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A modified quechers method for analysis of five pesticides residues in cow's milk by GC-MS analysis

In this study, cow's milk samples were used to determine the presence of residues substances (amitraz, fluazuron, eprinomectin, chlorpyrifos, cypermethrin and dichlorvos). The first stage was carried out to validate the QuEChERS (modified) method of extraction and the second stage involved characterization and quantification of the residues of these chemical substances contained in cow's milk from the Unicesumar experimental farm. The methodology was validated through the analysis of average recoveries greater than 90%, excellent precision (<20%) and low detection limits (0.023 to 1.79 µg L-1) and quantification (0.05 to 3.13 µg L-1). The milk collected from groups of dairy cattle (Dutch black and white) showed high amounts of residues on days 0, 1 and 2 after application, with the highest concentration being chlorpyrifos on day 0 (632.2 µg L-1). The QuEChERS methodology combined with gas chromatography coupled with mass spectrometry proved to be an easy to apply and highly efficient method for the development of routine analyzes in the quality control of cow's milk for the detection of residues of veterinary drugs used to combat ticks in cattle dairy.

Keywords: Chemical analysis; Veterinary waste; Quality control.

Um método quechers modificado para análise de cinco resíduos de pesticidas no leite de vaca por análise GC-MS

Neste estudo, amostras de leite de vaca foram utilizadas para determinar a presença de substâncias residuais (amitraz, fluazuron, eprinomectina, clorpirifós, cipermetrina e diclorvos). A primeira etapa foi realizada para validar o método de extração QuEChERS (modificado) e a segunda etapa envolveu a caracterização e quantificação dos resíduos dessas substâncias químicas contidas no leite de vaca da fazenda experimental da Unicesumar. A metodologia foi validada através da análise de recuperações médias superiores a 90%, excelente precisão (<20%) e baixos limites de detecção (0,023 a 1,79 µg L-1) e quantificação (0,05 a 3,13 µg L-1). O leite coletado de grupos de gado leiteiro (preto e branco holandês) apresentou grande quantidade de resíduos nos dias 0, 1 e 2 após a aplicação, sendo a maior concentração o clorpirifós no dia 0 (632,2 µg L-1). A metodologia QuEChERS aliada à cromatografia gasosa acoplada à espectrometria de massas mostrou-se um método de fácil aplicação e altamente eficiente para o desenvolvimento de análises de rotina no controle de qualidade do leite de vaca para detecção de resíduos de medicamentos veterinários utilizados no combate a carrapatos em bovinos leiteiros.

Palavras-chave: Análise química; Resíduos veterinários; Controle de qualidade.

Topic: **Química Agrícola e Ambiental**

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INTRODUCTION

Cow's milk is considered a very rich food because it contains proteins, lipids, carbohydrates, minerals and vitamins, as well as to provide nutrients and immunological protection for humans. Milk also offers anticarcinogenic elements presented in fat, such as conjugated linoleic acid, butyric acid, beta-carotene, vitamins A and D and among others (RAZA et al., 2018; LIU et al., 2019).

Over the years, the human population has been increasing considerably, leading to a considerable increase in the demand for food. However, due to the unwanted occurrence of pests, fungi and bacterial diseases, the use of veterinary drugs and pesticides has also grown on a large scale (MORAIS et al., 2018; RAZA et al., 2018; MANAV et al., 2019). In most cases, these veterinary drugs and pesticides are applied in an indiscriminate and inappropriate, which can cause contamination of products (food), soil, water and even cause deaths (CRISTOFANI et al., 2009; KLAINBART et al., 2019).

From a technological point of view, the quality of raw material is one of the biggest obstacles to the development and consolidation of the dairy industry in Brazil. In general, milk quality control in recent decades has been restricted to adulteration prevention of the *in nature* product, based on the determination of acidity, cryoscopic, index, density, fat percentage, defatted dry extract and specific tests for illegal substance detection and antibiotic residues (LIU et al., 2019).

The use of pesticides is growing more and more in the management of dairy cattle to prevent diseases in the herd. Pesticides are commonly used to prevent flies, ticks and pests, even when they a have high toxicity. Many classes such as organochlorines, organophosphates, pyrethroids and carbamates are the most used for this purpose (MENEGHINI et al., 2014; JAWAID et al., 2016; SORIA et al., 2018).

Pesticides can be absorbed through the skin, by ingestion and inhalation, they can cause acute intoxications such as seizures, vomiting and nausea as well as more serious and chronic problems such as infertility, liver and kidney diseases, carcinogenesis and even chromosomal changes (MENEGHINI et al., 2014; JAWAID et al., 2016). A measure that is commonly used to determine pesticide toxicity is the 50% Lethal Dose (LD50). This measure represents the amount (single dose) of the compound needed to kill 50% of the animals tested under experimental conditions (GUTIÉRREZ et al., 2012).

According to research carried out by Anadón et al. (2009), problems such as excessive salivation, hypersensibility and tremors are signs presented by both animals and humans within 24 to 72 hours after pyrethroid exposure. Anadón et al. (2009) also concludes that the use of compounds such as carbamates or organophosphates can cause enzymatic induction or inhibition in animals (small or large) or in the population that consume their products or derivatives.

Although pesticides generate toxic waste, they are fully applied during production and post-production processes worldwide. Thus, the distribution of food products treated with these compounds must be carefully controlled in order to conserve human, animal and environmental health (CRISTOFANI et al., 2009; LEE et al., 2011; JAWAID et al., 2016). The standard adopted by the global food trade (ANVISA, 2003; CODEX, 2012) was to control pesticide residues in food through the maximum residue limit (MRL).

The QuEChERS technique (quick, easy, cheap, effective, robust and safe) was adapted from Anastassiades et al. (2003) in order to overcome many disadvantages and limitations of traditional extraction techniques for multiresidue analysis. Thus, the method allows quick and viable extraction from acidic, basic and neutral analytes, allowing excellent performance due to high analyte recoveries and at low cost compared to other methods (ARIAS et al., 2018; MESA et al., 2018; MORAIS et al., 2018; MUHAMMAD et al., 2018; SORIA et al., 2018; MANAV et al., 2018).

The method is based on an initial extraction with acetonitrile in buffered medium, followed by a cleaning step with solid phase extraction with a primary-secondary amine (PSA) combined with C18 to retain lipid substances (KOESUKWIWAT et al., 2008; PRESTES et al., 2009; LEE et al., 2011; MESA et al., 2018). In the extraction and quantification process, many aspects must be taken into account for the selection of analytes, such as selectivity during extraction, partition, analyte extraction capacity, cost, compatibility with the chosen analytical technique and environmental aspects (CUNHA et al., 2007; MANAV et al., 2019; PRESTES et al., 2009).

After the extraction process, the analyte must be characterized by chromatographic analysis (DI CORCIA et al., 2002; VAN RHIJN et al., 2002; CINQUINA et al., 2003; MAKESWARAN et al., 2005; MSAGATI et al., 2007; MCGLINCHEY et al., 2008; CRISTOFANI et al., 2009; MAMANI et al., 2009; SPISSO et al., 2009; JUAN et al., 2010) in accordance with standards established by the Brazilian inspection agency, ANVISA - National Health Surveillance Agency (ANVISA, 2003; CODEX, 2012). Thus, the study was carried out in order to validate the method developed and to detect and quantify the presence of multiresidues of pesticides (cypermethrin, amitraz, dichlorvos, chlorpyrifos, fluazuron and eprinomectin) in bovine milk using the modified QuEChERS technique combined with analysis by gas chromatography coupled with mass spectrometry.

MATERIALS AND METHODS

Reagents and instruments

All reagents and solventes used in this research were from analytical grade and HPLC grade. Acetonitrile (MeCN), dichloromethane and metyl acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The d-SPE and PSA adsorbents, namely C18 and primary-secondary amine, respectively, were purchased from Supelco (Bel-Lafone, PA, USA).

Analytical standards for Amitraz (N,N'-[(Methylimino)dimethylidyne]di-2,4-xylidine), Cypermethrin, Chlorpyrifos (0,0-Diethyl-O-[3,5,6-trichloro-2-pyridyl]phosphorothioate), Dichlorvos (2,2-dichloroethenol dimethyl phosphate), Eprinomectin (4"-epi-acetylamino-4"-deoxy-avermectin B1a) and Fluazuron (N-[[4-chloro-3-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]oxyphenyl]carbamoyl]-2,6-difluorobenzamide) were purchased from Sigma-Aldrich (Supelco, Bel-alfonte, PA, USA). Standard stock solutions (1 mg/mL) were prepared in MeCN and kept in the freezer at -20 ° C, avoiding exposure to light. The intermediate standard solutions were prepared from the standard stock solutions and their subsequent dilution with MeCN. Standard intermediate solutions were stored at 4°C and equilibrated at room temperature before use.

Standard working solutions were prepared from standard intermediate solutions by diluting in MeCN according to the desired concentration level.

For sample preparation, a centrifuge Splabor A model 4000, mechanical stirrer Kasvi K45-2810 and a Genie 2 vortex were used. A pH meter was used in order to adjust the pH of the solutions, a resolution of ± 0,01 pH unit was settled.

Sample preparation

In milk analysis, the sample preparation is an essential step in the elimination of interferences that may compromise the analysis. For optimization and validation of the method, samples of UHT milk purchased from a local supermarket (Maringá, Brazil) were used. Samples (1L each) from the same manufacturer were identified, recorded and stored at -18 °C until the time of the analysis. Before the treatment of the sample, the content of each milk was mixed, homogenized and subsequently in the aliquots of the milk samples were adjusted to the desired concentrations of the pesticides and subjected to the extraction process. The volume of each rate depended on the sample treatment applied.

Extraction of the analyte from the matrix - QuEChERS method

Aliquots of the milk sample (10 g) and 15 mL MeCN (extraction solvent) were placed in 50 mL polypropylene centrifuge tubes. The samples were vortexed for 30 seconds. Then, a mixture of salts (1.0 g NaCl, 4.0 $MgSO_4$, 1 g tri-sodium citrate and 0.5 g hydrogen di-sodium citrate) was added to each sample and subsequently were mechanically agitated for 1 minute and centrifuged for 5 minutes at 6000 rpm at 25 ° C. Consequently, phase separation occurred and the organic phase (upper phase) was fully collected and transferred to a clean polypropylene centrifuge tube (15 mL). The aqueous phase (lower phase) of the sample was discarded. To clean the sample, a mixture of 250 mg of $MgSO_4$, 50 mg of C18 and 25 mg of PSA (cleaning absorbent) was added to the supernatant and that was mechanically stirred (2 minutes) and centrifuged (5 minutes, 8000 rpm, 25 ° C). Finally, 0.5 mL of each layer was collected, filtered, transferred to a 2 mL vial and evaporated to dryness under a stream of nitrogen (40 ° C). All extraction methods were performed in triplicate.

Derivatization

After the phase for extracting of the analytes from the matrix, 50 µL of DMF (dimethylformamide) and 50 µL of BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) + 1% TMCS (Trimethylchlorosilane) were added to the obtained above dried extract. The vial was capped, mixed, and heated in a temperature adjustable oven at 80 °C for 1 h. After the derivatization, the vial was cooled to room temperature and dried under an stream of nitrogen. Finally, 1 mL of EtAc was added to the vial, well mixed and the analyzed by GC-MS.

Chromatographic conditions GC-MS

The GC-MS analyzes were performed on a gas chromatograph (model Agilent 7890B) coupled to mass spectrometer (model Agilent 5977A MSD) equipped with HP-5MS UI Agilent columm with 5% phenyl methyl siloxane phase (30.0 m x 250 µmd.i. x 0.25 µm film thickness), with automatic injector (CTC PAL Control). In order to perform a proper separation of the analytes in the GC-MS system, 2µL of extract were injected into the columm using Split injection mode in a 1:50 ratio, under the following oven conditions: initial temperature of 70 °C maintained for 2.5 minutes, then ramp from 20 °C min^{-1} to 175 °C maintained for 13 minutes, and ramp from 20 °C min^{-1} to 290 °C and maintained to for 15 minutes. The other conditions of the analysis method were: injection volume of 1.0 µL, flow of carrier gas (He, purity 99.99999%) euqal to 1.2 mL min^{-1}, ionization by electronic impact of 70 eV, ionization source temperature of 230 °C, quadrupole of 150 °C, transfer line of 280 °C and injector of 250 °C. Data acquisition was performed using the MassHunter software and qualitative analysis of the mass spectra by NIST 11 library.

Validation procedures

The validation tests were performed by GC-MS following the parameters describe by Brazillian and Internacional Standards (BULUT et al., 2011; MENEGHINI et al., 2014; ANVISA, 2017). In order to set linearity (external standardization), standard solutions of different concentrations were used to each pesticide (Table 1) and injected in triplicate.

A blank sample was included in each group of spiked samples to confirm that the analyte was not detected. Through these tests, selectivity, accuracy, precision and robustness were evaluated. To determine the detection limits (DL) and quantification limits (QL) of the method, tests were performed with forfified samples in decreasing concentrations for each pesticide.

Table 1: Concentration of pesticides standards added in milk.

Substances	C1 µg/L	C2 µg/L	C3 µg/L	C4 µg/L	C5 µg/L	C6 µg/L
Dichlorvos	2.25	5.75	6.75	9	11.54	22.5
Cipermethrin	0.25	0.625	0.75	1.0	1.25	2.5
Amitraz	1.56	1.875	2.5	3.125	6.25	
Chlorpyrifos	3.125	3.75	5.0	6.25	12.5	
Eprinomectin	0.05	0.125	0.15	0.2	0.25	0.5
Fluazuron	0.125	0.3125	0.375	0.5	0.625	1.25

C = concentration.

Separation of cows and application of treatment

In order to apply the described methodology of monitoring pesticides residues (marketed in Brazil) present in milk, twenty-four dairy cattle (Dutch Black and White) from Unicesumar research center farm, were separated into 6 treatment groups (with four animals each) for the application of pesticides in the control of ticks and more the group (tank) representing the total volume of milking on the day (beginning 09/16/2019). The first group (T1) was treated with the dichlorvos and cypermethrin substances, the second (T2) with chlorpyrifos and cypermethrin, the third group (T3) received amitraz treatment, while the fluazuron and eprinomectin compounds were the chosen substances for T4 and T5 group, respectively. Two more

groups were adopted for analyses, T6 group corresponds to the milk removed from the main tank, where all samples were mixed and, T7 group corresponds to the control group (no treatment - N.T).

The pesticides (marketed in Brazil) application in its respective treatment group occurred in the morning, being carried out for all animals in the same way, through pour-on. The product was applied over the upper midline of the cow's body, in a narrow band that goes until the lumbosacaral joint, in a dose of 1 mL for each 10 kg of live weight, providing a dose of 500 µg kg^{-1} of live weight, according to the recommended dose.

On the same day of application (day 0), milk was already collected for analysis and thus continued for another 7 days (day 0 to day 7). All treatment groups (T1, T2, T3, T4, T5 and T7) were milked during these 8 days and after collecting the samples, thrown into the tank (T6). The average daily production in the tank was around 530 liters in the morning and 380 liters of milk in the afternoon per day. The samples were collected in triplicates (50 mL per container), totaling 168 samples collected for analysis.

RESULTS AND DISCUSSION

Optimization of QuEChERS method

The QuEChERS method was optimized using samples of milk fortified with pesticides (Table 1). In each experiment, three samples were treated under the same conditions, in order to verify the reproducbility of the method. The sample quantity was adjusted to 10 g and the solvent volume was settled as 15 mL, as it allowed to collect quantitatively 2 mL of MeCN after sample extraction (ARIAS et al., 2018; MANAV et al., 2019). Method optimization is based on maximizing the efficiency of the process, which takes into account the influence on the analyte in the sample treatment procedure evaluated by means of the recovery efficiency previously describe by Matuszewski et al. (2003).

The QuEChERS extraction method was evaluated as a simple method for analysis of pesticide residues in cow's milk, the determinant step being the elimination of interferents that may compromise the analysis. The QuEChERS method, adapted from Anastassiades et al. (2003) was satisfactory in order to extract the analyte from the matrix, after the optimization of different parameters that affected the extraction and the sample cleaning. Tri-sodium citrate salts and di-sodium hydrogen citrate were used in order to promote salting out effect, increasing the recovery of polar analytes as well as helping to regulate the solution's pH.

PSA sorbent addition (primary secondary amine) promotes a chelating effect dure to its structure with primary and secondary amino group, resulting in a retention of free fatty acids and other polar compounds that are in the matrix, thus making mosto f remaing solids decantate from the solution (PRESTES et al., 2011). In this case, the PSA can act as a normal mphase sorbent and a weak anion exchanger, so that it can retain polar compounds and anions that show negative charge at pH 8 or less. On the other hand, the C18 silica addition, which is a reverse phase sorbent that reatins a wide variety of compounds (non-polar to moderately polar compounds) due to Van Der Waals interaction, combined with PSA it certifies that lipid residues are eliminated, ensuring that there is no contamination to the chromatographic system and that the

matrix effect is reduced. Thus, i tis necessary to use sorbents that interract with the matrix's components but not with the analytes, enabling cleaning recoveries and elevating extraction rate.

Table 2 shows the extraction efficiency combined with the matrix effect and the process efficiency for the best experimental condition (50 mg of C18 and 25 mg of PSA). The amount of cleaning sorbent was investigated in the mass range of 0 - 100 mg. This parameter influences the effect of the recovery of the analytes and there was no variation in the matrix effect.

Proportion changes of the cleaning sorbent (PSA and C18) were tested the effect decreased to 50.1% with a decrease in the proportion (25 mg of C18 and 12.5 mg of PSA). However, the increase in the proportion does not increase the recovery, for exemple, in the proportion of the cleaning sorbents (50 mg of C18 and 25 mg de PSA) the effec, the effect ofrecovery reached 15.7%. The results obtained for the analyte recovery effect in the absence of sample cleaning procedure were very low (<10%). Thus, the cleaning step with C18 and PSA sorbents in order to improve sample recovery treatment was necessary to perform QuEChERS procedure, providing the greatest recoveries and the best process efficiencies.

Table 2: Results of detection limits quantification limits and recovery of the method for the determination of pesticides in milk.

Substances	Detection Limits µg L^{-1}	Quantification Limits µg L^{-1}	% Recovery
Dichlorvos	1.79	2.25	89.7
Cypermethrin	0.10	0.25	96.3
Amitraz	1.15	1.56	86.6
Chlorpyrifos	1.406	3.125	91.3
Cypermethrin	0.101	0.75	96.3
Eprinomectin	0.023	0.05	85.8
Fluazuron	0.061	0.125	96.7

As the method's name says, this technique has the advantage of easy and quick realizaion since the principle is based on extraction, partition and cleaning (MESA et al., 2018). According to the evaluated validation parameters, the reliability of the analytical results was assured, aiming the reduction of any errors.

Calibration curves and method validation

QuEChERS method combined with GC-MS was validated in terms of linearity, detection limit, quantification limit, recovery efficiency, matrix effect, process efficiency and precision. The detection limit is the lowest concentration of a substance that can be identified by an analytical procedure with a specified confidence level, or that can still be statistically differentiated from noise. The limit of quantification is the lowest concentration that can be measured with adequate precision, obtained by adding each pesticide to milk in decreasing concentrations (POONIA et al., 2016).

In order to compare the analytical characteristics performance, calibration curves were obtained from the analysis of spiked samples treated by modified QuEChERS method. The calibration curves were prepared in 6 different concentration levels for dichlorvos, cypermethrin, eprinomectin and fluazuron and in 5 different concentration levels for amitraz and chlorpyrifos (Table 1) and after treatment analyzed by GC-MS. Blank samples were also analyzed. No co-elution of any matrix compound was observed in relation to the selected analytes. The peak área corresponding to each analyte was selected as a response signal, being

linearly dependent on its concentration levels. In all cases, good linearity was obtained ($R^2 > 0.9969$).

The detection and quantification limits, shown in Table 2, were calculated from the minimum concentration of the analyte produced by the signal/noise ratio for the identification ion equal to four and ten times, respectively, where the signal/noise ratio was estimated based on at the height of the peak. The most important analytical parameters are related to recovery efficiency, matrix effect and, consequently, process efficiency that must always be evaluated when selecting a sample treatment. These parameters were estimated for each analyte and at all concentration levels established for the calibration curves and calculated solutions as described by Mesa et al. (2018).

The proposed analytical method was validated in terms of detection and quantification limits for performing analysis of non-permitted pesticides in bovine milk samples (ANVISA, 2003), following the procedure of the calibration curve according to ISO 11843 (ISO 11843-4, 2003), ensuring the reliability of analytical results and making it possible to consider that the method expresses results proportional to the concentration of the analyzed analytes. Therefore, the QuEChERS treatment method combined with GC-MS can be used for routine analysis to detect traces of pesticide (Amitraz, Cypermethrin, Chlorpyrifos, Dichlorvos, Eprinomectin and Fluazuron) in bovine milk.

Figure 1 represents the chromatographic profile of dichlorvos, eprinomectin, fluazuron, chlorpyrifos, amitraz, and cypermethrin after QuEChERS extraction of the samples of cow's milk. The selectivity of the method proved to be efficient for the multiresidue determination of these pesticides in cow's milk.

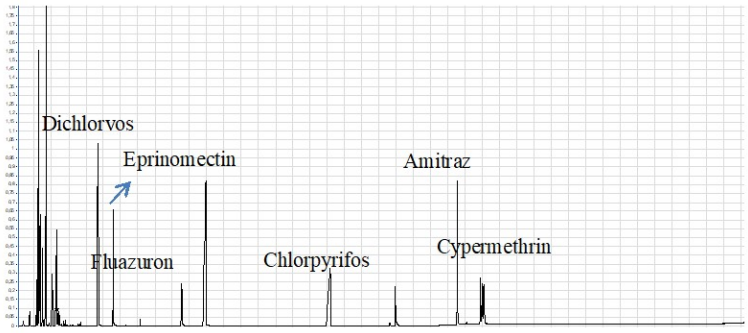

Figure 1: Chromatogram obtained from the extraction of pesticide residues (Dichlorvos, Cypermethrin, Amitraz, Chlorpyrifos, Eprinomectin and Fluazuron) in spiked cow's milk samples.

Linearity researches were performed and the concentration levels were selected based on the concentration range selected for the calibration curves from the establishment of maximum residue limits (MRLs) and maximum permitted residues limits (LMRs) for each pesticide (ANVISA, 2019; ANVISA, 2018) and according to the limits of detection and quantification (Table 2) established in the present work.

For each substance, a standard calibration curve (Supplementary material, S1 and S2) was established with different concentrations depending on the chromatographic characteristic of each substance, identified in Table 1. Each concentration point identified on the calibration curve corresponds to the average of the values of the analyzes performed in triplicates. No outliners were observed in any situation presented in all analytical curves. For all compounds, good linearity and repeatability were observed.

Linearity data demonstrate that there is a correlation between the concentrations of pesticides and

the respective areas of the chromatographic peak. The linearity for the tested method was verified by reading the analytical curve, according to the expressed linear relationship.

The validated method in the present research demonstrated to be qualified to verify the presence of residues of pesticides in bovine milk, being easy to execute and short in analysis time. The veracity of the analysis was evaluated in blank samples of spiked cow's milk at five different concentration levels. Thus, it was possible to observe that the regression was significant while not expressing deviation from linearity in the curve evaluated for the six target substances of this research.

Characterization and quantification of pesticides residues in bovine milk

For the application of the developed methodology, groups of animals that received the treatment with the respective pesticides were selected. After application, samples were collected at the Unicesumar experimental farm, followed by extraction and analysis by GC-MS.

The samples were collected on day 0 (day of application) until the seventh day after application (day 7), with the presence of pesticide residues in groups G1 to G6 being identified for days 0, 1 and 2. The greater intensity of the chromatographic signal was identified on day 0 and the smallest, close to the detection limit of the method, on day 2. On the other collection days (3rd to 7th day), it was not possible to observe defined chromatographic peaks (through the minimum signal/noise ratio) for the retention times of the respective pesticides.

As shown in Figure 2 and Table 3, the residues of all substances can be seen in their respective treatment groups. The G6 group (tank), where we have the presence of all substances used in cattle dairy management. After the characterization and identification of the substances, quantification was performed on days 0,1 and 2 in order to determine the concentrations of respective residues and to compare their values with the MRLs established by ANVISA (2018; 2019) and CODEX (2012).

Table 3: Determination of pesticides in milk after treatment protocol applied to 6 groups of black-white Holstein cows at the Unicesumar school farm.

Treatment groups	**Substances**	**Day 0 (µg L^{-1})**	**Day 1 (µg L^{-1})**	**Day 2 (µg L^{-1})**	**Day 3 - 7 (µg L^{-1})**
G1	Dichlorvos	453.6	254.66	75.1	u.d
	Cypermethrin	32.2	24.97	3.15	u.d
G2	Chlorpyrifos	632.2	499.08	137.37	u.d
	Cypermethrin	96.6	74.91	9.47	u.d
G3	Amitraz	369.16	268.55	65.68	u.d
G4	Fluazuron	15.17	7.96	2.77	u.d
G5	Eprinomectina	13.23	4.95	2.31	u.d
G6 Tank	Dichlorvos	11.34	8.75	2.30	u.d
	Cypermetrhin	3.22	2.27	0.63	u.d
	Chlorpyrifos	15.8	12.22	3.31	u.d
	Amitraz	8.39	6.55	1.61	u.d
	Fluazuron	0.322	0.177	0.063	u.d
	Eprinomectin	0.278	0.115	0.055	u.d
G7 Blank	N.T	u.d	u.d	u.d	u.d

N.T = No treatment (cows that did not get treatment); u.d = Undefined.

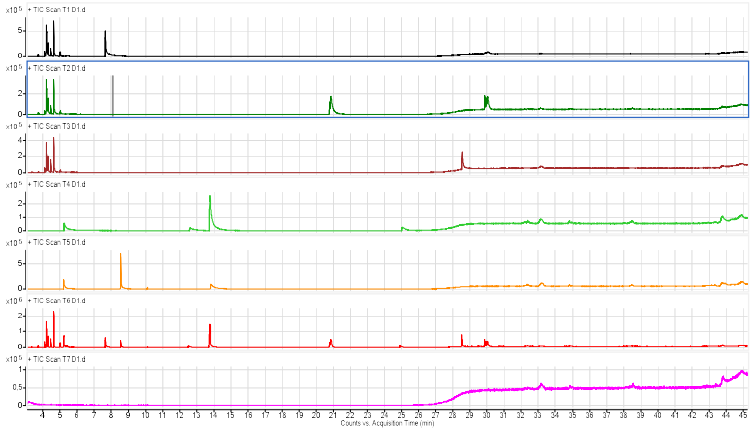

Figure 2: Chromatogram of cow's milk samples after the treatment of cattle with pesticides (day 1).

The dichlorvos and cypermethrin substances form the basis of some veterinary products marketed in Brazil to combat ticks and have a grace period of 1 day after the application of bovine dairy management. Through the data in Table 3, the presence of these residues in bovine milk was identified until the second day after its application. Quantification of dichlorvos showed a residual charge of 75.1 µg L^{-1} on the second day (day 2, after application), where the maximum allowable limit for this residue is 10 µg L^{-1} (ANVIVA, 2019; ANVIVA, 2018; CODEX, 2012).

Similar behavior was observed for the compounds chlorpyrifos and cypermethrin that also make up the standard formula of several veterinary products. Some products containing chlorpyrifos have a withdrawal time of three to ten days after application to consume the bovine milk. Chlorpyrifos was the substance that showed the highest concentration identified in milk on days 0, 1 and 2 and on day 2 137.37 µg L-1 was quantified (Table 3) of this residue in the milk sample (G2), a value that is very higher than allowed by the legislation of 20 µg L^{-1} (ANVIVA, 2019; ANVIVA, 2018; CODEX, 2012). When comparing the MRL with the sample collected in the storage tank (G6) we can observe that the concentration of the chlorpyrifos residue is below the concentration allowed by the legislation.

Cypermethrin compound is present in two different formulations of veterinary products marketed in Brazil for tick control in dairy cattle and these products have a maximum grace period of 1 day (ANVIVA, 2019; ANVIVA, 2018) after application. Table 3 shows that this compound was identified and quantified on days 0, 1 and 2 for the specific treatment groups (G1 and G2) and in the tank (G6). Thus, the identification of cypermethrin in the bovine milk sample from the second day (day 2) of treatment is in disagreement with the guidelines presented by the Brazilian regulatory agency, even though it has a concentration below the MRL of 100 µg L-1.

The amitraz residues (a withdrawal time of 1 day after application) reached 268.55 µg L^{-1} (Table 3) on day 1 and, even with its reduction to 65.68 µg L^{-1} on day 2 (G3), its concentration is higher than MRL of 10 µg L^{-1} (ANVIVA, 2019; ANVIVA, 2018; CODEX, 2012) allowed for bovine milk samples. When we observe the concentration of this substance in the tank, we can find its concentration lower than the MRL, this fact does not minimize the impact of the presence of this substance on day 2 after treatment, which also presents contradictions regarding the withdrawal time and disposal of milk.

As described in the veterinary product use guidance, eprinomectin has a maximum grace period of 1

day for tick control in dairy cattle (ANVIVA, 2019; ANVIVA, 2018) after application. Through the analyzes, it was possible to identify and quantify the presence of this substance after the second day of treatment (Table 3). All values found in this research for eprinomectin are below the MRL of 20 µg L^{-1}. The presence of this substance on day 2 points out to a probable failure in dairy cattle management.

According to ANVISA (2019; 2018) and CODEX (2012), fluazuron is a substance forbidden to be used in dairy cows, it does not have an established residue load MRL, so fluazuron-based products are strictly forbidden in dairy cattle management (CODEX, 2012). Finally, we can see that if this substance is applied to dairy cattle even with the tank's dilution its residue will be present until day 2, after its application to the dairy cow.

CONCLUSIONS

The QuEChERS technique combines with GC-MS demonstrated satisfactory efficiency for all analytes, low matrix effect, and detection limit. The proposed methodology proved to be a simple method, of low cost and easy application to development routine analysis, besides being an analytical method of extraction that uses little solvent. The analytical method was validated in the present research and proved to be efficient and safe for the determination and quantification of the residues of veterinary drugs (Amitras, Cypermethrin, Chlorpyrifos, Dichlorvos, Eprinomectin and Fluazuron) present in cow's milk samples.

Most of the substances contained in the milk samples have been shown to leave a residual chemical load on the food. For some substances described in this work, in addition to the residual load being above the MRL imposed by Codex, these residues were also quantified after the grace period for the application of the veterinary product in dairy cattle, as described in the guidelines for use.

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REFERENCES

ANADÓN, A.; MARTÍNEZ-LARRAÑAGA, M. R.; MARTÍNEZ, M. A.. Use and abuse of pyrethrins and synthetic pyrethroids in veterinary medicine. **The Veterinary Journal**, v.182, n.1, p.7-20, 2009. **DOI:** http://doi.org/10.1016/j.tvjl.2008.04.008

ANASTASSIADES, M.; LEHOTAY, S. J.; SAJNBAHER, D.; SCHENECK, F. J.. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and 'dispersive solid-phase extraction' for the determination of pesticide residues in produce. **Journal of Aoac International**, v.86, p.412-431, 2003. **DOI:** http://doi.org/10.1093/jaoac/86.2.412

ANVISA. Agência Nacional de Vigilância Sanitária. Resolução da Diretoria Colegiada - RDC nº 51/19, 19/12/2019. **Limites máximos de resíduos (LMR), ingestão diária aceitável (IDA) e dose de referência aguda (DRfA) para insumos farmacêuticos ativos (IFA) de medicamentos veterinários em alimentos de origem animal**. Brasília: ANVISA, 2019.

ANVISA. Agência Nacional de Vigilância Sanitária. **Limites Máximos de Resíduos de Medicamentos Veterinários em Alimentos de Origem Animal**. Brasília: ANVISA, 2018.

ANVISA. Agência Nacional de Vigilância Sanitária. **Resolução da Diretoria Colegiada - RDC nº 166/17, 24/07/2017**. Guia para validação de métodos analíticos. Brasília: ANVISA, 2017.

ANVISA. Agência Nacional de Vigilância Sanitária. **Programa Nacional de Análise de Resíduos de Medicamentos Veterinários em Alimentos Expostos ao Consumo - PAMVet**. Brasília: ANVISA, 2003.

ARIAS, J. L. O.; SCHNEIDER, A.; ANDRADE, J. A. B.; VIEIRA, A. A.; CALDAS, S. S.; PRIMEL, E. G.. Chitosan from shrimp shells: A renewable sorbent applied to the clean-up step of the QuEChERS method in order to determine multi-residues of veterinary drugs in different types of milk. **Food Chemistry**, v.240, n.1, p.1243-1253, 2018. **DOI:** http://dx.doi.org/10.1016/j.foodchem.2017.08.041

BULUT, S.; AKKAYA, L.; GOK, V.; KONUK, M.. Organochlorine pesticide (OCP) residues in cow's buffalo's and sheep's milk from Afyonkarahisar region, Turkey. **Environmental Monitoring and Assessment**, v.181, p.555- 562, 2011. **DOI:** http://doi.org/10.1007/s10661-010-1849-x

CINQUINA, A.; LONGO, F.; ANASTASI., G.. Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline in bovine milk and muscle. **Journal of Chromatography A**, v.987, n.1-2, p.227 - 233. 2003. **DOI:** http://doi.org/10.1016/S0021-9673(02)01446-2

CODEX. Codex Alimentarius Comission - FAO and WHO. **Maximum residue limits for veterinary drugs in foods.** Updated as at the 35th session of the Codex Alimentarius Commission. CODEX, 2012.

CRISTOFANI, E.; ANTONINI, C.; TOVO, G.; FIORINI, L.; PIERSANTI, A.; GALARINI, R.. A confirmatory method for the determination of tetracyclines in muscle using high-performance liquid chromatography with diode-array detection. **Analytica Chimica Acta**. v.637, n.1-2, p.40-46, 2009. **DOI:** http://doi.org/10.1016/j.aca.2008.10.029

CUNHA, S. C.; LEHOTAY, S. J.; MASTOVSKA, K.; FERNANDES, J. O.; OLIVEIRA. M. B. P. P.. Evaluation of the QuEChERS sample preparation approach for the analysis of pesticide residues in olives. **Journal of Separation Science**, v.30, p.620 - 632, 2007. **DOI:** http://doi.org/10.1002/jssc.200600410

DI CORCIA, A.; NAZZARI, M.. Liquid chromatographicemass spectrometric methods for analyzing antibiotics and antibacterial agents in animal food products. **Journal of Chromatography A**, v.974, n.1-2, p.53-89. 2002. **DOI:** http://doi.org/10.1016/S0021-9673(02)00905-6

GUTIÉRREZ, L.-F.; HAMOUDI, S.; BELKACEMI, K.. Lactobionic acid: A high value-added lactose derivative for food and pharmaceutical applications. **International Dairy Journal**, 26(2):103-111. 2012. **DOI:** http://doi.org/10.1016/j.idairyj.2012.05.003

ISO. **ISO 11843-4:2003.** Capability of detection - Part 4: Methodology for comparing the minimum detectable value with agiven value. ISO, 2003.

JAWAID, S.; TALPUR, F. N.; NIZAMANI, S. M.; KHASKHELI, A. A.; AFRIDI, H. I.. Multipesticide residue levels in UHT and raw milk samples by GC-µECD after QuEChER extraction method. **Environmental Monitoring and Assessment**, v.188, n.230, 2016. **DOI:** http://doi.org/10.1007/s10661-016-5222-6

JUAN, C.; MOLTÓ, J. C.; MANES, J.; FONT, G.. Determination of macrolide and lincosamide antibiotics by pressurised liquid extraction and liquid chromatographytandem mass spectrometry in meat and milk. **Food Control**, v21, n.122, p.1703 - 1709, 2010. **DOI:** http://doi.org/10.1016/j.foodcont.2010.05.004

KLAINBART, S.; GRABERNIK, M.; KELMER, E.; CHAI, O.; CUNEAH, O.; SEGEV, G.; AROCHACD, D. I.. Clinical manifestations, laboratory findings, treatment and outcome of acute organophosphate or carbamate intoxication in 102 dogs: A retrospective study. **The Veterinary Journal**, v.251, p.105349, 2019. **DOI:** http://doi.org/10.1016/j.tvjl.2019.105349

KOESUKWIWAT, U.; SANGUANKAEW, K.; LEEPIPATPIBOON, N.. Rapid determination of phenoxy acid residues in rice by modified QuEChERS extraction and liquid chromatography-tandem mass spectrometry. **Analytica Chimica Acta**, v.626, n.1, p.10-20, 2008. **DOI:** http://doi.org/10.1016/j.aca.2008.07.034

LEE, S. W.; CHOI, J.; CHO, S.; YU, H.; EL-ATY, A.; SHIM, J.. Development of a new QuEChERS method based on dry ice for determination of 168 pesticides in paprika using tandem mass spectrometry. **Journal of Chromatography A**, v.1218, n.28, p.4366-4377, 2011. **DOI:** http://doi.org/10.1016/j.chroma.2011.05.021

LIU, Q. H.; WU, J. X.; SHAO, T.. Roles of microbes and lipolytic enzymes in changing the fatty acid profile, α-tocopherol and β-carotene of whole-crop oat silages during ensiling and after exposure to air. **Animal Feed Science and Technology**, v.253, p.81-92, 2019. **DOI:** http://doi.org/10.1016/j.anifeedsci.2019.04.004

MAKESWARAN, S.; PATTERSON, I.; POINTS J.. An analytical method to determine conjugated residues of ceftiofur in milk using liquid chromatography with tandem mass spectrometry. **Analytica Chimica Acta**, v.529, n.1-2, p.151-157, 2005. **DOI:** http://doi.org/10.1016/j.aca.2004.07.042

MAMANI, M. C. V.; REYES, F. G. R.; RATH, S.. Multiresidue determination of tetracyclines, sulphonamides and chloramphenicol in bovine milk using HPLC-DAD. **Food Chemistry**, v.117, n.3, p.545-552, 2009. **DOI:** http://doi.org/10.1016/j.foodchem.2009.04.032

MANAV, Ö. G.; DINÇ-ZOR, Ş.; ALPDOĞAN, G.. Optimization of a modified QuEChERS method by means of experimental design for multiresidue determination of pesticides in milk and dairy products by GC-MS. **Microchemical Journal**, v.144, p.124-129, 2019. **DOI:** http://dx.doi.org/10.1016/j.microc.2018.08.056

MESA, M. H.; CAMPAÑA, A. M. G.; BLANCO, C. C.. Development and validation of a QuEChERS method for the analysis of 5-nitroimidazole traces in infant milk-based samples by ultra-high performance liquid chromatography-tandem mass spectrometry. **Journal of Chromatography A**, v.1562, p.36-46. 2018. **DOI:** http://doi.org/10.1016/j.chroma.2018.05.050

MCGLINCHEY, T. A.; RAFTER, P. A.; REGAN, F.; MCMAHON, G. P.. A review of analytical methods for the determination of aminoglycoside and macrolide residues in food matrices. **Analytica Chimica Acta**, v.624, n.1, p.1-15, 2008. **DOI:** http://doi.org/10.1016/j.aca.2008.05.054

MENEGHINI, L. Z.; RÜBENSAM, G.; BICA, V. C.; CECCON, A.; BARRETO, F.; FERRÃO, M. F.; BERGOLD, A M.. Multivariate Optimization for Extraction of Pyrethroids in Milk and

Validation for GC-ECD and CG-MS/MS Analysis. **International Journal Environmental Research Public Health**, v.11; n.11, p.11421-11437, 2014. **DOI:** http://doi.org/10.3390/ijerph111111421

MORAIS, E. H. C.; COLLINGS, C. H.; JARDIM, I. C. S. F.. Pesticide determination in sweet peppers using QuEChERS and LC-MS/MS. **Food Chemistry**, v.249, n.30, p.77-83, 2018. **DOI:** http://doi.org/10.1016/j.foodchem.2017.12.092

MSAGATI, T. A. M.; NINDI, M. M.. Determination of b-lactam residues in foodstuffs of animal origin using supported liquid membrane extraction and liquid chromatographyemass spectrometry. **Food Chemistry**, v.100, n.2, p.836-844, 2007. http://doi.org/10.1016/j.foodchem.2005.09.021

MUHAMMAD, N; SUBHANI, Q.; WANG, F.; LOU, C.; LIU, D. J.. Simultaneous determination of two plant growth regulators in ten food samples using ion chromatography combined with QuEChERS extraction method (IC-QuEChERS) and coupled with fluorescence detector. **Food Chemistry**, v.241, p.308-316, 2018. http://doi.org/10.1016/j.foodchem.2017.08.112

POONIA, A.; JHA, A.; SHARMA, R.; SINGH, H. B.; RAI, A. K.; SHARMA, N.. Detection of adulteration in milk: A review. **International Journal of Dairy Technology**, v.69, p.1-20, 2016. **DOI:** http://doi.org/10.1111/1471-0307.12274

PRESTES, O. D.; FRIGGI, C. A.; ADAIME, M. B.; ZANELLA, R.. QuEChERS - Um método moderno de preparo de amostra para determinação multirresíduo de pesticidas em alimentos por métodos cromatográficos acoplados à espectrometria de massas. **Química Nova**, v.32, n.6, p.1620-1634, 2009. **DOI:** http://dx.doi.org/10.1590/S0100-40422009000600046

RAZA, N.; KIM, K.. Quantification Techniques for Important Environmental Contaminants in Milk and Dairy Products. **Trends in Analytical Chemistry**, v.98, p.79-94, 2018. **DOI:** http://doi.org/10.1016/j.trac.2017.11.002

SORIA, F.; IRIBARNE-DURÁN, L. M.; MUSTIELES, V.; JIMÉNEZ-DÍAZ, I.; FERNÁNDEZ, M. F.; OLEA, N.. QuEChERS and ultra-high performance liquid chromatography-tandem mass spectrometry method for the determination of parabens and ultraviolet filters in human milk samples. **Journal of Chromatography A**, v.1546, p.1-9, 2018. **DOI:** http://doi.org/10.1016/j.chroma.2018.02.060

SPISSO, B. F.; ARAUJO JUNIOR, M. A. G.; MONTEIRO, M. A.; LIMA, A. M. B.; PEREIRA, M. U.; LUIZ, R. A.; NÓBREGA, A. W.. A liquid chromatographyetandem mass spectrometry confirmatory assay for the simultaneous determination of several tetracyclines in milk considering ketoeenol tautomerism and epimerization phenomena. **Analytica Chimica Acta**, v.656, n.1-2, p.72-84, 2009. **DOI:** http://doi.org/10.1016/j.aca.2009.10.012

VAN RHIJN, J. A.; LASAROMS, J. J. P.; BERENDSEN, B. J. A.; TH, U.; BRINKMAN, A.. Liquid chromatographicetandem mass spectrometric determination of selected sulphonamides in milk. **Journal of Chromatography A**, v.960, n.1-2, p.121-133, 2002. **DOI:** http://doi.org/10.1016/S0021-9673(02)00329-1