


Figure 4: Concentration of ivermectin in milk from treated dairy cow.

The findings presented in figure 4 with regard to the concentration pattern of ivermectin in milk from treated dairy cows with time are consistent with previous works done by Escribano *et al.*, 2012 and Anastasio *et al.*, 2002, who reported similar trends. Equally, these studies detected occurrence of ivermectin residues in milk sampled from dairy cows after the administration of the approved dosage of 200 µg/kg through percutaneous or subcutaneous routes (Canga *et al.*, 2009; Anastasio *et al.*, 2002; Alvinerie *et al.*, 1993). However, it is worth noting that other related research suggested that the largest concentrations of ivermectin in milk were observed on the 3rd day after subcutaneous administration (Alvinerie *et al.*, 1993). Occurrence of ivermectin residues in milk can be accredited to its lipophilic properties, size, and relative insolubility. These characteristics, along with its pharmacokinetic properties, contribute to its distinct distribution and slow elimination from the body (Cerkvenik *et al.*, 2004, Dahiya *et al.*, 2010). Additionally, both the composition and the route of administration have a direct impact on its pharmacokinetics, with subcutaneous administration resulting in the highest bioavailability, followed by oral administration. As a result, the low plasma clearance and the ability of ivermectin to accumulate in adipose tissue lead to its prolonged retention in the body. It is worth noting that ivermectin tends to accumulate in subcutaneous tissues due to its limited solubility in water, which results in delayed absorption from the injection site. This accumulation in the tissues prolongs its presence in the bloodstream (González *et al.*, 2009). Similar observations were made by Fernandez *et al.*, (2009) when excretion profile of ivermectin was investigated against time in cattle dung, which is a different matrix. Furthermore, studies indicated that the increased levels of ivermectin residues present in milk was associated with the higher content of fat present in milk during lactation (Canga *et al.*, 2009; Lobato *et al.,* 2006; Anastasio *et al.*, 2002; Alvinerie, *et al.*, 1993). These findings supported the notion that the concentration of ivermectin in milk was influenced by factors such as milk composition as well as the physiological state of lactating animals.

CONCLUSION AND RECOMMENDATIONS

Conclusions

This study evinced that the ivermectin molecule was effectively extracted by QuEChERS, whilst the +ESI LC-MS-MS analytical approach effectively detected, quantified, and validated the existence of ivermectin residues in milk. Qualitative analysis yielded precise chromatographic peaks within a specific retention time range, while quantitative analysis showed low LOD and LOQ values, indicating excellent sensitivity. Besides, the absence of interfering matrix components and the satisfactory recovery values for the analyte further supported the validity of the method used. Ivermectin residues appeared in milk within 12 hours of treatment, peaking on day two, and remained in milk for 17 days. Nevertheless, no residues occurred on day 18 going

forward, demonstrating that the 18-day withdrawal time may be recommended as a suitable strategy to safeguard safety of milk for human feeding even in circumstances of off-label use.

Recommendations

According to the study findings, it is recommended to utilize the QuEChERS extraction method together with the +ESI LC-MS/MS analysis technique for detection, quantification, and confirmatory analysis of ivermectin residues in milk. Consequently, it is recommended that routine monitoring of ivermectin residues in all milk available for consumption be done to protect public safety and prevent exposure to this pollutant. Additionally, it is imperative to put in place control measures on the use and sale of ivermectin, as well as consider use of safer alternative methods for tick control, such as use of eprinomectin or biological methods. Further research is also recommended to investigate the depletion of ivermectin residues in various animal products that included blood, beef and edible internal organs. Moreover, it would be crucial to examine whether ivermectin residues persist in other common products derived from contaminated milk, such as cheese and yogurt, after undergoing industrial processes.

Data Availability

The data that underlies this study are presented and accessible within this article.

Conflicts of Interest

The authors declare no conflicting interest that could potentially influence the objectivity or impartiality of the research.

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