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LC-MS/MS determination of chloramphenicol in food of animal origin in Brazil

Determinação de cloranfenicol em alimentos de origem animal no Brasil empregando LC-MS/MS

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Abstract

Chloramphenicol is a highly efficient antibiotic with broad spectrum activity. It has been banned from food producing animals because of serious adverse effects to human health. Nevertheless, it is still being used in some countries because of its high efficacy and relatively low price. There is currently a minimally required performance limit (MRPL) defined at 0.3 µg/kg. This is the reason why chloramphenicol has often been analyzed by highly efficient and sensitive single residue methods. The objective of this review is to provide the state-of-art scientific knowledge on chloramphenicol, the LC-MS/MS methods used for its analysis and its occurrence in foods of animal origin in Brazil.

Keywords: antibiotic, milk, fish, honey, liquid chromatography, mass spectrometry.

Resumo

O cloranfenicol é um antibiótico de amplo espectro e elevada eficiência. Devido à ocorrência de efeitos adversos graves à saude humana, este antibiótico teve seu uso banido em animais destinados à alimentação humana. No entanto, seu uso ainda é comum em muitos países, devido à alta eficácia e baixo custo. Atualmente, existe um limite mínimo de desempenho requerido (LMDR) de 0,3 µg/kg e, por essa razão, o clorofenicol tem sido frequentemente analisado por métodos altamente eficientes e sensíveis. O objetivo desta revisão é apresentar o estado-da-arte sobre o conhecimento científico a respeito do cloranfenicol, métodos baseados em LC-MS/MS usados para sua análise e ocorrência em alimentos de origem animal no Brasil.

Palavras-chave: antibiótico, leite, peixe, mel, cromatografia líquida, espectrometria de massas.

1. Introduction

Antibiotics are widely used in intensive agriculture. They can be a therapeutic agent in the treatment of animal diseases, a prophylactic agent to avoid or prevent sickness, and also a feed additive to promote growth and increase feed efficiencies. However, their widespread use in food producing animals can be a potential hazard to human health due to the possibility of causing bacterial resistance and potential allergic reactions to the antibiotic. Special concern has been raised with regard to chloramphenicol, which, besides the inherent problems with antibiotics, it can cause fatal health problems, among them, bone marrow aplasia, aplastic anemia and gray baby syndrome. Due to the potential harmful effects to human health, the use of chloramphenicol has been prohibited for the treatment of food-producing animals in several countries[1-3].

However, the use of chloramphenicol to treat food-producing animals remains a possibility due to its high efficiency, broad spectrum of activity, prompt availability and low cost. The occurrence of chloramphenicol in foods can be the result of authorized use but lack of compliance with the withdrawal time period, unauthorized use and also unintentional or cross-contamination[3,4]. Therefore, there is a need to constantly evaluate the occurrence of this antibiotic in food.

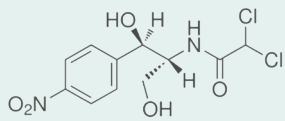
The control of chloramphenicol in foods can be performed by screening or confirmatory procedures. Screening methods only provide semi-quantitative analysis and can give rise to false positives, but they are used due to simplicity in sample preparation, sensitivity, speed and low cost. On the other hand, confirmatory methods, such as those employing liquid chromatography (LC) coupled to mass spectrometry (MS) are the approaches of choice for determination of antibiotics, because they allow definitive identification, quantitative determination at very high level of specificity and sensitivity[4,5]. The objective of this review is to provide updated information on the occurrence and concentrations of chloramphenicol in food in Brazil determined by LC-MS/MS.

2. Characteristics and antimicrobial activity of chloramphenicol

Chloramphenicol is a naturally occurring, broad-spectrum antibiotic with excellent antibacterial and pharmacokinetic properties. Its formula, structure, chemical names and numbers as well as physico-chemical and spectral characteristics are described in Table 1. Chloramphenicol was isolated in 1947 from *Streptomyces venezuelae,* a soil bacterium, but it has been synthetically produced for a long time. Different trade names are available and there are three common forms for systemic therapy: a free base form, chloramphenicol palmitate and chloramphenicol succinate. Other formulations are also available for topical use[2,6].

Chloramphenicol has a wide spectrum of antimicrobial activity. It is effective against Gram-positive and Gram-negative cocci and bacilli (including anaerobes), *Rickettsia*, *Mycoplasma*, *Chlamydia*, among others. It is usually bacteriostatic, but at higher concentrations it can be bactericidal. It acts by diffusing through the bacteria cell wall, binding to the bacterial 50S ribosomal subunit and inhibiting protein synthesis and cell proliferation[1]. It was widely used as a human antibiotic and also as a veterinary drug. Nowadays, its use in human medicine has been restricted to ophthalmic and some serious infections (*Salmonella typhi* and other forms of salmonellosis, staphylococcal brain diseases and life threatening infections of the central nervous system and respiratory tract). The veterinary use of chloramphenicol includes administration to pets, farm and aquaculture animals. In therapy and prophylaxis, the main infectious diseases treated with chloramphenicol are enteric and pulmonary infections, skin and organ abscesses and mastitis. It is also used in infections caused by anaerobic bacteria or those that are resistant to other antimicrobial agents[1,3].

Table 1. Characteristics of chloramphenicol.

Parameter	Characteristics
CAS number	56-75-7
EC number	200-287-4
IUPAC name	2,2-dichloro-N-[(1R,2R)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl] acetamide
Names	Chloramphenicol; chlornitromycin; chloromycetin; levomycetin; chlorocid; globenicol
Molecular formula	$C_{11}H_{12}Cl_2N_2O_5$
Structure	
Molar mass (g/mol)	323.12938
Melting point (°C)	150.5-151.5
pka	11.03
Log P	1.103
Physical description	White to greyish-white or yellowish-white fine crystalline powder or fine crystals, needles or elongated plates
Taste	Bitter to taste
Spectral properties:	Specific optical rotation: +18.6˚ at 20 ˚C (ethanol); -25.5˚ at 25 ˚C (ethyl acetate). IR: u 5174; UV: 385 nm; Mass: 236
Solubility	Very soluble in methanol, ethanol, butanol, ethyl acetate, acetone, chloroform; Water solubility - 2500 mg/L (at 25 °C)
Stability	Neutral and acid solutions are stable on heating; In solution, chloramphenicol undergoes a number of degradative changes related to pH, temperature, photolysis and microbiological effects

CAS[7]; Pubchem[8].

3. Toxicological aspects and current legislation

The widespread use of antibiotics in food-producing animals can be a potential hazard for human health. Furthermore, the indiscriminate use of chloramphenicol can lead to bacterial resistance, allergic reactions, disruption of the balance of the gastrointestinal microbial flora, and hemotoxic effects, such as aplastic anemia, bone marrow depression and gray baby syndrome. Since it undergoes biotransformation to the inactive metabolite chloramphenicol glucuronide in the liver, individuals with subnormal liver function and infants are also at risk. Aplastic anemia is an irreversible side effect that is not dose-related; this side effect is probably the result of the reduction of its *p*-nitro group to the highly toxic nitroso metabolite. It is a rare but often fatal condition with no treatment. Another side effect is bone-marrow depression, suppressing bone marrow and its production of red and white blood cells and platelets. This effect is reversible if the treatment is discontinued. Also, infants, especially premature babies, when exposed to high levels of chloramphenicol, can develop the 'gray baby syndrome'. This probably occurs because the liver enzymes of an infant are not fully developed, and any chloramphenicol received across the placenta or in breast milk remains intact in the body, inducing hypotension, hypothermia, flaccidity, cardiovascular collapse, cyanosis and death within hours. There are also indications that chloramphenicol is genotoxic *in vivo* and could cause cancer. Although the evidence is considered limited, chloramphenicol has been categorized by the International Agency for Research on Cancer (IARC) as probably carcinogenic in humans, classified as group 2A[1,9].

Based upon scientific reports about chloramphenicol, an acceptable daily intake (ADI) has never been allocated and a maximum residue limit (MRL) has not been assigned[1]. Chloramphenicol was banned for use in food-producing animals in the European Union and in many other countries including Brazil as a means to eliminate it from the food production chain and related goods[10,11]. A zero tolerance provision was established and a minimum required performance limit (MRPL), which is the concentration that laboratories should be able to detect and confirm, of 0.3 µg/kg for chloramphenicol was set by the European Commission and adopted by several countries for analytical methods to be used in testing for chloramphenicol in products of animal origin[11-14].

To warrant national public health safety and to maintain competitiveness in international trade, food producers have to ensure that the products traded are in compliance with the safety and quality criteria required by consumers. Among actions undertaken by Brazil to warrant safety and quality control, the Ministry of Agriculture, Livestock and Food Supply of Brazil created a food safety program called National Residue Control Plan (NRCP). It has the purpose of generating reliable analytical results, monitoring residues and contaminants involved in food production, including antibiotics[15]. The Brazilian Agency of Sanitary Surveillance (ANVISA) from the Ministry of Health also created a National Program for the analysis of veterinary drug residues in food available for consumers[16]. Therefore, it is of great importance to have sensitive methods for the determination and confirmation of residues and contaminants in foods.

4. LC-MS/MS methods for the analysis of chloramphenicol in foods

Several methods are available for the determination of chloramphenicol in foods, both for screening or quantification purposes. Screening methods are cost-effective and have a high sample throughput[2,17]. However, the effective control of chloramphenicol in foods requires very sensitive and reliable analytical methods to comply with the stringent requirement established for a banned compound. Due to the chemical properties of chloramphenicol, quantitative methods using gas chromatography with mass spectrometry (GC-MS) require transformation of chloramphenicol into a stable volatile compound, which lengthens analysis time and may not be reproducible at trace levels[4,18]. Many of the reported liquid chromatography/ultraviolet (LC-UV) methods did not reach the required sensitivity and selectivity to meet the current MRPL. The power of a mass spectrometer as a chromatographic detector results from its capacity to determine, by means of the molecular weight, the precursor ion and its fragments, which provide structural information. The combination of liquid chromatography with tandem mass spectrometry (LC-MS/MS) allows definite identification and quantification of trace chloramphenicol in complex food matrices due to the specificity and sensitivity associated with this technique[4,5,19,20]. In this context, only methods and results for chloramphenicol based on LC-MS/MS will be described.

According to Table 2, several studies were undertaken on the analysis of chloramphenicol in foods by LC-MS/MS. In most of them, analysis was carried out in the multiple reaction monitoring (MRM) mode via electrospray ionization operated in the negative mode. Deuterated chloramphenicol (d_5-chloramphenicol) was used as the internal standard. The transitions used for chloramphenicol quantification and confirmation varied among studies. However, the $[M-H]^-$ ion and at least two product ions are monitored. For example, Guidi et al.[21] used m/z 320.9→152.1 and m/z 320.9→256.9 for chloramphenicol in fish analysis. The monitored ion for the internal standard was m/z 326.015→157.0. Matrix-matched calibration curves were used. In most of the studies, the method was validated according to the criteria established by the EC Commission Decision 657/2002[22].

Table 2. Methods for the extraction and separation of chloramphenicol in food of animal origin in Brazil by LC-MS/MS.

Reference / Food	Extraction technique	LC column & mobile phase	Recovery (%)	Limit of detection
Monteiro et al.[23]				
Fish	LLE - acetonitrile:water & SPE - Captiva cartridge	C18 (3x100 mm, 3.5 µm) & 0.1% formic acid:acetonitrile with 0.1% formic acid	93.2	1 µg/kg
Taka et al.[24]				
Honey	LLE - ethyl acetate	C18 (2.1x50 mm, 5 µm) & 2 mM ammonium acetate:methanol	>97	0.04 µg/kg
Nicolich et al.[25]				
Milk	LLE - 10 mM formic acid & ethyl acetate	C18 (2x100 mm, 5 µm) & 0.1% formic acid:acetonitrile with 0.1% formic acid	95-97	0.09 µg/L
Barreto et al.[26]				
Honey	LLE - ethyl acetate	C18 (4.6x150 mm, 5 µm & 2.1x100 mm, 3.5 µm) & acetonitrile:water	85.5-115.6	0.02 µg/kg
Fish	LLE (acetonitrile, chloroform)		89-97	0.06 µg/kg
Shrimp			87-100	0.06 µg/kg
Martins Junior et al.[27]				
Honey	LLE - ethyl acetate	C18 (2.1x50 mm, 3 µm) & 5 mM ammonium acetate:(methanol:water, 95:5) with 5 mM ammonium acetate	83	0.00052 µg/kg
Milk	LLE (acetonitrile, chloroform) & SPE		83	0.00052 µg/L
Guidi et al.[21,28] & Tette et al.[29]				
Milk	LLE - 10 mM formic acid & ethyl acetate	C18 (2x50 mm, 5 µm) & 0.1% formic acid:acetonitrile with 0.1% formic acid		
Fish	LLE - ethyl acetate		82.7	0.019 µg/kg
Honey				
Rocha Siqueira et al.[30]				
Fish	Phosphate extraction solution + LLE ethyl acetate	C18 (2.1x100 mm, 4 µm) & water:methanol	101-104	0.03 µg/kg
Shrimp			103-109	
Bovine meat			100-106	
Pork meat			102-104	
Poultry meat			87-97	
Egg			105-111	

LLE – liquid-liquid extraction, SPE – solid-phase extraction.

LC separation of chloramphenicol was obtained by reverse phase C18 columns from different brands (Table 2). Columns dimensions varied from 50 to 150 mm length, 2 to 3 mm internal diameter and 2 to 5 µm particle size. Different mobile phases were used in gradient elution, among them methanol:water, acetonitrile:water, acetonitrile:water acidified with formic acid, and ammonium acetate:methanol. In every method, except for one, the limits of detection and quantification were below 0.3 µg/kg, which is the MRPL established for chloramphenicol. Moreover, high sensitivity was obtained, in the ng/kg or ng/L range. Therefore, the majority of the methods were appropriate for the purpose.

Prior to LC-MS/MS analysis, sample preparation is needed to properly extract chloramphenicol from the food matrix. Concentration of the analyte and removal of interfering compounds may also be needed[31]. According to Table 2, sample preparation for chloramphenicol analysis involved mostly liquid-liquid extraction (LLE),

even though solid-phase extraction (SPE) was also used in a few studies. Representative and homogeneous samples were extracted for chloramphenicol by LLE. In most of the methods, a simple extraction procedure using ethyl acetate provided good recoveries of chloramphenicol from honey, milk and fish samples. The sample was spiked with the internal standard, vortexed for a few seconds and allowed to equilibrate. The extracting solvent was added and sample was mixed (several minutes), centrifuged, the supernatant was transferred and the sediment was extracted once more. The supernatants were mixed and evaporated to dryness under nitrogen flow and they were dissolved in the mobile phase, vortexed for a few seconds, allowed to equilibrate and injected into the LC.

In the extraction of chloramphenicol from honey, dissolution of the sample in water (1:1, w/v) was needed prior to a simple LLE procedure with ethyl acetate[24,26,27,29]. During extraction of chloramphenicol from milk, Nicolich et al.[25] and Guidi et al.[28] added water acidified with 10 mM formic acid prior to LLE with ethyl acetate. However, Martins Junior et al.[27] proposed two sequential LLE procedures, the first with acetonitrile and the second using chloroform. The supernatant was dried under nitrogen flow, dissolved into methanol, water and Na_2HPO_4 and submitted to SPE using a Supelclean™ ENVI™ Chrom P (Supelco, Bellefonte, PA, USA). By using this more sophisticated procedure, a detection limit in the ng/kg range was obtained.

Different procedures were used for the extraction of chloramphenicol from fish. Guidi et al.[21] used a simple LLE procedure with ethyl acetate and obtained good recoveries. Barreto et al.[26] used two LLE procedures, the first with acetonitrile and the second with chloroform, achieving similar results for fish and shrimp samples, improving recoveries. Monteiro et al.[23] used a more sophisticated procedure involving LLE with acetonitrile:water, followed by ultrafiltration (SPE) using a Captiva cartridge to remove protein and particulate matter; however, these researchers focused on multiresidue analysis of 12 drugs of different antimicrobial classes. Such a detailed procedure would not be necessary for a single antibiotic analysis. Rocha Siqueira et al.[30] proposed a method based on the extraction of chloramphenicol using a phosphate extraction solution (containing NaCl, KCl, Na_2HPO_4 and KH_2PO_4) and ultrasound bath for 15 minutes prior to LLE with ethyl acetate. They validated this method for fish, shrimp and also for meat (bovine, pork and poultry) and egg.

Several sophisticated and complex sample preparation techniques have been used in the analysis of chloramphenicol, by using different sorbents (Oasis, molecularly imprinted polymers and multi-walled carbon nanotubes) or techniques, like QuEChERS[18,32-35]. However, efficient extraction of chloramphenicol from food matrices for LC-MS/MS analysis can be undertaken by a simple LLE procedure. The use of additional steps may not be necessary. Furthermore, they can be time-consuming, require larger quantities of chemical reagents, involve extensive manual procedures, and use cleanup columns (SPE) that increases the analysis time and cost.

5. Occurrence of chloramphenicol in food

Even though the use of chloramphenicol in food producing animals was banned several years ago, it was detected in some foods of animal origin, as indicated in Table 3. Four studies focused on honey (total of 43 samples) indicated that samples from different regions of Brazil, from different beekeepers, floral sources and colors did not contain chloramphenicol[9,25,27,29]. Eighty six samples of fish were analyzed in four different studies, and tilapia was the main type of fish analyzed. Chloramphenicol was only detected in one sample at levels below the MRPL[9,22,24,30]. No chloramphenicol was found in shrimp (14 samples)[30]. Samples of meat (556 from bovine, pork and poultry) and eggs (60) were also analyzed and none of them contained chloramphenicol[30].

Table 3. Occurrence of chloramphenicol in food of animal origin by LC-MS/MS in Brazil.

Food	Samples analyzed (% Positive)	Concentration in positive samples	Reference
Honey			
Different beekeepers and floral	5 (0%)	nd	Barreto et al.[26]
Brands from SP market	4 (0%)	nd	Martins Junior et al.[27]
Different regions, floral & colors	22 (0%)	nd	Taka et al.[24]
Samples from MG market	12 (0%)	nd	Tette et al.[29]
Milk			
Milk (brands from market)	4 (25%)	4.73 ng/L	Martins Junior et al.[27]
Dried milk (brands from market)	3 (66.6%)	5.9 – 6.10 ng/L	
Suspect (Elisa) milk	41 (0%)	nd	Nicolich et al.[25]
Farm samples (raw)	49 (41%)	0.10 – 13.9 µg/kg	Guidi et al.[28]
Fish			
Nile tilapia (4 farms)	36 (0%)	nd	Monteiro et al.[23]
Aquaculture fish (Pintado, tilapia, matricha, saint peter, tambaqui, tambacu)	13 (7.7%)	0.063 µg/kg	Guidi et al.[21]
Sarotherodon niloticus (farms)	21 (0%)	nd	Barreto et al.[26]
Fish	16 (0%)	nd	Rocha Siqueira et al.[30]
Other foods			
Bovine meat	149 (0%)	nd	Rocha Siqueira et al.[30]
Pork meat	199 (0%)	nd	
Poultry meat	208 (0%)	nd	
Shrimp	14 (0%)	nd	
Egg	60 (0%)	nd	

nd – not detected.

Milk was the food product with the highest occurrence of chloramphenicol. Among studies undertaken, only the one by Nicolich et al.[25] failed to detect chloramphenicol in the 41 milk samples which were positive by ELISA. However, the samples had been stored for a long period of time prior to analysis, which could have affected the results. Martins Junior et al.[27] observed 42% occurrence of chloramphenicol in pasteurized and dried milk (total of 7 samples) at levels varying from 0.0047 to 0.0061 µg/kg. Guidi et al.[28] found similar prevalence (41%), at levels ranging from 0.10 to 13.9 µg/kg in samples obtained from dairy farms. Indeed, it is more likely to find antibiotics in farm samples prior to their dilution by the mixture with milk from other farms.

The NRCP has also been generating results for chloramphenicol and other residues in different foods of animal origin. Among the many different samples analyzed every year, only a few positive samples for chloramphenicol have been found, among them poultry meat (1 out of 76 samples from 2014, containing 0.39 µg/kg) and fish (1 out of 77 samples, containing 75.6 µg/kg)[36]. NRCP results for milk were negative for chloramphenicol in 120 samples of milk analyzed in 2009 and 2010. Results from PamVet[16] on chloramphenicol in milk also indicated no detectable levels in dried milk (139 samples) and 0.6% occurrence in UHT milk (464 samples) at levels ranging from 0.3 and 0.8 µg/kg.

Even though the number of samples analyzed was very limited, the outcome is good considering the low percentage of foods of animal origin containing

detectable levels of chloramphenicol. However, the illegal utilization of chloramphenicol to treat food-producing animals remains a possibility, either by administration of prohibited antibiotics, or failure to respect the proper withdrawal periods. The problem is more visible with milk due to its role in infant and overall human nutrition and its widespread consumption. Furthermore, chloramphenicol in milk can be transfered to dairy products, specially those rich in fat[17,35,37]. Therefore, it is important to ensure milk quality. Brazil has a quality control program aimed at milk from individual dairy farms. Antibiotic analysis of these milk samples should be performed to be able to detect the source of contamination and to implement educational programs to warrant milk quality.

It is also important to consider that there could be other sources of food contamination with chloramphenicol. Its use as a human medicinal antimicrobial can result in its release into the environment through waste streams by which food products may be contaminated during production. For instance, chloramphenicol has been detected in the aquatic environment such as effluents of sewage treatment plant and in surface water. Another source of this as well as other antimicrobials could be the natural occurrence in soil by bacteria (e.g., *Actinomycetes*), which can result in a large biomass per hectare in topsoil and subsequent uptake by crops and transfer of plants to feed[3,35,38,39].

6. Conclusion

Several methods have been developed for the analysis of chloramphenicol in food by LC-MS/MS. Extraction of chloramphenicol from food can be undertaken by simple LLE procedures without requiring any sophisticated clean-up technique. The methods were validated according to the criteria of Commission Decision 2002/657/EC and were found appropriate for the analysis of chloramphenicol with limits of detection way below the MRPL of 0.3 μg/kg. However, a very limited number of samples have been analyzed using this method, which became common in the last 10 years. Most of the studies performed focused on honey, milk and fish followed by shrimp, meats and egg. Chloramphenicol was detected in raw milk samples at levels above the MRPL and in trace amounts in fish. Even though chloramphenicol has been banned for use in food-producing animals for many years, it is still being detected. Therefore, monitoring and educational programs are needed to warrant safety of consumers and international trade.

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