Article

Determination of Eleven Veterinary Drugs in Chicken Meat and Liver

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Featured Application: Our results can provide occurrence trends of veterinary drugs from poultry products in Brazil and help regulatory agencies to established safe protocols regarding the number of samples that need to be annually analyzed.

Abstract: Brazil chicken production is around 13 million tons and about a third is exported to over 150 countries, placing Brazil as the world largest chicken meat producer, and therefore it is crucial to follow the legislation of all importer markets. This study conducted a survey by chance in 45 meat industries able to export. Therefore, 2580 chicken meat samples were collected and submitted to 11 analyte extraction and chromatographic verification of compliance in an accredited laboratory. Ten chemical residues (amoxicillin, bacitracin, colistin, dinitolmide + zoalene, spectinomycin, roxarsone, tiamulin, tylosin, trenbolone acetate and virginiamycin) were investigated in chicken meat and one (halofuginone hydrobromide) in chicken liver. The results showed that no compound exceeded the maximum residue limits established by seven legislations. All residue concentrations found were below the method quantification limit, thereby confirming the capability of Brazilian chicken meat industries in complying to foreign markets.

Keywords: broiler; feed additives; LC-MS/MS; meat legislation; meat safety; poultry meat; veterinary drugs

1. Introduction

Brazil is the world leader in chicken meat exports (4.2 million tons comprising whole carcass and chicken cuts), serving more than 150 countries, followed by the United States (3.2 million tons) and the European Union (1.5 million tons). With respect to chicken production, Brazil also plays a significant role as the second largest producer (13.2 million tons), being overcome by the United States (19.9 million of tons) and close to the European Union (12.4 million tons), occupying 13% of the world market in chicken meat production [1].

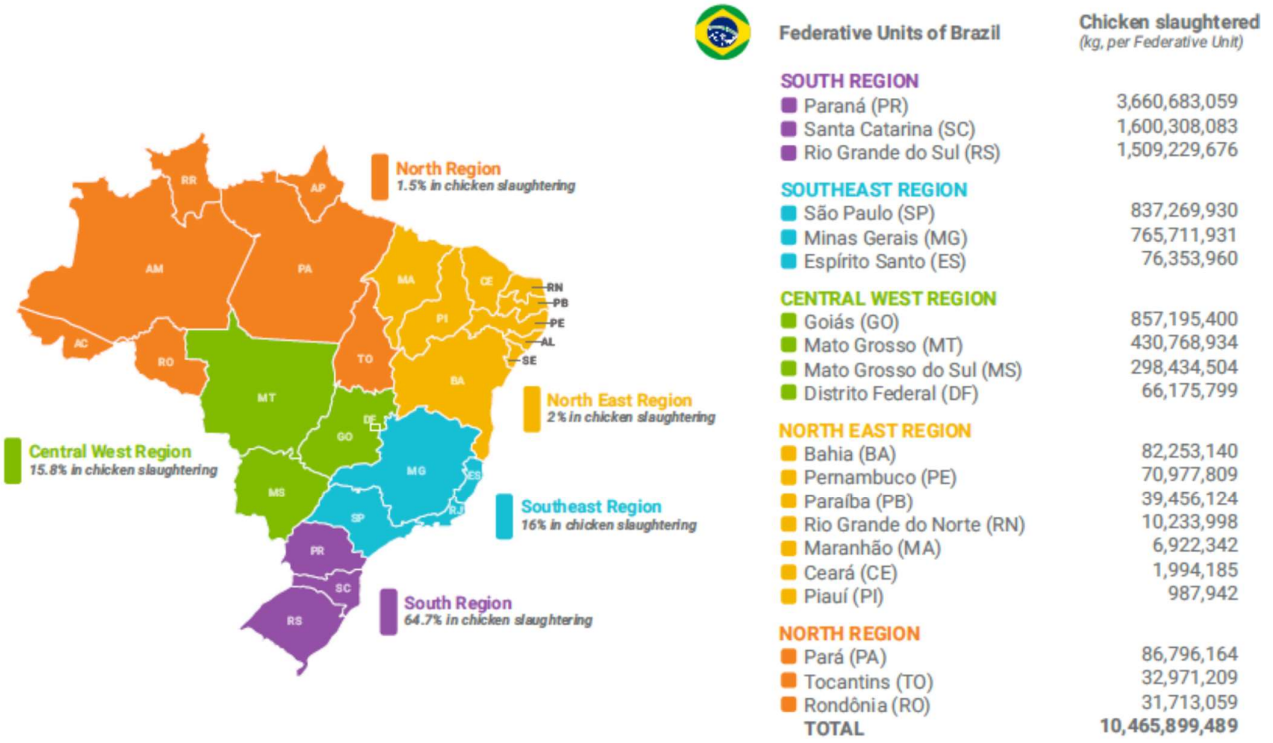
From the total amount of 13.2 million tons of chicken meat produced by Brazilian industries, 68% remains in the country and 32% is exported [1]. Figure 1 shows all the Federative Units in Brazil that export chicken meat, mainly represented by the South and Southeast Regions which together comprise 80.7% of the country production.

Regulatory agencies and authorities have established maximum residue limits (MRL) in various food matrices of animal origin to support governments and food operators in the monitoring of veterinary drug residues in the food chain. About 200 veterinary drug residues with antibiotic, antiparasitic or anti-inflammatory activities are regulated in a variety of food matrices [2]. As veterinary drugs and feed additives are used in animal

farming, there are concerns about residues in edible tissues [3]. Considering that poultry products are among the main protein sources consumed worldwide, it is though necessary to keep monitoring the meat safety to assure consumer protection.

In this sense, exporter food companies must follow the rules of the importing countries. Such demands may be difficult to attend and depend on advanced laboratory techniques, such as liquid-chromatography coupled to mass spectrometry to detect extremely low amounts of a given veterinary drug. At the end, the objective is to demonstrate that an exporter country can produce meat in such required circumstances.

Therefore, it is important to continuously control and monitor the quality of chicken meat and products, investigating if the industry complies with the agreed international standards. Moreover, it is recommended that the statistical reassessment of the National Sampling Plan undergoes a review, if necessary, to evaluate residues of some substances. Therefore, the objective of this study was to randomly evaluate 45 different Brazilian exporter companies regarding eleven compounds usually used in poultry farming and check whether residues of these chemicals comply with different regulations. A complementary purpose of this study was to assist in foreign missions to prove that Brazilian chicken meat is regularly complying with the international standards by providing scientific data, which are scarce, about chemical residues in these matrices.



Federative Units of Brazil	Chicken slaughtered (kg, per Federative Unit)
SOUTH REGION	
Paraná (PR)	3,660,683,059
Santa Catarina (SC)	1,600,308,083
Rio Grande do Sul (RS)	1,509,229,676
SOUTHEAST REGION	
São Paulo (SP)	837,269,930
Minas Gerais (MG)	765,711,931
Espírito Santo (ES)	76,353,960
CENTRAL WEST REGION	
Goiás (GO)	857,195,400
Mato Grosso (MT)	430,768,934
Mato Grosso do Sul (MS)	298,434,504
Distrito Federal (DF)	66,175,799
NORTH EAST REGION	
Bahia (BA)	82,253,140
Pernambuco (PE)	70,977,809
Paraíba (PB)	39,456,124
Rio Grande do Norte (RN)	10,233,998
Maranhão (MA)	6,922,342
Ceará (CE)	1,994,185
Piauí (PI)	987,942
NORTH REGION	
Pará (PA)	86,796,164
Tocantins (TO)	32,971,209
Rondônia (RO)	31,713,059
TOTAL	**10,465,899,489**

Figure 1. Chicken meat exporting Federative Units (kg/Federative Unit) during 2019–2020.

2. Materials and Methods

2.1. Sampling Plan

The Brazilian Association of Animal Protein (ABPA) is the national institutional organization on poultry and pork production in Brazil. The mission of ABPA is to represent the industry in national and international forums, ensuring product quality, food safety and sustainability, and promoting the integration of the entire production chain.

Every year, Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) monitors the compliance of chicken meat with respect to chemical residues, among other

issues that are beyond the scope of this study. From March 2019 to March 2020, 45 industries were randomly selected by ABPA to undergo a survey regarding eleven important chemical residues that are used as feed additives to raise poultry, then export the meat for over 150 countries.

All these 45 meat industries must be registered under the Federal Inspection System (SIF) allowed by MAPA. After the total of 45 industries were selected and identified, the collection of the samples took place. For one year, a total of 2580 chicken meat and liver samples were collected (Figure 2) and had 11 chemical residues evaluated through LC-MS/MS. Briefly, sample collection must follow the guidelines of the National Residues and Contaminants Control Plan (PNCRC) established by MAPA Sample Collection Manual, which established that 500 g is sufficient to perform the chemical residue analysis of each specified matrix. The sample sent to analysis must be frozen, which is the ideal condition for sample preservation before being submitted to chemical residue analysis. The Quality Assurance Sector of the companies are responsible for sampled collection and sending. This process is supervised by the Official Veterinary of the meat-producing establishment.

Figure 2. Chicken meat sampling and chemical residues determination by LC-MS/MS.

2.2. Chicken Samples Preparation and Chromatographic Analysis

The samples were immediately identified, processed, and submitted to an accredited laboratory, Eurofins, located in Indaiatuba, SP, Brazil. The sample preparation and analyte extraction procedure were as follows:

a) Internal Method POP-QV028/3 (applied to tiamulin, virginiamycin, tylosin, spectinomycin, amoxicillin, trenbolone acetate, halofuginone hydrobromide, dinitolmide + zoalene): The samples were weighed (2.00 ± 0.05 g) and internal standard solution was added. The samples were extracted with acetonitrile:water:acetic acid (70:29:1)v/v then the mixture was manually stirred or vortexed. In case, if further homogenization was needed, ceramic homogenizer or disposable glass sphere were added to the mixture. Afterward, the mixture was placed on a shaking table for 20 min at 200 rpm and then centrifuged at 4000 rpm for 5 min. Then, 500 µL from the extract and 500 µL deionized water were transferred to 15-mL Falcon tubes. These tubes were vortexed, and the solution was filtered through 0.22 µm membrane directly into vial, which were then submitted to LC-MS/MS analysis;

b) Internal Method POP-QV013/3 (applied to bacitracin and colistin): The samples were weighed (5.00 ± 0.05 g) and internal standard solution was added to these samples, as well as to the calibration curve and blank samples. The samples were extracted with MeOH:H20 (25:75) and 1 mol/L sulfuric acid. Afterward, the mixture was stirred to

homogenize it properly. Then, the mixture was placed on a shaking table for 20 min at 200 rpm and then centrifuged at 4000 rpm for 5 min. Then, the samples were frozen (−18 °C) and allowed to rest for 20 min. After that, samples were centrifuged for another 20 min at 4000 rpm and the supernatant was immediately separated to another 50-mL polypropylene tube. The samples were submitted to vacuum-controlled SPE extraction, so that the analytes were allowed to interact with the stationary phase. Afterward, clean-up was performed with deionized water and the cartridges were dried under vacuum in this step. The 15-mL polypropylene tubes were placed under the cartridges and the analytes were eluted with MeOH:Formic acid solution (75:25). The mixture was stirred and filtered through 0.22 μm membrane directly into borosilicate vial, which were then submitted to LC-MS/MS analysis;

c) AOAC Official Method 2013.06 applied to roxarsone extraction and quantification is well established in the literature [4]. Briefly, the sample was submitted to pressure digestion which occurs using nitric acid in a closed vessel with elevated temperature and pressure by conventional or microwave-assisted heating. Determination occurs using inductively coupled plasma (ICP)/MS [4].

In order to determine the chemical residues by chromatographic analysis, a C8 column (150 mm × 3 mm × 4 μm) was used for analytes separation in a system consisting of an HPLC (Sciex Exion LC), coupled to an ABSciex API 5500 mass detector, with a binary pump, a column oven, and an auto-injector. The mobile phase for methods a) and b) were the same and consisted of (phase A) an aqueous solution with 0.1% formic acid (v/v) + 5 mM ammonium formate and (phase B) methanol with 0.1% formic acid (v/v) + 5 mM ammonium formate. The gradient elution ramp was set as follows: 98% A (0 min), 100% B (0.0 to 7.5 min), 100% B (7.5 to 9.5 min), 98% A (9.5 to 9.6 min), and 98% A (9.6 to 17.0 min). The injection volume was 20 μL, the flow rate was 700 μL/min, and the column temperature was 40 °C.

2.3. Statistical Analysis

In the sampling plan protocol, the prevalence of 1% non-compliance and 95% confidence coefficient for substances allowed in national poultry farming was considered, totaling an annual sampling of 300 samples for each of the most known compounds (see Table 1, third column – number of samples). For the remaining compounds (dinitolmide + zoalene, halofuginone hydrobromide and trenbolone acetate) not registered by MAPA/Brazil in poultry farming, a higher prevalence of non-conformity (5%) was considered, resulting in 60 samples annually, at 95% confidence coefficient. Therefore, the sampling protocol was 300 samples for 8 residues and 60 samples for the remaining 3 residues; thus (300 × 8) + (60 × 3) = 2580 samples needed.

3. Results and Discussion

3.1. Feed Additives

Table 1 shows the eleven evaluated antimicrobials in this study in chicken meat and liver as demanded by the Gulf Technical Regulation to attend Middle East requirements [5]. All complied to the international standards as well as the concentration found in this study for all analytes were below the limit of quantification (LQ). Regarding the domestic market, Brazilian MAPA collects samples annually from Federal Inspected (SIF) meat industries and monitors the feed additive residues through the PNCRC for the chains of beef, pork, goat, mutton, equine, rabbit, poultry, ostrich meat, and milk, fisheries, honey, and eggs.

Usually, MAPA monitors 7 from the 11 antimicrobials displayed in Table 1 (see columns 5, 6 and 7), however sometimes in a different matrix, not only chicken meat. For instance, bacitracin, colistin and tiamulin are often monitored in eggs, however MAPA prohibited the import and manufacture of colistin, through Normative Instruction 45/2016, for the purpose of a zootechnical additive that improves animal performance,

while the others are allowed; and spectinomycin is monitored in kidneys, either for internal or external market.

Table 1. Results of the eleven compounds analyzed in chicken meat and liver, number of samples evaluated for each matrix; Brazilian legislation (matrix, number of samples, MRL) compared to worldwide MRL legislation regarding these same residues in meat.

This Study			MRL in Meat (µg/kg)								
Compound	Residues (µg/kg) *	Number of Samples **	Brazil [a]			Middle East [b]	EU [c]	China [d]	Canada [e]	USA [f]	CODEX [g]
			Matrix	Number of Samples	MRL						
Amoxicillin	<10	300	Chicken meat	600	50	10	50	50	10	10	50
Bacitracin	<75	300	Egg	300	500	500	150	500	500	500	-
Colistin	<75	300	Egg	300	300	150	150	150	-	-	150
Dinitolmide and zoalene	<5	60	-	-	-	3000	-	3000	3000	3000	-
Spectinomycin	<50	300	Kidney	600	5000	100	300	500	100	100	500
Halofuginone hydrobromide	<3	60	-	-	-	100	10	10	10	-	-
Roxarsone	<70	300	-	-	-	500	-	500	-	-	-
Tiamulin	<25	300	Egg	300	1000	100	100	100	100	-	-
Tylosin	<25	300	***	600	100	200	100	200	200	200	100
17-α-Trenbolone	<2	60	Swine urine	60	2	2000	-	-	2	-	2
Virginiamycin	<20	300	-	-	-	200	10	100	100	100	-

[a] BRASIL [6]; [b] Gulf Technical Regulation [7]; [c] EU [8]; [d] MOA [9]; [e] Health Canada [10]; [f] Code of Federal Regulation [11]; [g] Codex Alimentarius [12] * Residues found in this study refer to LQ, while LD where half the concentration of LQ for all compounds, except for bacitracin (LD = 20 µg/kg) and colistin (LD = 35 µg/kg); the recovery rates for all compounds were between 80 and 110%; ** all samples evaluated herein were from chicken meat, except for halofuginone hydrobromide, which was analyzed in liver; *** Tylosin has MRL for chicken meat, kidney, egg, milk, and honey.

Nevertheless, when Brazil exports chicken meat to other countries, the legislation of the specific importer country must be followed and sometimes other veterinary drugs are demanded in different matrices, as happened in the present research. For instance, Brazilian MAPA published an ordinance (87/2018) recognizing the private protocol of the Gulf Technical Regulation to attend Middle East requirements [5,7].

Most of the chemical residues monitored in chicken meat production by Brazilian PNCRC (Table 1) refer to antibiotics used as prophylactic agents, so these compounds are demanded and analyzed by the main importing countries. For instance, tylosin residues were below the LQ = 25 µg/kg in all 300 chicken meat samples analyzed herein. Although this compound is regulated for meat worldwide, varying from 100–200 µg/kg, in Brazil, a lot of matrices are analyzed regarding tylosin residues, such as chicken meat, kidney, egg, milk, and honey. Tylosin is a macrolide antibiotic, added to feed or to water and is registered exclusively for veterinary use (pigs, chickens, turkeys, and cattle) in many countries, being effective against gram-positive bacteria, Mycoplasma and Chlamydia spp [3].

For all 300 samples of amoxicillin (AMX) analyzed in this study, all were below LQ (10 µg/kg). Usually, Brazilian MAPA analyzes 600 samples per year of this additive residue in chicken meat samples collected throughout the country. AMX is a semisynthetic β-lactam antibiotic which belongs to the aminopenicillin group. AMX has a broad antimicrobial spectrum, low toxicity when compared to other veterinary drugs and a relatively good absorption rate. It is of low cost and applied in poultry farming. However, AMX

residues may be deposited in tissues and this antibiotic is considered in most monitoring plans in Latin America [13].

Colistin residues in this study showed concentration values below 75 μg/kg in chicken meat, while MAPA has in its PNCRC scope 300 samples to be analyzed in eggs with MRL = 300 μg/kg. Other legislations worldwide establish MRL in meat to be MRL = 150 μg/kg, while Canada and USA do not use this additive, nor Brazil in meat-producing animals. Colistin is one of the most effective antimicrobial agents and is able to inactivate 100% Salmonella spp. in poultry [14].

Regarding tiamulin, all 300 samples showed residue levels below LQ (25 μg/kg) in chicken meat. Although MAPA only analyzes this residue in eggs (MRL = 1000 μg/kg), the amount allowed by other legislations in meat, as displayed in Table 1, is 100 μg/kg for this type of residue. Tiamulin is approved to be used in chicken and turkey production to reduce the severity of disease caused by mycoplasma, while in swine tiamulin is FDA-approved to treat pneumonia and dysentery [15].

Bacitracin residues in this study showed concentration values below 75 μg/kg in chicken meat, while MAPA has in its PNCRC scope that 300 samples need to be analyzed in eggs (MRL = 500 μg/kg). In other legislations, bacitracin residues vary from 150 to 500 μg/kg for this type of residue. Bacitracin is indicated for the prevention of necrotic enteritis caused, for instance, by Clostridium spp [16].

Spectinomycin residues determined in 300 samples of chicken meat were found to be below LQ < 50 μg/kg. MAPA scope foresees spectinomycin determination only in chicken kidneys with MRL = 5000 μg/kg in 600 samples collected annually. Other legislations demand MRL values varying from 100 to 500 μg/kg in chicken meat. Spectinomycin is an aminocyclitol antibiotic, distinct from the aminoglycosides and has a broad-spectrum antibiotic applied in veterinary medicine for mycoplasma infections and for treating organisms with multiple antibiotic resistance [17,18].

Roxarsone residues in this study determined in 300 chicken meat samples showed concentration values below 70 μg/kg. This compound is not covered by Brazilian legislation. Roxarsone is an arsenic-basic veterinary drug, which was banned since 2014 and withdrawn from the market [19]. Although arsenic speciation is an important issue to be considered, once the organic form is less harmful than the inorganic one, FDA banned its commercialization [20]. Therefore, if this compound is found in feed or food matrices, it violates legislation, except for China and Middle East, which accept this additive when residues are below the MRL of 500 μg/kg in chicken meat.

Virginiamycin residues in all 300 samples showed values below LQ (20 μg/kg) in chicken meat. Virginiamycin is used as a feed additive to enhance animal growth in cows, swine, poultry, and fish, and contains lactonic rings that are primarily composed of two anti-bacterial factors, namely M1 and S1 [21]. Only Codex forbids its use [12] and Brazil follows this criterion, although in other legislation it is allowed at MRL = 10–200 μg/kg, as shown in Table 1.

Halofuginone hydrobromide residues were found to be LQ < 3 μg/kg in all 60 analyzed chicken meat samples. It is not authorized by Codex, and Brazil as well as the USA does not use this compound, although it is allowed by other legislations at MRL varying from 10 to 100 μg/kg in meat. Halofuginone hydrobromide is an antiparasitic agent, which acts against protozoa [8], and is more common to be analyzed in liver.

Trenbolone acetate residues in all 60 samples showed values below LQ (2 μg/kg) in chicken meat. In Brazilian MAPA this compound is only evaluated in swine urine, while other countries have MRL for meat, varying from 2 μg/kg (Codex, Canada) to 2000 μg/kg (Middle East) in meat. Trenbolone acetate is a synthetic progestational agent, used mainly as implants under the animal skin of feedlot steers [22]; and usually determined in beef [12] (MRL = 2 μg/kg) and liver (MRL = 10 μg/kg); however anabolic steroids are forbidden for food-producing animals in the EU [8], China [9], USA [11] and Brazil [6], although within Brazilian legislation, the MRL = 2 μg/kg (Table 1) corresponds to the Minimum Required Performance Limit (MRPL) for the analytical method in swine urine.

Dinitolmide residues were below LQ = 5 μg/kg in all 60 evaluated chicken meat samples. It is a nitro amide coccidiostat used in poultry feed and may be harmful to the environment and human health [23] and it is not authorized in Brazil, EU and nor recommended by Codex [6,8,12], however it is allowed by Canada, China, USA, and Asian legislation at 3000 μg/kg (Table 1).

Halofuginone hydrobromide, trenbolone acetate and dinitolmide + zoalene are not registered by the national Ministry (MAPA); the country does not have validated methodologies to evaluate such residues as a routine. Therefore, our assays are performed by accredited laboratories, only when demanded by certain importing countries, whose headquarters lays abroad.

To maintain the poultry production and exports, it is imperative to continuously educate farmers, by teaching them notions about chemical residue legislation, knowledge about meat inspection, biosecurity measures and animal vaccination, so as to ensure food safety, food security, and public health [24].

3.2. Statistical Analyses to Evaluate the Need of Decreasing the National Sampling Plan and Increasing Limit of Non-Compliance

The sampling procedures are specific to each type of problem to be addressed. With respect to PNCRC applied to products of animal origin, Discrete Probability Models (DPM) are usually adopted. The main discrete models used in the PNCRC are based on the Hypergeometric and Binomial Distribution Models.

The Hypergeometric Model aims to describe the number of successes (for example, a sample violating the MRL of a given residue displayed at the PNCRC) in a sequence of "n" extractions from a finite population of small size, without replacement. In this case, the effect of sampling without replacement significantly impacts in the coming probabilities of a new successful sample. For this situation, the sample distribution must be based on the Hypergeometric Distribution.

In populations of larger sizes (N > 5000 units, for instance), the effect of sampling without replacement is negligible. Thus, the Binomial Distribution can be used to determine an appropriate number of samples to be used, as the number of samples for a high confidence coefficient defined previously, will be constant for populations over 5000 evaluated units [25]. PNCRC for poultry, swine, and cattle (and other species) fall into this category, given the number of animals normally slaughtered along one year.

For adoption of the Binomial Distribution Model, initially a certain residue/contaminant is quantified in a particular number of samples and, subsequently, each sample is classified as a Binary Variable as: 0 = does not violate the MRL for market "X" or 1 = violates the MRLs for the same "X" market. Thus, each sample behaves like a success and failure test, characterized as a classic Bernoulli distribution model, where the sum of "N" Bernoulli results in the Binomial Probability Distribution. Thus, the Binomial model is more likely to be suitable for PNCRC because the populations of interest (cattle, poultry, swine, etc.) consist of thousands of individuals and/or units.

When non-compliance results are detected (for example, MRL violations of a given residue "X"), it is possible to obtain an estimate of likely prevalence of each residue in the general population. However, when no non-compliant results are found or the behavior of residue "X" is unknown, as is the case for some residues not yet assessed by the PNCRC, any claims about prevalence should be stated with a defined confidence coefficient, so that the prevalence of non-compliant results does not exceed a specified percentage. In this case, a prevalence of 1% is usually adopted when the behavior of residue "X" is unknown. The number of samples needed to provide a high coefficient of confidence to detect at least one non-compliant result is around 300 samples [25].

For a 95% probability of detecting a violation, if it occurs in 1% of the population, the survey should be done in 299 units of that population, a number that is rounded to 300, for practical purposes as recommended by MAPA [26]. That means that 300 samples per evaluated compound are a reasonable amount to be dealt with when referring to chemical

residues in poultry farming. This minimum amount is important for the establishment of plans/protocols that are being implemented for the first time and whenever necessary, the sampling plan can be revised.

Therefore, considering our satisfactory results and that more evaluations of each compound are carried out annually, making the Brazilian protocol increasingly robust, it is possible to increase the non-compliance limit from 1.0% to 1.5% for the substances allowed in national poultry farming (that means all residues evaluated in 300 samples according to the third column in Table 1). In this situation, the sampling calculated by the binomial model to guarantee (with 95% confidence) that there would be at least one sample above the MRL would be 199 per year, rounded up to 204 samples for convenience, representing 17 monthly samples. Therefore, instead of 300 samples analyzed annually, this number can be reduced to 204 samples per year. For substances not allowed in Brazilian poultry farming (for instance, those described in item 2.3), sampling must be maintained according to the ongoing protocol (60 samples/year).

4. Conclusions

Feed additives or veterinary drugs when used inappropriately, without obeying legislation and withdrawal periods may result in residue concentrations in edible tissues that exceed the corresponding maximum residue limits (MRL), therefore it is important to continuously monitor and control the presence of residues in the production chain to assure food security. From all the eleven chemical residues studied, no one showed concentration values above legislation maximum residue limits. Therefore, based on this study, Brazilian chicken meat does not violate any international regulatory limits considered herein (Middle East, EU, China, Canada, USA, CODEX and Brazil). With the adoption of good manufacturing and production practices, the risk of chemical residues in products of animal origin are minimized. Nevertheless, a continuous, broad, and robust monitoring plan is advised to maintain the quality, safety, and competitiveness of the meat products.

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