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https://doi.org/10.22239/2317-269x.00970

Determination of veterinary antibiotic residues in foods of animal origin by liquid chromatography

Determinación de residuos de antibióticos veterinarios en productos de origen animal mediante cromatografía líquida

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ABSTRACT

Introduction: The presence of certain infectious agents makes necessary the use of antibiotics to ensure the welfare of animals destined for human consumption; however, the withdrawal time must be considered and respected since there is the possibility of finding residues above the permitted levels, which could constitute a risk to public health. **Objective:** Present a collection of information based on how is performed the detection and quantification of antibiotic residues in various products of animal origin using chromatography methods. **Method:** Review of databases in Elsevier, SciELO, Springer, Hindawi, FAO, EFSA, Senasa and Sanipes, using keywords such as "liquid chromatography", "mass spectrometry", "antibiotic residues" and "products of animal origin" in Spanish and English. **Results:** They were selected 71 references among articles, book chapters, norms and regulations published between 2000 and 2017, which it is emphasized that chromatographic methodologies for antibiotic residues monitoring must be sensitive, reproducible, reliable and identify volumes in mg/kg; likewise, they must follow the requirements of international standards for the maximum residue limits detecction. **Conclusions:** Liquid chromatography coupled to a mass spectrometer is the most used technique to allow the separation of complex matrices based on the molecular weight of the compound (antibiotic) or its fragments; however, It is complex, expensive and requires highly trained personnel.

KEYWORDS: Antibiotic; Liquid Chromatography; Mass Spectrometry; Veterinary Drug Residues

RESUMEN

Introducción: La presencia de ciertos agentes infecciosos hace necesario el uso de antibióticos para asegurar el bienestar de los animales destinados a consumo humano; sin embargo, hay que considerar y respetar el tiempo de retiro ya que existe la posibilidad de encontrar residuos por encima de los niveles permitidos, hecho que podría constituir un riesgo para la salud pública. **Objetivo:** Presentar una recopilación de información basada en cómo se realiza la detección y cuantificación de residuos de antibióticos en diversos productos de origen animal mediante métodos cromatográficos. **Método:** Revisión de bases de datos en Elsevier, SciELO, Springer, Hindawi, FAO, EFSA, Senasa y Sanipes, utilizando palabras clave como "cromatografía líquida", "espectrometría de masas", "residuos de antibióticos" y "productos de origen animal" en idioma español e inglés. **Resultados:** Se seleccionaron 71 referencias entre artículos, capítulos de libros, normas y reglamentos publicados entre el 2000 al 2017, de las cuales se destaca que las metodologías cromatográficas para el monitoreo de residuos de antibióticos deben ser sensibles, reproducibles, confiables e identificar volúmenes en mg/kg; asimismo, deben cumplir con las exigencias de las normas internacionales para la detección de límites máximos de residuos. **Conclusiones:** La cromatografía líquida acoplada a espectrómetro de masas es la técnica más utilizada ya permite la separación de matrices complejas en base del peso molecular del compuesto (antibiótico) o sus fragmentos; sin embargo, es compleja, costosa y requiere personal altamente entrenado.

PALABRAS CLAVE: Antibióticos; Cromatografía Líquida; Espectrometría de Masas; Residuos de Fármacos Veterinarios

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Received: May 26, 2017
Approved: Jan 23, 2018

INTRODUCTION

The presence of antibiotics residues for veterinary use in foods of animal origin is one of the most important problems related to food safety[1]. These products have been used in veterinary medicine since 1950 for therapeutic purposes and as growth promoters, since that time having a relationship with food waste and the environment, becoming a public health problem when residues of the drug and/or its metabolites reach the consumer at levels that can be harmful to their health causing allergic reactions, toxicity and teratogenicity[2]. Besides that, bacterial resistance can be generated and even cause technological problems[3].

Due to these prerogatives, the control of antibiotic residues is of paramount importance. For this, organisms such as the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have established a maximum residue limit (MRL) for food. According to Table 1, where some of the MRLs of antibiotics for products of animal origin are shown, more emphasis should be placed on the MRLs of products like muscle (meat) and milk; however, these values are equal or lower if compared with other tissues such as liver, kidneys and fat. This is very important because in different parts of the world these tissues are consumed and are a fundamental part of the diet.

"Screening methods" such as Enzyme-linked Immunosorbent Assay (Elisa) which has the advantage of being low cost, of operational ease, fast and able of handling a large number of samples, have been proposed for the identification of antibiotics. However, many times this technique does not differentiate between antibiotics of the same kind and/or provides semiquantitative information of the residues, so for the necessity establishing if wheter the residues are above the recommended MRLs, Elisa would not be the most appropriate[4]. For these reasons and before declaring that the samples analyzed contain residues of antibiotics, it is necessary to carry out an adequate confirmation of the identification and its quantification[5]. Thus, the chromatographic analysis in liquid phase (High Performance Liquid Chromatography, HPLC) or gas (Gas Chromatography, GC) coupled to different types of detectors (Ultraviolet, UV, Array diodes, DAD, fluorescence and mass spectrometry (MS) is a technique that responds to these demands[6], providing information on the analyte under evaluation[7,8,9].

Despite being a reliable tool, chromatographic methods are not without drawbacks such as the cost of equipment implementation, technical training, laboratory infrastructure, trained personnel, cost per analysis, time between collection and the

Table 1. Maximum permissible limits of antibiotics in meat of various species and milk.

Antibiotic	Bovine	Porcine	Ovine	Chicken	Turkey	Rabbit	Milk
Amoxicillin	50	50	50	-	-	-	4
Avilamycin	-	200	-	200	200	200	-
Benzylpenicillin	50	50	-	50	-	-	4
Ceftiofur	1.000	1.000	-	-	-	-	100
Colistin	150	150	150	150	150	150	50
Chlortetracycline/Oxytetracycline/ tetracycline	200	200	200	200	-	-	100
Danofloxacin	200	100	-	200	-	-	-
Streptomycin	600	600	600	600	-	-	200
Erythromycin	-	-	-	100	100	-	-
Flumequine	500	500	500	500	-	-	-
Gentamicin	100	100	-	-	-	-	200
Lincomycin	-	200	-	200	-	-	150
Neomycin	500	500	500	500	500	-	1.500
Pirlimycin	100	-	-	-	-	-	100
Sarafloxacin	-	-	-	10	10	-	-
Spectinomycin	500	500	500	500	-	-	200
Spiramycin	200	200	-	200	-	-	200
Sulfadimidine	100	-	-	-	-	-	25
Tilmicosin	100	100	100	150	100	-	-
Tylosin	100	100	-	100	-	-	100
Chloramphenicol/ Furazolidone/ Nitrofuran/ Olanquindox	Absence						

Values expressed in µg/kg for meat and µg/L for milk.
Source: FAO/WHO[7].

result, among others. Because of this, it is necessary to know the various chromatographic techniques to be able to choose the one that best suits the needs and viability of each laboratory.

METHOD

The purpose of this narrative work is to provide information and explore the different chromatographic techniques and their application in the determination of residues of veterinary antibiotics in products of animal origin based on a literature review of book chapters and indexed scientific articles, of which the most consulted data bases were Elsevier, Scientific Electronic Library Online (SciELO), Springer and Hindawi. In addition, various rules and regulations of international databases such as FAO, European Food Safety Authority (EFSA), European Union, Andean Community; and other nationals such as the National Agricultural Health Service (Senasa) of Peru, National Health Service Fisheries (Sanipes). All of them are available on the internet freely and/or at a low cost. It is important to note that initially the on-line search was based on mixed reference terms in Spanish involving words such as: products of animal origin, antibiotics and liquid chromatography and mass spectrometry. This helped us to know a little the reality. However, the search was not very up-to-date, so it was later used for terms such as: "Liquid chromatography", "chromatography", "mass spectrometry", "LC/MS", "antibiotic residues", "meat", "milk", "fish", "food" and "animal origin". The bibliographic search period was from January, 2016 to December, 2017. During this time, 164 references could be found, of which only 71 were used to carry out this review. Within the criteria of inclusion of the selected references, it was tried as much as possible to use references from the last 7 years, however it was necessary to extend this range, as we found references that were interesting and necessary, especially some rules and regulations that come since the year 2000.

RESULTS AND DISCUSSION

Panorama on the control and monitoring of residues of veterinary antibiotics

The importance of the use of antibiotics in the breeding of production animals is well known. However, its indiscriminate use, lack of information about its kinetics in different species or even not respecting withdrawal periods increases the risk of residues above tolerable levels. This could lead to the appearance of allergic symptoms and the development of bacterial resistance transferred from food to man[10]. All foods are susceptible to contain drug residues, an example is the products from cattle (milk, meat and derivatives), which are associated with the presence of antibiotic residues to the treatment of frequent infectious diseases such as mastitis, pneumonia or podophyllitis[11,12]. Although the countries of the European Union (EU) banned the use of antibiotics as growth promoters since 2006 and the United States has proposed its gradual withdrawal, many countries continue with this practice that contributes to the generation of waste[13]. In a society that is globally interconnected, it is necessary that food importing and exporting countries are continuously monitoring their products. Aquaculture products are one of the most important productions in recent years due to consumer preference. Table 2 shows MRLs of some antibiotics established by different countries for aquaculture products.

In the EU, Regulation n°37/2010, of December 22, 2009, describes the monitored veterinary substances and drugs and the procedures to establish the MRLs for veterinary products in products of animal origin[14]. Based on this, the monitoring carried out in 2014 by the EFSA showed that 0.03% of samples (n = 736,907) presented residues of Group A antibiotics (prohibited substances such as chloramphenicol, nitrofurans, nitroimidazoles) and only 0.18% of the samples analyzed showed values above those permissible for Group B antibiotics (Residues of veterinary antibiotics), with honey samples being the most involved[15].

In the case of countries members of the Andean Community, livestock and agro-industrial development is being promoted, one of its objectives being to achieve a greater degree of food security. All this within the framework established by the Cartagena Agreement established to adopt common standards and programs of plant and animal health. Thus, Decision 483 establishes a standard for the registration, control, commercialization and use of veterinary products in all the member countries of the Andean Community[16].

Based on this decision, if observed what happens in Peru in relation to antibiotic residues, it is found an agrifood safety regulation and a national program for monitoring contaminants in primary agricultural foods and feed by Supreme Decree n° 004-2011-AG. It also establishes that the primary agricultural foods consumed in the national market, including imported foods, must not exceed maximum permissible limits of chemical residues and other contaminants, fixed in the national standard, or established by FAO/WHO[17]. This country has three institutions in charge of control and monitoring of drug residues: Senasa, General Directorate of Environmental Health (Digesa) and Sanipes[18,19]. These institutions establish annual plans to carry out sampling by regions, define the type of food to be evaluated, the number of samples to be analyzed and the analytical procedures;

Table 2. Maximum permissible limits of antibiotics for aquaculture products accepted by different countries.

Antibiotic	PERU	USA	EUROPE	JAPAN
Chloramphenicol	Absence	-	-	-
Nitrofurans	Absence	-	-	-
Amoxicillin	50	Absence	50	50
Ciprofloxacin	100	Absence	Abscence	200
Enrofloxacin	100	Absence	100	100
Erythromycin	200	Absence	200	200
Florfenicol	1.000	1000	1.000	200
Sulfonamides	100	Absence	100	100
Oxytetracycline	100	2000	100	200

Values expressed in µg/kg.
Source: National Fisheries Health Service (Sanipes)[2]

in addition, establish the necessary procedures for collection and sending for determination of veterinary drug residues[20,21].

Chromatography in the determination of veterinary residues

Examining a bit of history, it is found that the first experiments on chromatography were carried out in 1906 by the Russian botanist Mikhail Tswett who managed to separate some pigments (chlorophyll and xanthophyll) from leaves of plants using a glass column packed with $CaCO_3$. The separated species appeared as colorful bands on the column, which explains the name of Greek origin *chroma* = color and *graphein* = describe, with which the method was named. According to the International Union of Pure and Applied Chemistry (Iupac), chromatography is defined as a physical separation method in which the components are separated and distributed between two phases, one that is fixed (stationary phase) while the other moves in an established direction (mobile phase), which can be gas, gel or liquid[24,25]. This principle is fundamental for the separation and analysis of specific molecules in complex matrices such as food, so its application in the determination of drug residues has been widely explored.

In the review it can be verified that chromatography and its application in antibiotic residues have varied a lot over the years. In the beginning, GC was used coupled to electron capture detectors (ECD), nitrogen-phosphorus (NPD) and flame photometric (FPD). The applications carried out by LC were less common because the detectors used as UV, diodes and fluorescence presented a lower sensitivity and selectivity than those used in GC. The development that MS has undergone, which allows detecting levels below µg/kg and even ng/kg, has revolutionized this field to such an extent that today the detection and quantification of waste without the use of detectors can not be conceived without the use of MS[26]. MS detectors provide chromatographic methods with much higher sensitivity and power of confirmation, which was not possible with traditional detectors (UV and fluorescence)[27]. Taking this into consideration, we will now detail the procedures for performing the LC coupled to the MS detector.

High resolution liquid chromatography

Better known as HPLC for its acronym in English, High Performance Liquid Chromatography[28], this type of chromatography is based on a liquid mobile phase where solvents such as water, acetonitrile or methanol are used and a stationary phase or chromatographic column that can vary depending on the analyte. When introducing the sample to the chromatographic system, it interacts with both phases and it is in the stationary phase that the substances we intend to identify are retained. The retained particles have different affinity for the stationary phase, a property that makes the output of the system work at different paces, this corresponds to the retention time and is fundamental in the separation process. This can be done with greater speed and efficiency if the flow pressure of the mobile phase of a few hundred kilos per square centimeter is increased, so certain conditions are necessary in the equipment that supports these variations[29].

Normally, an HPLC system has 6 basic components: an injection system to introduce the sample, a pump that maintains constant the flow of the mobile phase, a mobile phase, a stationary phase, a detector and the integrator that processes the signals and transforms them into a language that we can interpret (Figure 1). The choice of the detection system for the HPLC system is very important for the selectivity and sensitivity of the analyte to be identified. Among the most used detectors it can be mentioned UV and DAD[4]. However, at present, the association of HPLC with detection by mass spectrometry (LC-MS) is the most preferred for the determination of residues of veterinary drugs[6]. It is also important to point out that the chromatographic methods used must be validated for a correct application, trying to meet specifications such as limits of detection/quantification, repeatability, linearity, robustness, among others[30].

The classification of LC can be made based on the chemical composition of the chromatographic column types of fillers, which have physical and chemical characteristics that produce different separation mechanisms. In the case of antibiotics, the most used is reversed phase LC, which is suitable for the analysis of polar and even ionic analytes, if ion pairs are used. The type of apolar material with which the reverse phase columns are filled is usually chemically modified silica (chains of C8, C18), that interact with a polar mobile phase (water combinations with organic solvents such as methanol or acetonitrile). The percentage and type of organic modifier in the mobile phase is the most determining factor in the retention of polar analytes, but not ionics. The interactions between the analyte and the solvent are those that determine the specificity of reverse phase chromatography, since the interactions of the analyte with the stationary phase are relatively weak, non-specific Van der Waals interactions[31].

Procedures used to perform high performance liquid chromatography

Conditioning of the samples

The conditions and procedures used will depend on the type of material to be analyzed (meat, milk, eggs, among others). In the case of solid samples such as meat, it is necessary to carry out a cutting, grinding and homogenization process[32]. A disadvantage of this is that sampling is destructive and takes place after the animal has been slaughtered[6].

Extraction of analytes from the sample

After conditioning, elements like proteins, lipids or others that may interfere with the reading of analytes should be eliminated. Most methodologies use organic solvents (acetonitrile, ethyl acetate, methanol, acetone or petroleum ether). The use of these solvents is accompanied by a process of agitation, homogenization or ultrasonication that allows a better interaction with the samples[33].

After this procedure, it is advisable to use a purification or cleaning technique called solid phase extraction (SPE). In this

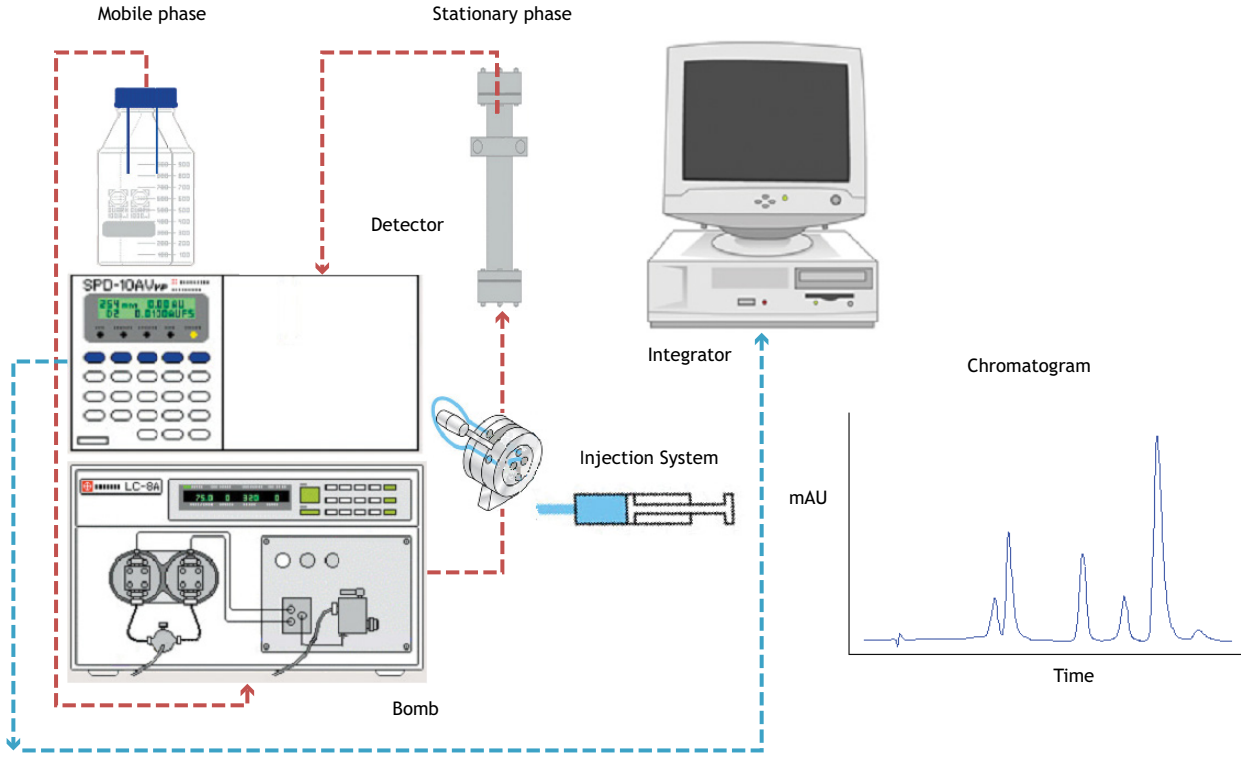

Figure 1. Schematic of a liquid chromatograph coupled to an ultraviolet detector.

part, the sample plus the organic solvent is subjected to a cartridge (syringe) that internally contains the same properties of the stationary phase (C18) and serves to retain the analytes of interest (antibiotic residues). To recover these residues, the cartridge is washed with solvents (acetonitrile/methanol and water) and then injected into the chromatograph[33]. The SPE is very effective for the detection of compounds with properties that are not very different; however, this technique, initially conceived with the stationary phase fixed in a cartridge, demanded even more preparation time, use vacuum pumps and cleaning for reuse. For this reason variations began to occur at the initial SPE[34].

Extraction in solid dispersed phase (SPE-d) is one of these variations, which consists in the addition of an adsorbent material to the crude extract followed by agitation, centrifugation and subsequent isolation of the adsorbent. At present, this method is called QuEChERS, acronym for the words in English Quick, Easy, Cheap, Effective, Rugged, and Safe. This technique, which is characterized by being quick, easy to perform, economical, effective, robust and safe, consists of two stages: 1) liquid extraction with solvents (water and acetonitrile) and hypertonic solutions (sodium chloride, magnesium sulfate), buffer agents) and 2) SPE-d in which an aliquot of the organic phase of the first stage is treated with various adsorbents to eliminate interferences from the matrix that could hinder subsequent instrumental analysis[33,35]. Recently, various materials have been experimented in order to improve extraction and purification using SPE. Columns with molecularly imprinted polymers (PIM) that selectively recognize molecules for which they were synthesized make the adsorption more selective[36].

The extraction process is fundamental to ensure the average life of the columns and optimize the maintenance and cleaning times of the chromatographic system. However, if the solvents in the filtration and purification processes are overused, we can lose part of the analytes (antibiotic residues). For this reason, the method to be used must be carefully chosen, according to the food and to the type of molecule[35].

Instrumentation

The fundamental parts of the chromatographic system consist of a pump, which supplies a constant flow (between 10 µL/min and 2 mL/min) and free of pulses of the mobile phase through the column. Apart from the capacity of the pump, the flow velocity depends on the diameter and material of the chromatographic column. Likewise, the pump must be manufactured with chemically inert materials, withstand high pressures and provide pulsation free flow[24]. Another fundamental part of the system is the column, where the separation of the analytes takes place. Most columns consist of cylindrical steel structures filled with chemically modified silica (C8 or C18). There are variations of the column based on the internal diameter, length, type of filling and size of the filler particle. The evolution of the column has prioritized the decrease in the size of the filler particle, which has resulted in an increase in selectivity and an improvement in the resolution of the chromatograms[31].

The detector of the chromatographic system must be sensitive to small analyte concentrations, give a wide linear response, have low background noise and be stable at the time the chromatographic run lasts. In addition, it must be able to withstand pressure, flow and percentages of the mobile phase (gradient). Some of the most used detectors are spectrophotometers that measure the absorbance at one or more wavelengths in the UV or visible spectrum; those of fluorescence, which measure the fluorescent emission by the analytes induced by a reactor located before or after the separation; others more complex, such as MS, can provide specific information that allows the unequivocal determination of compounds based on their molecular weight[29]. The following section details the parts and operation of the MS detector.

The volume of sample applied to the chromatographic system must be precise and must not disturb the circulation of the mobile phase. Due to this it is necessary to have an injection system which consists of high pressure rotary valves of several routes which can be manual or automated. These valves have two positions. In the filling position, the pump and the column are connected and the sample is introduced into a small tubular tank (loop) with the help of a microsyringe. The loop can be chosen in a different volume (5-500 µL). In the injection position, thanks to the rotation of the valve, the sample is dragged by the flow of the mobile phase and introduced into the column[29]. On the other hand, it is necessary that the solvents that enter the system are free of air. This makes it necessary to use a degasser, which can be operated by a vacuum pumping system, a distillation system, devices for heating and stirring the solvents or diffusion systems that allow the dissolved gases to be carried out of the solution by means of fine bubbles of an inert gas of low solubility. For example, a convenient way to treat solvents before being used as a mobile phase is to vacuum filter through a very small pore filter.

At present, the various methods favor that the interaction of the mobile and stationary phase take more time so the speed of the flow had to be increased in order to not affect the retention time. This resulted in the appearance of a new line of chromatographs capable of working at high pressures, using faster injectors, high-speed flows and detectors with higher capture speeds, which led to a reduction in chromatographic run time. This is how Ultra-Liquid Chromatography (Ultra Performance Liquid Chromatography, UPLC) and Ultra Fast Liquid Chromatography (UFLC)[37] appear.

Mass spectrometry

The MS coupled to LC (Figure 2) is one of the analytical techniques that has been most widespread in the last decades for the confirmation of antibiotic residues in products of animal origin. The success of its application is a consequence of the combination of a LC separation technique and the detection capacity by mass spectrum. The MS has been presented as an analytical technique of very high sensitivity, even up to pg / L levels. In addition, it is able to measure the ions with exact mass, providing information on the elemental composition, allowing to establish the fragmentation routes. It has even been possible to apply all these qualities in highly complex samples, reaching very high resolution levels[37,38].

The MS is based on the detection of ions from organic molecules in the gas phase; once these ions are obtained, they are separated according to the mass to charge ratio (m/z), and finally detected by means of a detector that converts the ion beam into an electrical signal that can be processed and stored. These analyzers can detect only the ions and therefore the molecules must be ionized in a source of ions before their separation and detection.

Ionization methods

The key that ensures the success of detection by MS is to convert neutral compounds, depending on the source of ions, into molecular ions, protonated, cationized, deprotonated, anionized molecules or fragments in the gaseous state through the application of electric fields and magnetic. The ionization is a property in which the molecules of the analytes pass to the gaseous state ioninzing; this state is achieved by adding or eliminating an electron or a proton. The excess energy provided in this step can also be transformed into a fragmentation of the molecule, generating fragment ions[24].

The first interfaces that were developed had as objective the elimination of the solvent (mobile phase) and try to get analyte molecules in gas phase before arriving at the source of ionization. Subsequently, during the development of these sources it was found that it was possible to favor the ionization of the analytes in the presence of the solvent and at atmospheric pressure without disturbing the mass analyzer. From these basic physical principles began to develop atmospheric pressure interfaces (Atmospheric Pressure Interface, API), including electrospray ionization (Electrospray Ionization, ESI) are the most used in the detection of antibiotic residues by LC -MS[37].

The ESI is produced by the application of a high voltage (3-6 kV) on a conductive capillary through which circulates a small flow

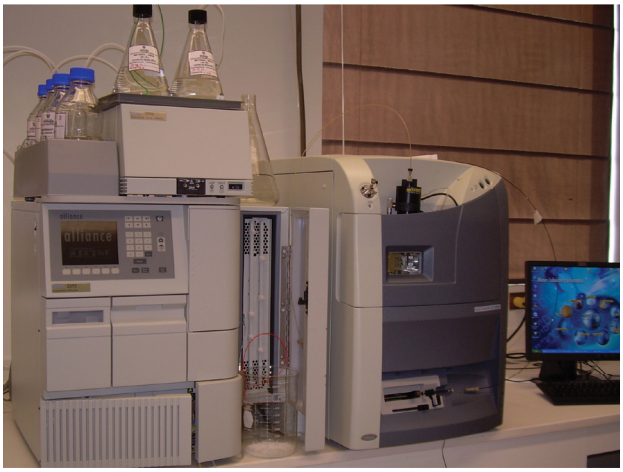

Figure 2. Liquid chromatograph coupled to tandem quadrupole mass spectrometer. (Courtesy Senasa).

of mobile phase at atmospheric pressure. The high potential difference creates an electric field that induces the accumulation of charges on the liquid surface at the end of the capillary, breaking into small drops of positively charged solvent (positive mode) or negatively (negative mode). These solvent droplets are dispersed as a consequence of the introduction of an inert gas flow coaxial to the flow of the mobile phase; this gas also causes the evaporation or loss the rest of the solvent in the droplets, since it circulates at high temperatures. The optimal flow from the LC should be of the order of 2 to 10 µL / min, however higher flows have been applied, up to 300 µL / min, using designs of sources with additional energy, temperature or gas flow, which allow a better dispersion of the drops from the capillary[37].

On the other hand, the Atmospheric pressure chemical ionization (APCI) is an interface that first vaporizes the mobile phase and then subjects the particles to a discharge to charge them to the analyte in the gas phase. This technique is used in compounds that do not ionize well with the ESI (often more stable, compounds with low molecular weights and non-polar compounds) but under more complex conditions it is likely that ESI causes the degradation of the sample, especially in thermolabile compounds. Apparently APCI have fewer problems for ionization than ESI. Because both ESI and APCI have different ionization mechanisms, the response and selectivity can vary between the two interfaces[39].

Ion separation

The mass analyzer is the most important part of the spectrometer; the physical principle is based on the dispersion and focusing of the ions as a function of the mass/charge ratio (commonly indicated as m/q or m/z), the variants of this process being the difference between the different instruments of mass spectrometry. Among the most used are: Quadrupole (Q), Quadrupole Ion Trap (QIT) and Time of Flight (TOF).

The quadrupole is the most used since it offers a wide range of masses (40 to 4000 u), reproducibility and precision for quantification, as well as high sensitivity. A quadrupole consists of four bars arranged in parallel with high precision, the poles are spaced around central axes. The bars located in opposite position are applied a direct current (DC) and a radio frequency (RF) voltage[38]. The ions are introduced in the quadrupole field through the application of a potential, with which they begin to oscillate in a plane perpendicular to the four bars. In this way, the ions describe a trajectory that directly depends on their m/z ratio, allowing a complete analysis of all the elements of the sample (Full scan). However, specifically, the quadrupoles are able to adjust a radio frequency to stabilize a specific m/z ratio that is directed towards the detector, discarding those m/z ratios higher or lower than the selected one (Selected Ion Monitoring). The quadrupole acts as a mass filter, so that, of all the ions coming from the source, only the selected ones are transferred to the detector, the rest being lost along the way. In this sense, to obtain a total mass sweep, the m/z must be coupled one by one, creating selective electric fields for each of them[37,38].

The quadrupole ion trap is a device formed by three electrodes, two of them hyperbolic, and among these, an electrode in the form of a toroidal ring. The system has the same foundation as the quadrupole analyzer. Simultaneously, a direct current and a radiofrequency potential are applied, in such a way that the ions generated are confined inside the toroidal ring. The ions are expelled from the chamber after the application of radiofrequency ramps. As the voltage increases, the amplitude of its oscillatory movement increases until it is expelled. Higher mass ions are destabilized as the radiofrequency voltage increases, so that the ions are detected sequentially, thus obtaining the spectrum as a function of voltage and mass[24].

The triple quadrupole (QqQ or TQ), where the first quadrupole (Q1) acts as a filter, selects and separates the charged molecules from the rest of the components that elute from the chromatograph. The third quadrupole (Q3) also acts as a filter, but in this case of the fragments produced by dissociation that arrive from the second quadrupole (Q2), allowing only those masses of selected fragments to pass to the detector. The dissociation process that occurs in Q2 is induced by an ionized and accelerated gas, so that it collides with the analyte molecules causing their fragmentation[24].

The TOF is based on the separation of the ions as a function of the time it takes to cross a flight tube of known length. This time depends on the m/z ratio because the less heavy ions will reach the detector more quickly than those with a higher value m/z ratio. One of the most important difficulties of this type of instruments when coupling them with LC is that the ions that arrive continuously from the interface have to be sent to the TOF by means of a pulse. To obtain a good measure of the time of flight of the ions, the time at which the flight of the ions begins must be very well controlled. The TOF works discontinuously, unlike the quadrupole analyzer, since the ions arriving at the analyzer are pulsed to the flight tube, wait for the time necessary for all to reach the detector before re-launching another pulse[37,38].

Traditionally had worked it with simple quadrupoles coupled to GC, because a large amount of structural information was obtained under an electronic impact ionization. In light of appearance of API ionization sources, which allow the coupling between LC and MS, a first drawback was seen related to the little fragmentation of the ionized molecules, which generates scarce structural information. For this reason the idea of coupling two mass spectrometry analyzers (MS/MS) to significantly increase the potential and the possibilities offered by the LC-MS arises. In this way, the MS in tandem (MS/MS) involves two stages: in the first, the selection of a precursor ion followed by an ionization of the molecules takes place by means of a dissociation process or by means of a chemical reaction. In the second stage, the analysis of the product ions obtained from the fragmentation process is carried out. This technique offers great selectivity, since it allows the possibility of isolating an ion in the collision cell, eliminating other ions or fragments that may interfere. The fragmentation in this case is produced by the collision of the selected ion[37,38].

Ion detection

A mass spectrum will be, therefore, two-dimensional information that represents a parameter related to the abundance of different types of ions as a function of the m/z ratio of each of them. Once the acquisition is made, the processing of the data is one of the most important parts to identify and quantify the amount of analytes present in the sample. It must be borne in mind that thousands of compounds, including those of interest, elude thousands of compounds, the determination of which is almost impossible due in part to the amount of compounds present and the insufficient databases for the compounds[35].

Most instrument providers offer chemometric software tools to analyze their own data formats. However, the determination of the compounds under analysis is carried out by comparison with standards. In addition, is of vital importance for analysis the creation of databases (libraries) with information about fragments, molecular formula, etc. Most of the programs provided by the manufacturers allow the generation of several molecular formulas from a particular m/z and its isotopic pattern[35].

The quantification using the MS/MS detection does not differ from the quantification used by other chromatographic techniques. Basically, the intensity of the signal generated by an analyte in a sample is compared to a standard with known quantities and concentrations. Based on this, a relationship between the signal and the concentration of the standard can be created, obtaining a linear equation (Figure 3). The MS in tandem has great practical advantages compared to simple MS, since the quality and quantity of information provided by MS/MS, increases the selectivity and the capacity of the technique for structural elucidation. In the majority of MS equipment, the quantification and determination of compounds can be addressed with different forms of analysis[37].

- All ions sweep (Full Scan), in this acquisition mode all molecules that are ionized at the interface reach the detector. In the QqQ, both the collision cell (q) and the second quadrupole (Q2) do not act in the selection process, making the ion sweep in one of the quadrupoles and obtaining a full scan spectrum.

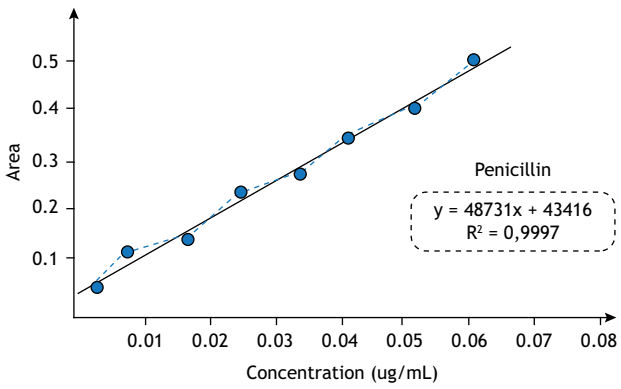

Figure 3. Representation of Linearity. Relationship between signal (area) and concentration of the standard for chromatographic quantification. In this case, eight different concentrations of the penicillin standard are presented.

- Acquisition of a selected ion (Single Ion Monitoring, SIM), the SIM acquisition is directed to the measurement of a single ion, which is selected in the first quadrupole. This type of acquisition derives from the use of simple Q, and its application in QqQ instruments is not usually very frequent.

- Product ions sweep (Product Ion Scan), the scanning of product ions is carried out by selecting in the first quadrupole (Q1) a concrete m/z called precursor ion, which is fragmented with an adequate energy in the collision cell; the second analyzer acquires in full scan mode so that the measurement of all the fragments of the precursor ion is obtained. These fragments are called product ions. This acquisition mode is ideal for obtaining the maximum possible structural information of the precursor ion.

- Acquisition of the selected reaction (Selected Reaction Monitoring, SRM), in the SRM acquisition mode, an ion is selected in the first quadrupole (Q1) called the precursor ion; the precursor ion is fragmented in the collision cell (q) in the presence of inert gas by applying an optimal collision energy; one of the ions fragment obtained in q is selected in the second quadrupole (Q2) labeled as product ion. The acquisition in SRM is the most used in quantitative analytical applications through QqQ, since it minimizes the presence of other interferers to the maximum and is presented as a tool of high sensitivity and selectivity.

- Precursor ion sweep (Ion Scan Precursor), in this acquisition mode, the first quadrupole (Q1) scans all the ions that come from the interface in the first quadrupole, fragmenting in the collision cell to a specific energy, of all the fragments obtained, only one is selected by the second quadrupole (Q2). The sweep of precursor ions makes sense in QqQ instruments, because a fragment ion must be selected from the collision cell in the second analyzer (Q2). The application to which this acquisition mode is associated is a group of compounds of the same family or metabolites from the same analyte, since the sweep of precursor ions to a common product ion is directly linked to a common chemical structure.

- Neutral loss sweep (Neutral Loss Scan) is a very specific acquisition mode of QqQ because it is necessary for two analyzers to work in coordination. The first quadrupole (Q1) and the second quadrupole (Q2) perform a sweep in phase shift, setting a mass value that differentiates the ions swept in Q1 and those swept in Q2, once they have been fragmented in the collision cell to an energy concrete In this way, only those analytes that present the neutral loss selected will be detected. As in the scanning of precursor ions, this mode of acquisition is ideal for the search of analytes from the same family or metabolites that share a common chemical structure.

Application of chromatography in the determination of antibiotics

Based on the polar nature and low volatility of veterinary antibiotic residues, LC is the choice technique for its identification and quantification; however, there are important points to consider

such as the diversity of antimicrobials used in animal husbandry; the low concentration of antimicrobial residues in food, often in µg/kg and the complexity of food matrices which may contain elements that interfere in the chromatographic reading, this leads to the development of various extraction methods[2,40,41]. Figure 4 shows the chemical structures of the main antimicrobials used in veterinary medicine.

As mentioned above in the sample extraction section of analytes, the antibacterials bind to various components of the food matrix (proteins) so that the samples must undergo a first process of deproteinization. This can be done with solvent like acetonitrile, ethyl acetate, dichloromethane or methanol. The SPE is another fundamental step in the extraction since it allows retaining various components of the food matrix that may interfere with the chromatographic system[2,42]. Below is present a summary of the separation and detection methods in different chromatographic techniques used for the monitoring of some antibiotics (Table 3).

Aminoglycosides

The most representative of this class are gentamicin, neomycin and streptomycin[43]. Although it is known that these drugs can cause nephro and ototoxicity, they are still used in the treatment of infectious diseases[6]. Several factors make difficult their determination. Its polar nature prevents its extraction and chromatographic separation, likewise, it does not possess the properties of chromophores or fluorophores and most drugs of this group have similar structures[6,43]. For its determination, extraction by ion exchange in high or low pH is preferred, it can also be used the ionic pairing in aqueous or methanol solution[6]. On the other hand, to facilitate its visualization, one of the most used derivatization agents is heptaldehyde (OPA) and a

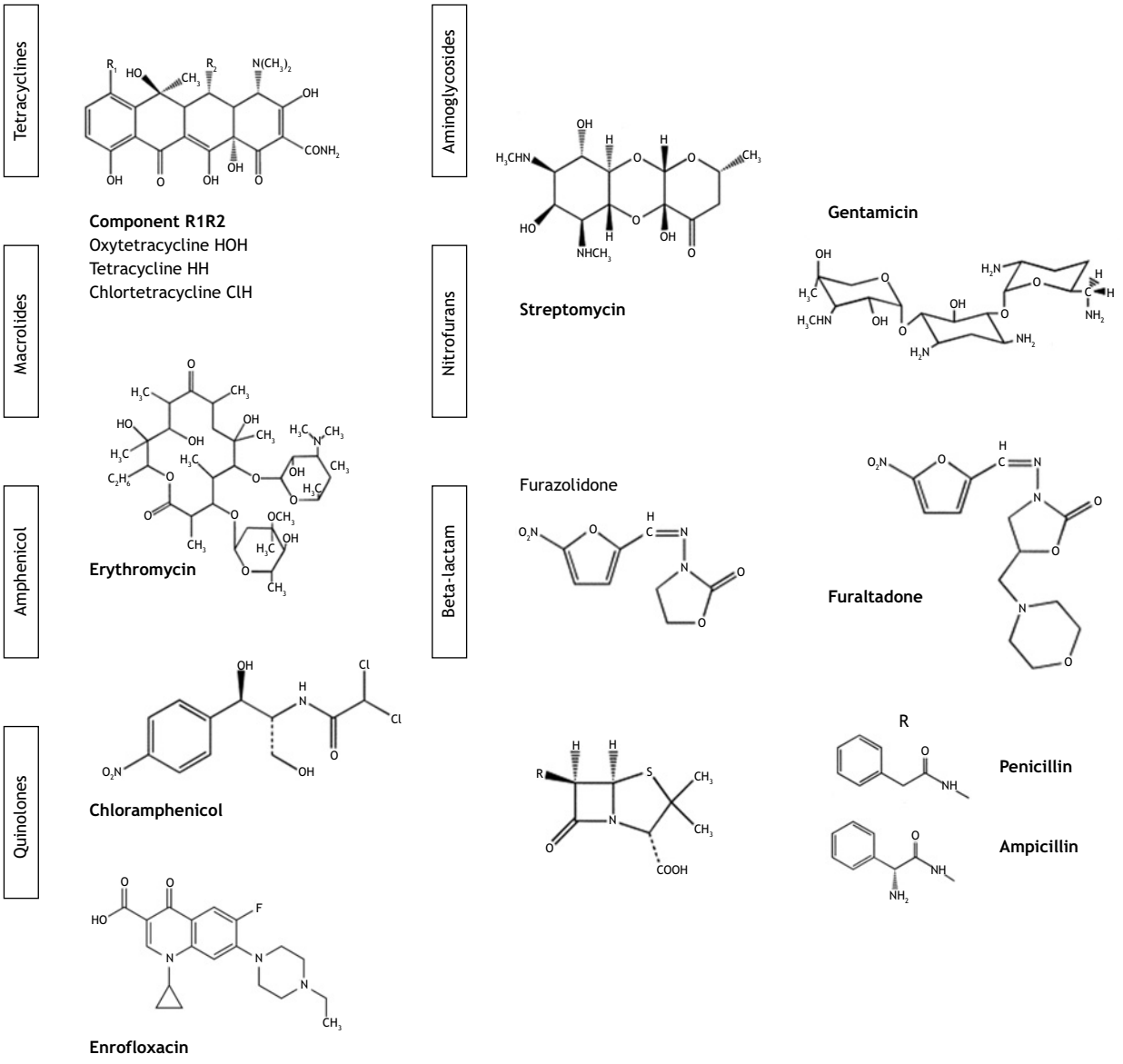

Figure 4. Chemical structure of the main veterinary antibiotics of importance in public health.

Table 3. Chromatographic techniques for the determination of residues of veterinary antibiotics.

Antibiotic	Sample	Extraction	Stationary phase	Mobile phase	Detection	Reference
Tetracyclines						
	Meat	Methanol/dichloromethane/Dispersive liquid phase	C18	ACE, FOR, AG	MS/MS	Mookantsa, Dube y Nindi[44]
	Meat, milk, eggs	Acetonitrile, Trifluoroacetic acid/ Solid phase with molecularly imprinted polymer	C18	ACE, TFA, AG	UV	Feng et al.[59]
	Honey, milk	Acetonitrile, acetic acid/Miniaturized solid phase	C18	FOR, MET	TOF/MS	Xu et al.[60]
B-lactams						
	Meat	Acetonitrile/Liquid phase	C18	ACE, AAC, AG	MS/MS	Li et al.[61]
	Milk, eggs	Acetonitrile/Liquid phase	C18	ACE, ACA, AG	UV	Shao et al.[49]
	Meat (pork)	Acetonitrile/dispersive solid phase	C18	ACE, FOR, AG	MS/MS	Huang et al.[62]
Macrolides						
	Meat (pork, chicken, bovine)	Sodium Borate and Ethyl Acetate/Solid Phase with Molecularly Printed Polymer	C18	ACE, FOR, AG	MS/MS	Song et al.[63]
	Meat (pork, chicken, bovine), milk	Acetonitrile/Liquid phase	C18	ACE, AG	MS/MS	Jank et al.[64]
Quinolone						
	Meat, eggs	Methanol, metaphosphoric acid/Solid phase	C18	ACE, FOR, AG	MS/MS	Annunziata et al.[65]
	Milk	Acetonitrile, methanol/dispersive solid phase	C18	MET, FOR	MS/MS	Dorival-García et al.[66]
Chloramphenicol						
	Milk, honey	Acetonitrile, acetic acid / Solid phase	C18	MET, ACE	MS/MS	Liu, Lin y Fuh[67]
	Meat (chiken, bovine, fish)	Ethyl acetate, sodium hydroxide	C18	ACE, ACA, AG	MS/MS	Barreto et al.[68]
Nitrofurans						
	Fish (diverse)	Hydrochloric acid/Solid phase	C18	ACE, ACA, AG	MS/MS	Zhang et al.[69]
	Meat (chicken, fish), milk, honey	Hydrochloric acid/Liquid phase	C18	MET, AG	MS/MS	Alkan, Kotan y Ozdemir[70]
	Meat (chicken)	Hydrochloric acid/Solid phase	C18	ACE, ACA, MET, AG	MS/MS	Kim et al.[71]

C18, octadecyl carbon chromatographic column bound to silica; MS, Mass spectrometer; UV, ultraviolet; TOF, time of flight; ACE, acetonitrile; FOR, formic acid; TFA, trifluoroacetic acid; MET, methanol; AAC, acetic acid; ACA, ammonium acetate; AG, water.

fluorescence detector. Within the extraction protocol the samples can undergo deproteinization with trichloroacetic acid or hydrochloric acid. Due to its polar nature, the purification is carried out with C8 or C18 columns, a mobile phase of acetonitrile/methanol and water is used, an ion exchange liquid chromatography system is preferred[6,43].

Tetracyclines

Antibiotics produced by Streptomyces spp. with activity against Gram positive and Gram negative bacteria including some anaerobes. They have affinity on the 30S ribosome and therefore inhibit the synthesis of proteins, producing a bacteriostatic effect. The most representative drugs of this group are oxytetracycline, tetracycline, chlortetracycline, doxycycline, demeclocycline; being associated with gastrointestinal problems, hypersensitivity and poor fetal development in humans with subchronic consumption[44]. As noted above, the MRLs for meat of various species and milk of this group are 200 and 100 µg/kg respectively; however, 400, 600 and 1200 µg/kg are accepted in eggs, liver and kidneys[7]. Besides that, for honey it should not be found, although some European countries accept MRL between 15 and 50 ng/g[45]. Extraction techniques for the determination of tetracycline residues by HPLC prefer the use of SPE, ultraviolet detection and different solutions depending on the food. Cinquina et al.[46] used trichloroacetic acid in milk and meat, while Andersen et al.[47] used succinic acid in shrimp. Conversely, for honey Viñas et al.[48] and Li et al.[45] proposed using a Na_2 EDTA buffer.

Beta-lactam

This group of antibiotics is widely used in cattle. Basically, penicillins and cephalosporins are divided into two classes[10]. Within the chromatographic methodology the extraction and purification part is one of the most complicated steps since it requires a large quantity of buffalo salts and organic solvents (sodium acetate, ammonium acetate, tetraethyl ammonium chloride, sodium tungsten, sodium citrate), trichloroacetic acid, sulfuric

acid or acetonitrile); likewise, the use of SPE (C18) and detection based on MS[49,43] is indicated. The MRL range from 4 µg/L for ampicillin in milk to 300 µg/kg for oxacillin, cloxacillin in tissue such as muscle, liver and kidneys[10].

Macrolides

They are a group of drugs with effects on Gram-positive bacteria, some Gram-negative and mycoplasma. They act by inhibiting the protein synthesis of sensitive microorganisms, reversibly binding to the 50S subunit of the bacterial ribosome. They do not bind to ribosomes of mammalian cells. Chromatographic methods are based on extraction with acetonitrile or methanol. The mobile phase is composed of water, acetonitrile/methanol and formic acid/acetate in different proportions. Also, the use of C18 columns coupled to a mass spectrometer is preferred. The most used form of ionization is the ESI because the macrolides are molecules that contain nitrogen atoms that are easily protonated[50].

Quinolone

It is an aromatic heterocyclic compound that shows an excellent activity against Gram positive and Gram negative bacteria by inhibiting the replication of bacterial deoxyribonucleic acid (DNA). It is often used in the livestock industry and aquaculture to combat infectious diseases of the lung, urinary and digestive types. Within the chromatographic techniques, the extraction of the analyte with acetonitrile at different pH and the identification and quantification with the HPLC and the fluorescence detector were the most used. However, in recent years LC-MS/MS4 is being more used[4].

Amphenicols

Chloramphenicol has been used for more than 60 years in veterinary medicine because of its broad-spectrum antibiotic activity against a variety of pathogens. However, in 1994 it was classified as a health risk drug and since then its use in livestock production has been banned. The most commonly used extraction methods involve the use of ethyl acetate or acetonitrile, purification with SPE (C18), separation in C18 columns and ultraviolet detection[43].

Nitrofurans

They are a particular group of antibiotics that were used as growth promoters in various systems of animal production and used primarily to treat histomoniasis and coccidiosis in birds, trichomoniasis in cattle or swine dysentery; however, they were banned due to the implication of their residues in food matrices and the generation of carcinogenesis and mutagenesis in humans. Nitrofurantoin (NFT), furazolidone (FZD), nitrofurazone (NFZ), nifursol (NFS) and furaltadone (FTD) are some of the drugs that were used. These were rapidly metabolized and certain metabolites were generated such as 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3,5-dinitro-salicylic acid hydrazine (DNSAH), semicarbazide (SEM), and 1-aminohydantoin (AHD)[51,52]. The extraction process has the use of acid solutions such as HCl (0.125 M), mobile phase with mixtures of water and methanol, SPE and detection by mass spectrometry.

Multiple antibiotic residues

At present, various chromatographic techniques have been developed and validated aimed at the simultaneous determination of drugs from different groups. This has been possible due to the enormous progress of MS[53]. The objective is to develop a technique that is easy, cheap and fast in the determination and quantification of antibiotic residues. The simultaneous analysis of residues of antibiotics veterinary has been carried out in various food matrices of animal origin such as eggs[53], honey[54,55] and milk[56,57,58].

CONCLUSIONS

After the review it can define that the most efficient technique to detect and quantify residues of antibiotics in products of animal origin is the high efficiency liquid chromatography coupled to the MS in tandem (LC-MS/MS). This technique is complex since it involves a series of steps, procedures and equipment in addition to a specialization level of the personnel in charge. Nevertheless, it has become an essential tool since it allows the separation of complex matrices and structural information based on the molecular weight of the compound or its fragments. This makes it ideal for control and monitoring plans in various countries. It is clear that in recent years there have been advances in resolution, performance and automation in this technique which has achieved greater sensitivity and accuracy. So it is, the review tries to give scope of the methodologies applied in the detection of residues of different groups of antibiotics, especially those that have a direct implication in public health.

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Acknowledgments

The authors would like to express their gratitude to the Vice-Rector for Research and Postgraduate Studies of the Universidad Nacional de San Marcos and the Fundação Carlos Chagas Filho for Amparo à Pesquisa do Estado do Rio de Janeiro (Faperj), process number E-26/201.185/2014. C.A. Conte-Junior also grateful the support of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), process number 311422/2016-0.

Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.