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Validation of a Method for Analysis of Avermectins Residues in Bovine Milk by HPLC-Fluorescence Detector

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Abstract The presence of veterinary drug residues in bovine milk has raised the need to adapt their detection methodologies in milk for human consumption. The avermectins, antiparasitic class have been detected by liquid chromatography with fluorescence detection after derivatization reaction. The method validated in this work is fast and accurate for the determination of these residues in bovine milk according with limits establishment by *Codex Alimentarius*. The limit of quantitation (LOQ) for Abamectin, Doramectin and Ivermectin is 5 μg L^{-1} with a great recovery associated with a low coefficient of variation.

Keywords Abamectin, Doramectin, Ivermectin, Lactation cows, Veterinary drugs

1. Introduction

Brazil is the fifth largest cow's milk producer after the European Union, India, United States and China. Currently, the Brazilian milk production chain shows high growth potential, and the Ministry of Agriculture, Livestock and Food Supply (MAPA) projections to 2020 indicate that the production and consumption will be 37.75 and 33.27 billion liters, respectively [1, 2]. The use of veterinary drugs accompanies the growth of the Brazilian herd in order to ensure animal health and consequently increase livestock production. However, the indiscriminate use of these drugs during cow´s lactation can lead to the presence of residues in milk, even above the maximum residue levels (MRLs) [3, 4].

Avermectins belong to the group of macrocyclic lactones and are produced by *Streptomyces avermectilis*. The main avermectins are Abamectin (ABA) which is a mixture of avermectin B1a and B1b, Ivermectin (IVE) is a mixture of two compounds 22,23-dihydroavermectin H2B1a and H2B1b and Doramectin (DOR) (Fig. 1) [5].

These compounds are antiparasitic agents widely used against endo and ectoparasites affecting livestock and domestic animals, as well as humans [3, 6]. They act by inhibiting the neural transmission causing the death of the parasites [7, 8]. The avermectins are liposoluble and can remain in the milk of several species, including bovine, for weeks after application.

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The *Codex Alimentarius* proposed MRLs for IVE and DOR the values of 10 μg kg^{-1} and 15 μg kg^{-1}, respectively. For ABA is not established MRL [9].

In Brazil, the National Plan of Waste Management in Animal Products (PNCRC) provides 10 μg L^{-1} as the reference limit in milk for IVE and ABA and 15 μg L^{-1} to DOR [10].

The National Health Surveillance Agency (ANVISA - Brazil) also develops in this country the National Program for Veterinary Drug Residue Analysis in Foods of Animal Origin (PAMVet), and for bovine milk matrix, the presence of avermectin residues is checked by high performance liquid chromatography with fluorescence detection (HPLC-FD) [4].

The aim of this study is to present the an intralaboratorial methodology of validation based on the literature, but with modifications related with decrease amount sample and solvents volume which allow the simultaneous quantification of Ivermectin, Abamectin and Doramectin in bovine milk.

2. Materials and Methods

2.1. Standards and Reagents

Standards of ABA, IVE and DOR were purchased from Sigma-Aldrich, with 99% of purity. The stock standard solutions were individually prepared in acetonitrile and stored at -20°C. The working standard solutions were prepared by diluting the stock solutions with methanol/ water/acetonitrile (40:5:55 v/v/v) in the concentration range of 2.5-25 μg L^{-1}.

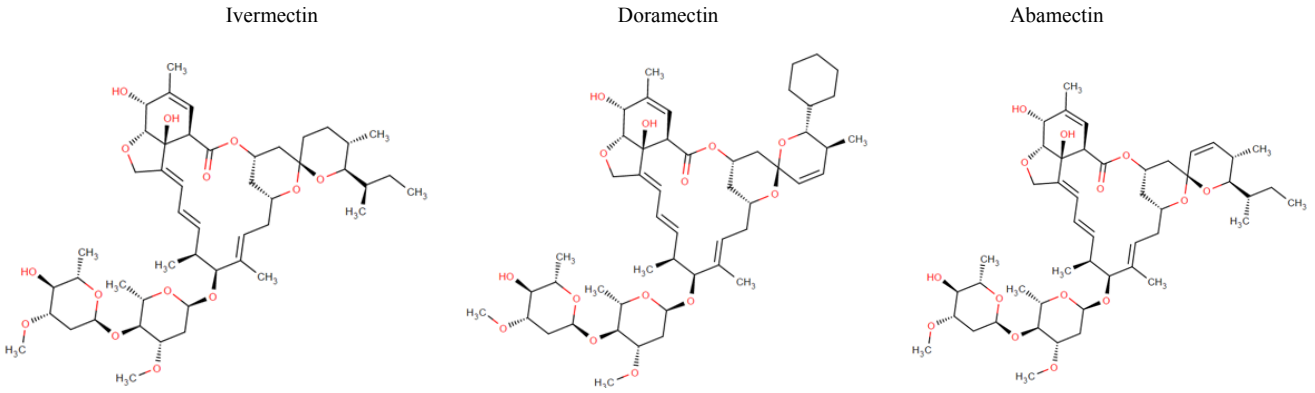

Figure 1. Chemical structures of avermectins

Acetonitrile and Methanol HPLC grade were purchased from JT Baker (USA). Methylimidazole (MI) and trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO), triethylamine (Vetec, Brazil) and trifluoroacetic anhydride (TFAA) (Merck, Darmstadt, Germany) were of analytical grade. Water was obtained from a Milli-Q water system (Millipore, Bradford, MA, USA).

2.2. Sample Collection and Preparation

The milk samples were purchased from a local organic farm and stored at -20°C. A total of 2.0 mL sample of milk was spiked by adding a standard solution to give fortification levels of 5, 10 and 15 µg L^{-1}.

Aliquots of 2 mL of milk were transferred to a 50 mL centrifuge tube and extracted with 5 mL of acetonitrile using a vortex mixer for 20 sec and then centrifuged for 10 min at 3000 g. The supernatant was transferred to a flask in which 13 mL of deionized water were added.

A SPE Strata-X cartridge (Phenomenex, Torrance, CA) was conditioned with 3 mL acetonitrile and 3 mL acetonitrile: water (3:7 v/v). The extract was transferred into the cartridge and washed with 5 mL acetonitrile: water (3:7 v/v) and the avermectins were eluted into an ambar glass tube with 5 mL of acetonitrile. The resulting extract was evaporated under nitrogen flow at 58 °C.

The dried extract was derivatizated with addition of 100 µL acetonitrile: MI (1:1), 150 µL acetonitrile: TFAA (2:1), 100 µL trimethylamine: acetonitrile (1:1) and 100 µL TFA: acetonitrile (1:1) using a micropipette. After, this solution was homogenizing in a vortex for 10 sec, filtered through a Millex HV filter (0.45 µm, Millipore) and analyzed by HPLC-FD [11].

2.3. HPLC-FD Analysis

The analyses were performed on a HPLC-FD model Waters 2695 (Milford, MA, USA) equipped with a fluorescence detector. The compounds were separated under isocratic conditions into a C18 column (Symmetry, 75 x 4.6 mm, 3.5 µm) at 30°C and mobile phase methanol: water: acetonitrile (40:5:55 v/v/v). The flow rate was 1.0 mL min^{-1} and the excitation wavelength 365 nm and emission wavelength 475 nm. The total running time was 15 minutes and data acquisition was performed by Empower Waters software.

2.4. Method Validation

The method validation was performed based on the criteria and recommendations of European Commission Decision 202/657/EC and also on good laboratory practice (GLP), supported in good scientific practice [12, 13]. The considered parameters were selectivity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), matrix effect, stability and robustness.

Selectivity was evaluated by analyzing 20 samples of blank milk. Linearity was determined in a range of 2.5-25 µg L^{-1} for ABA, IVE and DOR.

Precision and accuracy were evaluated by spiking blank milk in three fortification levels for each analyte in the range of 5-15 µg L^{-1} with six replicates each one.

Method precision was evaluated through the CV%. The analyses were performed on three different days by the same analyst and also by two different analysts at the same day.

Limits of detection and quantification were estimated based on parameters of the calibration curve [14].

Robustness was performed using Youden´s test. Matrix effect was evaluated preparing six replicates in blank matrix extract and compared with the external calibration curve. This comparison was performed employing Student´s and Fisher's test.

3. Results and Discussion

In determining the avermectin residues in bovine milk, the derivatization procedure requires care and attention. The avermectins containing tetrahydrobenzofuran ring that were derivatized with acylating reagent in the presence of a strong base, were converted to a chromophore group resulting in a fluorescent aromatic derivative Fig. 2 [11, 15].

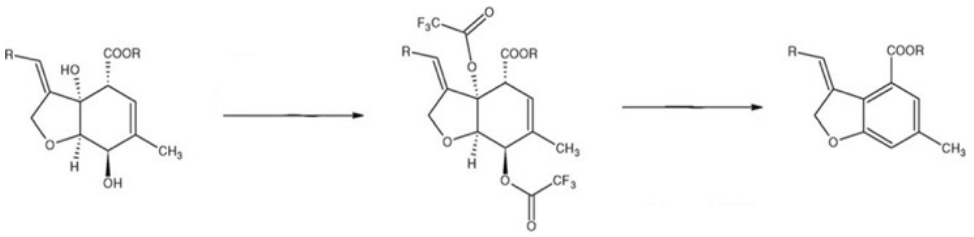

Figure 2. Reaction: formation of a fluorescent chromophore [11]

Thus, low detection limits are achieved when the reaction conditions are optimized, since it is possible the formation of two derivatization products for each avermectin. The derivatization reaction was studied for the compounds against the various compositions of the reagents and time stability of the derivatives. One study indicates the flu-OH derivative as a marker residue of avermectin [11]. Two compounds are formed primarily: flu-TFA and flu-OH. Thus, a better stability of the derivatives of ABA, IVE and DOR were achieved by adding 50 µL of TFA. Resulting in the instantaneous formation of the flu-TFA derivate (15 min at 70 °C) and inhibiting almost entirely flu-OH derivative. Therefore, it is very likely that the slow hydrolysis of the flow-TFA for the flu-OH is the cause of the reported cases of unstable derivative [11].

The time between injection and derivatization have also been studied and found that the derivatives are stable for up to 8 hours at room temperature in the dark. After checking the conditions of derivatization, we came to the conclusion to carry out the reaction and straightaway inject into the chromatograph.

Furlani *et al* analyzed the presence of macrocyclic lactones using the QuEChERS instead of SPE columns. To achieve the required levels of LOQ was necessary to increase the sample weight (10 mL) consequently the volume of extraction was increased [16]. While Gianetti *et al* replace the cartridges by washing with hexane and used 2.5 mL sample [5]. In our method some conditions were adapted, for instance, the samples and the solvent extraction volume were decrease, respectly 2 mL and 5 mL. And we keep using the SPE cartridge aimed at the concentration of the analytes studied and cleaning extract. Thus the presence of residues in environment was minimized.

The separation of the standards and their retention times, as well as a representative chromatogram of the blank milk are shown in Fig 3. The chromatographic conditions allowed an adequate separation in 12 min (Fig 3a). The selectivity of the method is indicated by the absence of interfering compounds in the analytes retention time (Fig 3b). In Figure 3c, we present a spike milk sample.

The external calibration curve was obtained from six injections at concentrations ranging from 2.5 to 25 µg L^{-1}. The curves of the three avermectins were submitted to ANOVA test and no deviation linearity ($p < 0.05$) was showed. The correlation coefficients (R) were greater than 0.99. The residuals were randomly distributed and did not exceed 20% [17].

For LODs and LOQs calculate it was used the method described by Ferreira *et al* [14]. This method uses the confidence interval obtained in analytical curve. In our case the LODs were 1.1, 3.1 and 4.5 µg L^{-1} for IVE, ABA and DOR respectively. The LOQs were also different for the three avermectins, being the highest one obtained for DOR. Thus, we fixed in 5 µg L^{-1} for all of them to facilitate the work with the standards.

In Table 1 is summarized all date obtained in linearity's and limits' studies.

Table 1. Summary of validation dates

Compound	Linearity (R)	LOD (ppb)	LOQ (ppb)	MRL *Codex Alimentarius* (ppb)	MRL Brazil (ppb)
Abamectin	0.9958	3.1	5	-	10
Ivermectin	0.9957	1.1	5	10	10
Doramectin	0.9916	4.5	5	15	15

So, for precision and accuracy, milk samples were prepared in sixplicate and in three concentration levels, ranging from 5 to 15 µg L^{-1}. The recoveries varied between 76 to 105% and the CV from 2 to 10% that were considered satisfactory (Table 2) [17]. Using QuEChERS like as extraction and clean-up Furlani *et al* reached 96-109% and them CV was until 10% [16].

In this study there was no matrix effect interfering significantly ($p < 0.05$) in the analytical results.

The stability of avermectins was measured in standard solutions stored under two different conditions, at room temperature (22 ± 2°C) for 24 hours and in a refrigerator (5 ± 3°C) for 15 days.

ABA, IVE and DOR remained stable during 24 hours at room temperature.

Under refrigeration for 15 days, only ABA remained stable, while IVE and DOR declined about 20% and 15% of initial concentrations, respectively.

The robustness was performed changing the following parameters: sample volume, solvent extraction volume, water volume, solvent elution volume, column temperature, flow rate and time from reaction to injection. Table 3 shows the changes made during the robustness test.

Using the Youden' test, the effects were not statistically significant, indicating that the validated method is robust.

A comparison of different analytical methods available for the analysis of ivermectins may be seen on Table 4.

Junqueira *et al* obtained accuracy and precision analyzing ABA, IVE, DOR and eprinomectin (EPR) in bovine milk ranging to 10-30 μg L^{-1}. They also assessed the level of 5 μg L^{-1} however, according to the criteria chosen by the authors, the level studied showed a variation coefficient above accepted [18].

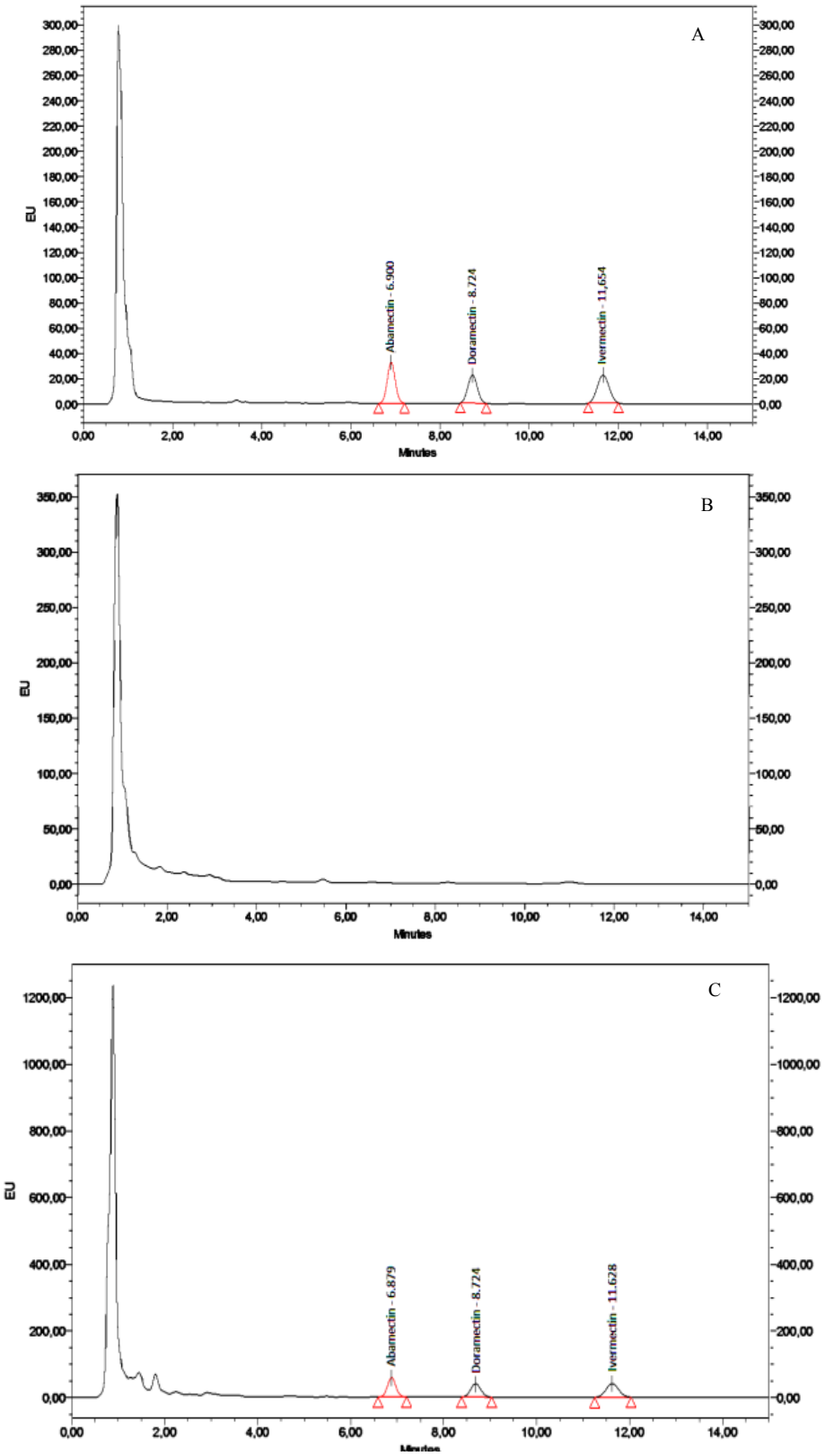

Figure 3. HPLC-FD chromatograms of (a) standard solutions ABA, IVE and DOR at 5 μg L^{-1} (b) blank milk sample (c) spike milk sample at 5μg L^{-1}

Table 2. Recoveries and precision for avermectins in milk

Compound	Level ($\mu g\ L^{-1}$)	Repetition			Precision intermediate	
		Recovery ± CV %				
		day 1	day 2	day 3	analist 1	analist 2
ABA	5	92 ± 5	92 ± 4	95 ± 9	93 ± 8	84 ± 6
	10	97 ± 3	94 ± 5	99 ± 5	97 ± 8	86 ± 4
	15	99 ± 5	93 ± 2	103 ± 5	90 ± 6	90 ± 6
IVE	5	93 ± 6	95 ± 5	99 ± 10	90 ± 8	90 ± 6
	10	98 ± 2	95 ± 4	101 ± 5	93 ± 8	94 ± 6
	15	100 ± 5	94 ± 3	105 ± 5	87 ± 6	89 ± 8
DOR	5	91 ± 7	92 ± 3	96 ± 9	84 ± 5	76 ± 7
	10	97 ± 3	94 ± 5	99 ± 5	90 ± 5	83 ± 2
	15	99 ± 5	94 ± 2	104 ± 4	85 ± 5	83 ± 2

The analysis were performed in sixplicates.

Table 3. Description of effects during robustness test

	Variables	1	2	3	4	5	6	7	8
Aa	Sample volume (mL)	2.1	1.9	1.9	2.1	1.9	2.1	2.1	1.9
Bb	Solvent extraction (mL)	5.5	5.5	4.5	4.5	5.5	4.5	5.5	4.5
Cc	Water volume (mL)	14	14	14	12	12	14	12	12
Dd	Solvent elution (mL)	4.5	5.5	5.5	5.5	4.5	4.5	5.5	4.5
Ee	Column temperature (ºC)	31	29	31	31	31	29	29	29
Ff	Flow rate ($mLmin^{-1}$)	0.95	1.05	0.95	1.05	1.05	1.05	0.95	0.95
Gg	Time from reaction (min)	0	0	5	0	5	5	5	0

Table 4. Summary of dates validation obtained by others authors

Compounds	LOD (ppb)	LOQ (ppb)	Range (ppb)	Recovery (%)	Reference
ABA, IVE, DOR, MOX, EPR and EMA	-	5	5-200	65-89	Gianetti *et al* [5]
ABA, IVE, DOR and MOX	0.3	ND	1-30	83-114	Schenck and Lagman [6]
ABA, IVE and DOR	0.1-1.2	0.2-7.5	ND	96-109	Furlani *et al* [16]
ABA, IVE, DOR and EPR	5	10	10-30	87.2-101.4	Junqueira *et al* [18]
IVE	0.037	0.043	0.5-100	73.5-79.5	Perez *et al* [19]
IVE	0.6	2	10-100	78	Lobato *et al* [20]
IVE	2.5	5	5-20	37-79	Machado *et al* [21]
AVE, IVE, DOR, EPR	0.05-0.68	0.17-2.27	2.5-200	63-104	Wang *et al* [22]

MOX = moxidectin EMA = emamectin ND= not disclosed

Schenck and Lagman reached average recovery in raw milk above 83% in the range of 1-30 $\mu g\ L^{-1}$ [6]. Perez *et al* evaluated only ivermectin in 5 replicates in the range of 0.5 to 25$\mu g\ L^{-1}$ obtaining recoveries varying from 73.5 to 79.5% [19]. Lobato *et al* also validated a method for ivermectin in milk. They found a LOQ of 2 $\mu g\ L^{-1}$ using a sample volume of 5 mL [20]. None of these authors explain the robustness like a validation item, leaving implicit that method is robust.

Recently, Machado *et al* conducted a validation for ivermectin in bovine milk obtaining recoveries variables (37-70%). The low recoveries values can be related to the derivatization reaction [21].

Wang *et al* analysed by liquid chromatography coupled to mass spectrometry, the residues of avermectins in various animal products among them bovine milk. They obtained LOQs of 0.17-2.3 $\mu g\ L^{-1}$ and recoveries of 62-104% [22].

The methodology optimized and validated in this work provides good results related to the applicability to a simultaneous analysis of ABA, DOR and IVE residues in bovine milk. All the validation parameters studied are in accordance to the criteria of European Commission Decision 202/657/EC.

4. Conclusions

This study demonstrates that methodology is applicable for the detection and quantification of Abamectin, Ivermectin and Doramectin residues in bovine milk. It is a low cost method, with good sensitivity and it uses a kind of detector accessible to any laboratory. The results showed that the use of small quantities of sample (2 mL) and organic solvent (5 mL) for extraction achieves the necessary accuracy and precision in compliance to the Brazilian legislation for monitoring of these residues. The use of small quantities of organic solvents also reduces the potentially toxic residues in the environment.

REFERENCES

[1] Brasil. Portal Brasil, 2015. [Online]. Avaiable: http://www.brasil.gov.br/economia-e-emprego/2015/10/rebanho-bovino-brasileiro-cresce-e-chega-a-212-3-milhoes-de-cabecas-de-gado.

[2] Brasil, Projeções do Agronegócio, 2015. [Online]. Avaiable: http://www.agricultura.gov.br/arq_editor/file/MAIS%20DESTAQUES/Proje%C3%A7%C3%B5es%20Agroneg%C3%B3cio%202009-2010%20a%202019-2020.pdf.

[3] Caldas, E.D., Souza, J.R., Silva, E.P., 2014, Resíduos de medicamentos veterinários em leite e ovos, Quimica Nova, 37, 111-122.

[4] Brasil, Agência Nacional de Vigilância Sanitária, 2015. [Online]. Avaiable: http://www.anvisa.gov.br/alimentos/pamvet/index.htm.

[5] Giannetti L, Georgi A, Necci F, Ferretti G, Buiarelli F, Neri B., 2011, Validation study on avermectine residues in foodstuffs. Analytica Chimica Acta, 700, 11-15.

[6] Shenck, F.J., Lagman, L.H., 1999, Multiresidue determination of abamectin, doramectin, ivermectin and moxidectin in milk using liquid chromatography and fluorescence detection. Journal of AOAC international, 82, 1340-1344.

[7] Danaher, M., Howells, L.C., Crooks, S.R.H., Cerkvenik-Flags, V., O'Keeffe, M., 2006, Review of methodology for the determination of macrocyclic lactone residues in biological matrices, Journal of Chromatography B, 844, 175-203.

[8] Souza, S.V.C., Silva, G., Diniz, M.H.G.M., Santos, E.V., Lima, J.A., Teodoro, J.C., 2003, Determinação de resíduos de avermectinas em fígado bovino por cromatografia líquida de alta eficiência, Ciênc. Tecnol. Aliment, 2, 54-8.

[9] Codex Alimentarius. Maximum residue limits (MRLs) and risk management recommendations (RMRs) for residues of veterinary drugs in foods CAC/MRL. 2–2015. Updated as at the 38th Session of the Codex Alimentarius Commission (July 2015).

[10] Brasil, Ministério da Agricultura, Pecuária e Abastecimento, Instrução Normativa n°- 13, 2015. [Online] Avaiable: http://www.agricultura.gov.br/animal/qualidade-dos-alimentos/residuos-e-contaminantes.

[11] Berendsen, B.J.A., Mulder, P.P.J., van Rhijin, H.J.A., 2007, The derivatization of avermectins and milbemycins in milk: New insights and improvement of the procedure, Analitica ChimicaActa.2007; 585:126-33.

[12] Commision Decision 2002/657/CE of August 2002 implementing Council Directive 93/23/EC concerning the performance of analytical methods and the interpretation of results. Official Publications of the European Communities 2002, L221, 8-36.

[13] Rodrigues, N.R., Souza, A.P.F., Watanabe, M., 2012, Implantação e implementação das normas das Boas Práticas Laboratoriais (BPL) no laboratório de análises de resíduos da Universidade Estadual de Campinas, Química Nova, 35, 1276-1280.

[14] Ferreira, C.M.M., Ribeiro, F.A.L., 2008, Planilha de validação: uma nova ferramenta para estimar figuras de mérito na validação de métodos analíticos univariados, Quimica Nova, 31, 164-171.

[15] Xie, X., Gong, S., Wang, X., Wu, Y., Zhao, L., 2011, Simplified RP-HPLC method for multiresidue analysis of abamectin, emamectin benzoate and ivermectin in rice, Food Addit. Contam., Part A, 28, 19-25.

[16] Furlani, R.P.Z., Dias, F.F.G., Nogueira, P.M., Gomes, F.M.L., Tfouni, S.A.V., Camargo, C.R., 2015, Occurrence of macrocyclic lactones in milk and yogurt from Brazilian Market, Food Control, 48, 43-47.

[17] Sanco, European Commission Health & Consumer Protection, 2013, Guidance document on analytical quality control and validation proce dures for pesticide residues analysis in food and feed. Sanco/12571/2013. [Online]. Avaiable: http://ec.europa.eu.

[18] Junqueira, R.G., Teodoro, J.C., Lima, J.A., Souza, S.V.C, 2007, Validação intralaboratorial de método quantitativo para determinação múltipla de resíduos de avermectinas em leite bovino por cromatografia líquida de alta eficiência com detecção de fluorescência, Ciênc. Tecnol. Aliment., 27, 823-836.

[19] Peréz, R., Peréz, L., Palma, C., Villegas, R., Veja, M., 2006, Metodología analítica y detección de resíduos de ivermectina em muestras de leche de rebaños de la província de Ñuble, Chile, Archivos de medicina veterinária, 38, 143-150.

[20] Lobato, V., Rath, S., Reyes, F.G., 2006, Occurrence of ivermectin in bovine milk from the Brazilian retail market, Food Addit. Contam., 23, 668-673.

[21] Machado, S.T.Z., Rezende, A.R., Gennari, S., Conte-Junior, C.A., Costa, M et al, 2016, Development of HPLC-fluorescence method for the determiantion of ivermectin residues in comercial milk, J. Exp. Food Chem, 2, 1-6.

[22] Wang, F., Chen, J., Cheng, H., Tang, Z., Zhang, G., Niu, Z., Pang, S., Wang, X., Lee, F.S., 2011, Multi-residue method for the confirmation of four avermectin residues in food products of animal origin by ultra-performance liquid chromatography-tandem mass spectometry, Food Addit Contam Part A, 28, 627-639.