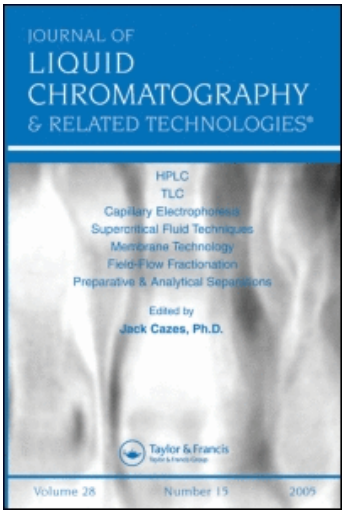

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Residue Analysis of Penicillins in Food Products of Animal Origin by HPLC: A Review

V. F. Samanidou[a]; S. A. Nisyriou[a]; I. N. Papadoyannis[a]
[a] Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece

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Residue Analysis of Penicillins in Food Products of Animal Origin by HPLC: A Review

V. F. Samanidou, S. A. Nisyriou, and I. N. Papadoyannis
Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece

Abstract: Penicillins have been widely used in veterinary medicine for more than 30 years and today still form the most important group of antibiotics. Since there is a potential impact of their residues in animal derived food on consumers' health, their monitoring is of paramount importance.

This review focuses on residue analysis of penicillins in food products of animal origin by high performance liquid chromatography. Emphasis is given to confirmatory methods since these comprise useful tools to regulatory agencies and identifications based on these methods can be used in support of regulatory action. Sample preparation for analyte extraction and cleanup have been also discussed.

Keywords: Penicillins, β-Lactams, Antibiotics, HPLC, Animal derived, Food products

INTRODUCTION

Veterinary drugs and especially antibiotics are required in the efficient production of meat, milk, and eggs in the frame of hygienic management of farms, in rational use. They can be dosed at low levels for growth promotion, at intermediate levels to prevent disease, and at high, therapeutic levels to treat infected animals. Although there is a continued debate and disagreement within the medical, veterinary, and regulatory communities as to whether the veterinary use of antimicrobials is a potential risk factor for the development of antimicrobial-resistant pathogens, much attention has been

Address correspondence to Victoria F. Samanidou, Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki 541 24, Greece. E-mail: samanidu@chem.auth.gr

focused on food-producing animals as a potential source of antimicrobial-resistant bacteria in humans, since these drugs can be found as residues in animal derived food products.[1,2]

Residue analysis is concerned with food safety, as it establishes whether food is safe or unsafe for human consumption. For chemicals used in farming, residue analysis is part of the monitoring programs of regulatory agencies, in order to ensure that residues are at levels that respect the established maximum residue levels (MRLs) or permitted limits. For prohibited substances, residue analysis aims to monitor compliance with the regulations. Regulatory demands for the control of chemical contaminants in food have increased dramatically and this has led to an increase in the demand for analytical methods for detecting residues matrices at low levels, e.g., below MRLs in foodstuffs.

Advances in analytical equipment and automation at every stage of the analytical process have provided invaluable assistance to the direction of increased speed, sensitivity, and specificity of the determination techniques applied to residue analysis. High-performance liquid chromatography (HPLC) is a versatile technique that has been commonly used to determine low concentration levels, which is fundamental to residue analysis. However, methods based only on chromatographic analysis without the use of spectrometric detection are not suitable on their own for use as confirmatory methods.

Confirmatory methods (meaning methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest) for organic residues or contaminants are those which can provide information on the chemical structure of the analyte.[3]

Antibiotics used in both veterinary and human medicine include: penicillins, cephalosporins, tetracyclines, fluoroquinolones, chloramphenicols, aminoglycosides, spectinomycin, lincosamide, macrolides, nitrofuranes, nitroimidazoles, sulfonamides, trimethoprim, and polymyxins.

Penicillins have been widely used in veterinary medicine for more than 30 years and today form the most important group of antibiotics.

It has been fifty years since the discovery of the penicillin nucleus, 6-aminopenicillanic acid, in 1957. This discovery gave birth to the development of semi-synthetic penicillins and other types of β-lactam antibiotics that became one of the most important groups of antibiotics in clinical practice. In the beginning, the penicillin family comprised just two compounds, penicillin G and penicillin V but, with 6-aminopenicillanic acid as the starting point for the preparation of new penicillins, the family grew to more than 20 different compounds in clinical use by the end of the 1970s.[4]

Several review articles on antibiotics, including penicillins or exclusively referring to penicillins, can be found in the literature covering the last two decades. These articles concern the analysis of different matrices, mainly food-animal tissues, by various analytical techniques.

Dewdney et al., in 1991, wrote on risk assessment of antibiotic residues of β-lactams and macrolides in food products with regard to their

immuno-allergic potential. Normal food constituents have been recognized to be a major cause of food intolerance problems and residues of veterinary drugs or food additives have been suspected to be the cause of allergic reactions. Although there have been extremely few well documented cases of humans being sensitized or exhibiting allergic reactions that definitely can be traced to animal drug residues, scientific evaluation of this possibility is required.[1]

In 1992, J. O. Boison reviewed the chromatographic methods of analysis for penicillins in food-animal tissues and their significance in the regulation of the use of these drugs in livestock production. In this review, the need to develop sensitive, accurate, and reliable methods to support regulatory programs to establish withdrawal times and to determine whether presumptive positive tissue samples from slaughtered animals intended for human consumption contain violative levels of penicillins to necessitate regulatory action is examined. Moreover, emerging techniques that could be taken advantage of, to improve the sensitivity and usefulness of current chromatographic methods for tomorrow's regulatory agency are taken into consideration.[5]

D. R. Bobbitt and K.W. Ng, in their review, discussed chromatographic methods for screening applications of antibiotic materials in food, concerning sample preparation, separation mode, and detection strategies associated with the food matrix.[6]

B. Shaikh and W. A. Moats, in 1993, reported on liquid chromatographic analysis of antibacterial drug residues, including penicillins, in food products of animal origin. The review covers clean-up procedures, such as, ultrafiltration, liquid-liquid partition, solid-phase extraction, immunoaffinity, and matrix solid-phase dispersion, for use as extraction, deproteination, and concentration steps for rapid automated analysis, as well as for direct screening of residues in meat and milk.[7]

A. Macri and A. Mantovani, in their review published in 1995, discussed the safety evaluation of residues of veterinary drugs in farm animal tissues and products. The paper reviews the EU criteria to establish admissible daily intakes (ADI) and maximum residue limits (MRL) for veterinary drug residues. In the toxicological evaluation, special attention is paid to pharmacologically active molecules (e.g., tranquilizers); potentials for subtle effects on reproduction, immunity, etc.; genotoxicity and carcinogenicity; and risks of severe effects in sensitive individuals.[8]

Four reviews were published in 1998. W. M. A Niessen reported on the current status of the application of liquid chromatography–mass spectrometry (LC-MS) in the analysis of antibiotics including β-lactams and antibacterial compounds in animal food products for human consumption covering the period 1987–1996. The main application area of LC–MS in this field is the confirmation of residue identity in animal food products for human consumption at maximum residue levels, set by the regulatory authorities. Currently available data on these compound classes are reviewed, with special emphasis on important aspects especially relevant to LC–MS and on the mass spectral information obtained.[9]

D. G. Kennedy et al., in 1998, reviewed the use of LC-MS in the analysis of residues of antibiotics, including penicillins, in meat and milk. According to this report, a massive increase in the number of publications describing quantitative methods for the analysis and confirmation of veterinary drug residues has been noticed. In this review, analytical methods for the determination of residues of each of the major classes of antibiotics are presented. The literature referring to penicillins covers 1987–1994.[10]

Two years later, O. Bruggemann et al. presented a review on the application of MIPs on the analysis of penicillins, in food products, by HPLC.[11]

M. H. Lee et al., in their review published in 2001, covered classification and usage or sources of chemical residues, their adverse effects, and chemical residue status of some countries. Issues are expanded to residue detection methodologies, toxicological and pharmacokinetic backgrounds of MRL, and withdrawal time establishments, as well as the importance of non-governmental activities with regard to reducing chemical residues in food.[12]

A. Di Corcia and M. Nazzari, in 2002, published a review focusing on both sample treatment and final LC-MS analysis of antibiotic and antibacterial agents in animal food products.[13]

E. L. Miller, in 2002, provided a review of the modes of action, spectrum of activity and adverse effects for the various classes of penicillin most often used in primary care.[14]

In 2003, G. Balizs and A. Hewitt described the principles, the current technology, and the application of HPLC and tandem mass spectrometry (LC–MS–MS) in the analysis of veterinary drug residues, including penicillins.[15]

W. F. Smyth and P. Brooks in 2004, published a critical review of applications of high performance liquid chromatography-electrospray ionisation-mass spectrometry (HPLC-ESI-MS) and capillary electrophoresis-electrospray ionisation-mass spectrometry (CE-ESI-MS) to the detection and determination of small molecules of significance in clinical and forensic science, including penicillins among other drugs, in the period 2000–2003. Information was taken from the Web of Knowledge database. Details are given on the fragmentations that these ionic species exhibit in-source and in ion-trap, triple quadrupole and time-of-flight mass spectrometers. Analytical information on sample concentration techniques, HPLC and CE separation conditions, recoveries from biological media, and limits of detection (LODs) are also provided.[16]

Recent advances in the analysis of antibiotics by capillary electrophoresis have been provided by C. García-Ruiz and M. L. Marina, in 2006. This review covers the literature dealing with the analysis of antibiotics by CE from the beginning of 2003 till May 2005. The experimental conditions employed to achieve the analysis of antibiotics by CE, main applications performed in the pharmaceutical, clinical, food, and environmental fields

making emphasis on sample preparation requirements needed in each case, and main conclusions and future prospects in this field are presented.[17]

In our recent review, published in 2006, we provided an extended and comprehensive review on the analytical techniques and methods for penicillins reported in the literature within the last two decades, with emphasis on chromatographic methods for the determination of these compounds in pharmaceuticals and biological samples. The state-of-the art in chromatographic methods was reviewed, focusing on sample pretreatment, chromatographic conditions, detection techniques, method validation, and application to real samples. Results of various published assays are presented comparatively, while an informative discussion of chemical structure, classification, spectrum of activity, and action mechanism of common penicillins has been given as well.[18]

In this review, we focus on up to date residue analysis of penicillins in food products of animal origin by high performance liquid chromatography. Emphasis is given to confirmatory methods, since these methods are of significant importance to regulatory agencies. Sample preparation for analyte extraction and clean up has been also discussed.

CHEMISTRY AND ANTIMICROBIAL ACTIVITY

Structure and Chemical Characteristics

Penicillins belong to the class of β-lactam antibiotics. They have the molecular formula R_1-$C_9H_{10}(R_2)N_2O_4S$, where R_1 is a variable side chain and R_2 is hydrogen, except for the case of esters of some members. Their basic structure as shown in Figure 1; it consists of three parts: a thiazolidine ring attached to a β-lactam ring, forming 6-aminopenicillanic acid and a side chain R_1 in the 6-position, which determines the stability and the antimicrobial activity of the different derivatives. Manipulation of the side chain R_1 has altered the antibacterial spectrum of penicillins to include both Gram-positive and Gram-negative bacteria.

Combinations of penicillins with other antibiotics and enzyme inhibitors (e.g., amoxicillin with clavulanic acid) are often used as being more effective against many bacterial infections.

Ampicillin was the first major development, which offered a broader spectrum of activity against Gram-positive and Gram-negative infections. Further developments led to amoxycillin, with improved duration of action as well as to β-lactamase-resistant penicillins, including flucloxacillin, dicloxacillin, and methicillin, active against β-lactamase-producing bacteria such as *Staphylococcus* species.

The mechanism of action of penicillins is via inhibition of bacterial cell wall biosynthesis. Penicillins are mostly bactericidal, resulting in cell death of the offending bacteria due to faulty production of the vital cell wall components.[18]

Classification

According to the United States Pharmacopoeia, penicillins are subclassified into four classes, based on their spectrum or activity. These are: 1. Natural penicillins, 2. Aminopenicillins, 3. Penicillinase-resistant penicillins

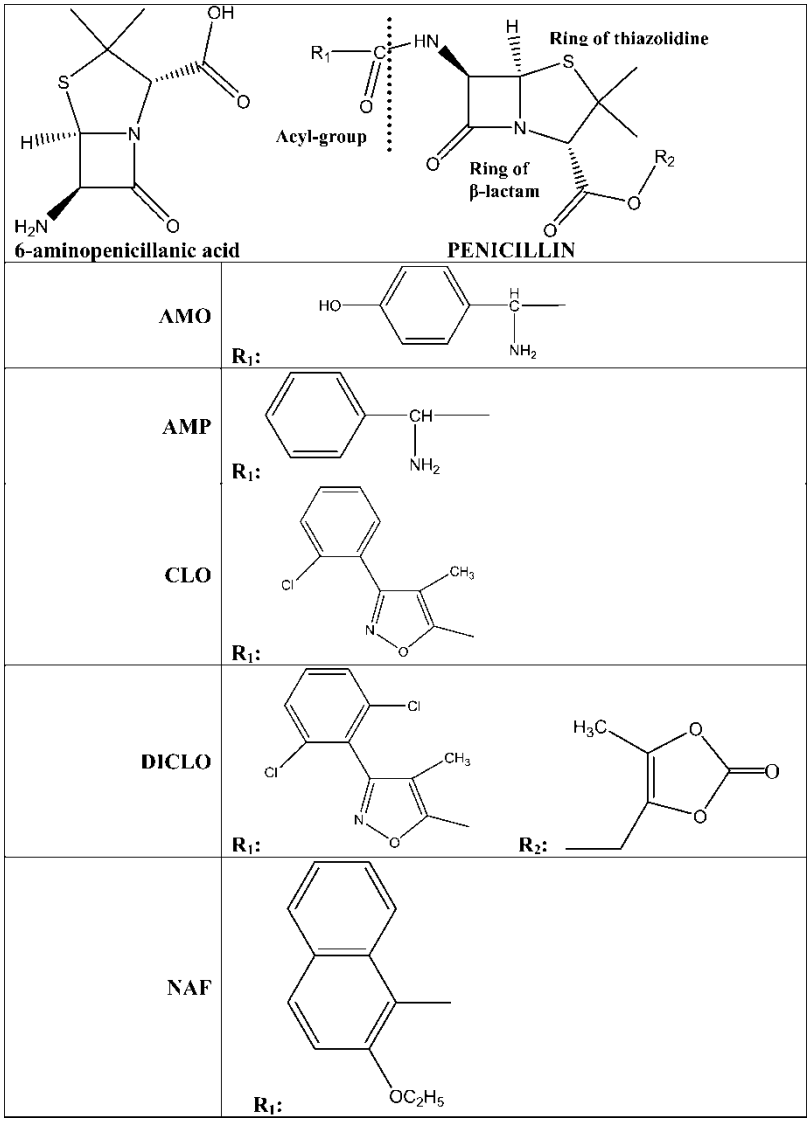

Figure 1. Chemical structures of main penicillins and 6-aminopenicillanic acid. When not otherwise mentioned, $R_2 = H$.

(*continued*)

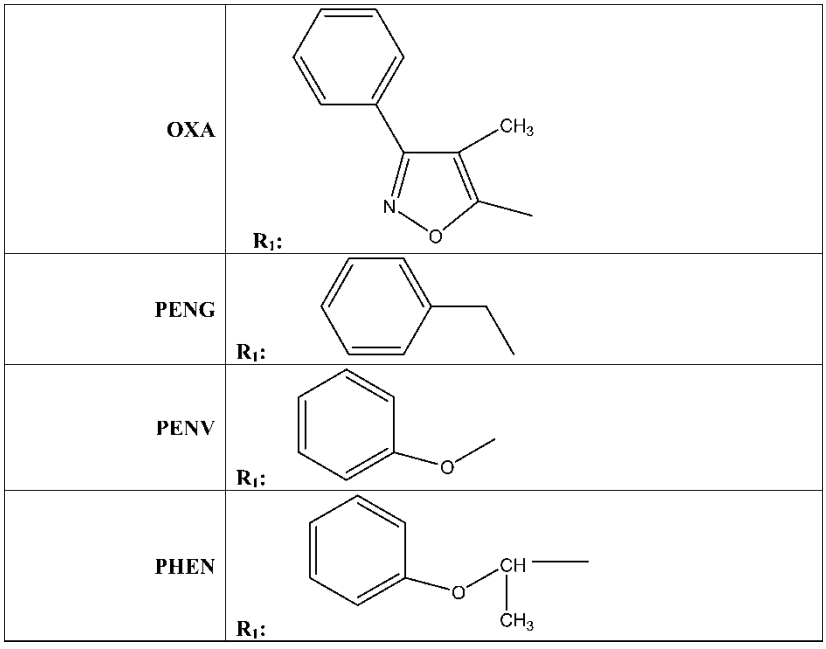

Figure 1. Continued.

(antistaphylococcal) and 4. Extended spectrum penicillins (antipseudomonal penicillins).[19]

Table 1 shows the classification of penicillins used in veterinary medicine according to their origin, as well as according to their resistance and activity.

Another classification scheme categorizes them into four groups of penicillin derivatives:

1. Natural penicillins or Penicillins of group G (benzylpenicillin). These substances act against Gram-positive micro-organisms and only in very high doses against Gram-negative micro-organisms. They are destroyed by the penicillinase and they are not active when administered orally. With various amines, penicillin G gives various forms of extended acting, such as procaine penicillin. The mixture of a salt of penicillin with penicillin of extended action constitutes a "dipenicillin".
2. Penicillins of group V (phenyl-methyl-penicillin type). These substances can be administered orally and act against Gram-positive and, to a small extent, against Gram-negative micro-organisms. They are susceptible to the action of penicillinase.
3. Penicillins of group M (methicillin type). These are resistant to the penicillinase.
4. Aminopenicillins or penicillins of group A (ampicillin type). These act against Gram-positive and Gram-negative micro-organisms when they

Table 1. Classification of penicillins used in veterinary medicine

Classification according to their origin	Generic name	Derivative	Classification according to their resistance and activity
Natural penicillin (metabolic products by molds of *Penicillium notatum* and *Penicillium chrysogenum)*	Penicillin G	Penicillin G (benzyl penicillin) Procaine penicillin G Benzathine penicillin G	
	Penicillin V	Phenoxymethyl penicillin (penicillin V) Phenoxyethyl penicillin (phenethicillin)	Acid resistant
Semi-synthetic (synthetic)	Phenethicillin		Acid resistant
	Ampicillin, amoxicillin, bacampicillin	(Aminoderivatives)	Moderate spectrum (gram positive and gram negative)
	Oxacillin, cloxacillin, dicloxacillin, flucloxacillion, methicillin nafcillin	(Isoxazolyl derivatives) a) Acid labile: Methicillin, nafcillin, cloxacillin, dicloxacillin	Narrow spectrum Penicilliase resistant (antistaphylococcal penicillins)
		(Isoxazolyl derivatives) b) Acid resistant: Flucloxacillin	
	Carbenicillin, ticarcillin	(Carboxypenicillin)	Extended spectrum penicillins (antipseudomonas penicillins)
	Piperacillin	(Piperazine penicillin)	
	Mezlocillin, azlocillin	Ureidopenicillins (alkylaminopenicillin)	
	Mecillinam, Pivmecillinam	Amidinopenicillins	
Penicillins with β-lactamase inhibitors:	Amoxycillin-clavulanic acid ticarcillin-clavulanic acid piperacillin and tazobactam ampicillin-sulbactam	Potentiated penicillins	Broad spectrum penicillins
Dipenicillins	Dicloxacillin-ampicillin		

are intravenously or orally administered and they are susceptible to the penicillinase.

Moreover, a fifth category can be discriminated, i.e., the combination of penicillins with inhibitors of β-lactamases as, for example, amoxicillin with clavulanic acid or ampicillin with sulbactam. The combinations, called *potentiated penicillins*, are used to treat susceptible bacterial infections in many species.[20–22]

Amoxycillin and clavulanic acid combination is used in veterinary medicine since 1980 for treating livestock and companion animals. Different formulations are in use: injectable, intra-mammary, oral tablets and bolus and oral drops.[23]

Use of Antibiotics in Veterinary Medicine

Antibiotics are vital drugs in veterinary medicine. The term "antibiotic" was originally applied to naturally occurring compounds, such as penicillin or semi-synthetic compounds, which attack disease-causing bacteria without harming the host. The terms "antibacterials" or "antiviral" drugs are used to describe synthetic compounds. However, very often, the term antibiotics is used including both naturally occurring and synthetic compounds.

Penicillin, the first antibiotic, was discovered by Alexander Fleming in 1928 and was purified and then synthesised for clinical use by Florey and Chain in 1940.

Antibiotic use in livestock production originated in the 1950's when waste from the fermentation process used in commercial chlortetracycline production was found to improve the growth rates of pigs, poultry, and cattle. Today, antimicrobials in livestock production are employed in three directions:

1. As *animal medicines*: for therapeutic purposes, to treat diseases in all animal species. In the case of intensive livestock systems, such as those for poultry and pigs, individual treatment is not usually feasible, so mass oral medication administered in the food or the drinking water is the only practical method of treatment.
2. As *Prophylactics (Preventatives)*: Prophylactic antimicrobial use is applied in intensive livestock production to protect animal welfare, protect uninfected animals thereby preventing epidemic spread of infectious animal diseases, to provide high efficiency of animal production, to prevent the transfer of zoonoses from animals to the human population, to warrant safety of food of animal origin (meat, milk or eggs), and to prevent food-borne diseases. Prophylactic measures in medicinal treatment are usually applied as mass medication in the food or water. For farm animals, the main infectious diseases treated are enteric and pulmonary infections, skin and organ abscesses, and mastitis.

3. As *Growth Promoters*: The use of antibiotics specifically for performance enhancement has been routine practice since the 1970's, though their modes of action in this respect are not well understood.[2,24]

Adverse Effects in Health and Safety

All bioactive compounds used in animal nutrition and veterinary medicine have intrinsic hazards. Antibacterials present two types of health and safety risk. The first is that some antibiotics, such as penicillins, can evoke allergic reactions, even though small amounts of them are ingested or exposed by parenteral routes. The second is the development of antibiotic resistance in gut bacteria of humans. Recently multi-resistant pneumococcal, glycopeptide-resistant enterococci, and gram negative bacteria with extended-spectrum β-lactamases have spread all over the world, and are now a serious therapeutic problem in humans.[2]

In most humans, the effect of residual antibiotics consumed through animal products is not immediate. However, in susceptible humans, the residue causes allergy (scratching, rash) or a severe reaction such as anaphylaxis.

One of the most studied phenomena on the delayed effect of antibiotic residue over time is the development of drug resistant organisms in humans and animals and their transfer to suitable host. Antibiotic resistant pathogens do not respond well to therapy by ordinary antibiotics. As a result, new antibiotics must be developed. Additionally, continuous feeding of antibiotics to animals tends to adversely effect development of immune responses.[18]

Food Safety

Food safety is of great importance, since food quality may adversely affect human health. Veterinary drugs are administered to food producing animals, they are accumulated in edible animal tissues, and finally result, through the food chain, to the final destination which is humans.

For this reason, only safe food should be consumed. However, a definition of food safety is hard to find. According to the USDA Food Safety and Inspection Service, safe food is "a suitable product which, when consumed orally, either by a humans or an animals, does not cause health risk to the consumer."

Although veterinary drugs in food animals can be dosed at low levels for growth promotion, at intermediate levels to prevent disease, and at high, therapeutic levels to treat infected animals, antibiotics should be provided only for treatment of the disease when necessay and not sub-therapeutically. To ensure human health, regulatory agencies make efforts to prevent antibiotic residue problems. Toxicological aspects and drug withdrawal times are taken into consideration when establishing maximum residue levels.

There are several reasons why antibiotic residues are detected in animal derived food. One of the reasons is "shot gun therapy," whereby an antibiotic

is used in a sick animal just to make it appear healthy before slaughter. The other reasons are: use of an antibiotic not cleared to be used in a species of food animal, excessive use of an otherwise cleared drug, improperly prepared and labeled feed, and non-observance of drug withdrawal time, where drug withdrawal time is the time period before slaughter when animals should not be allowed to receive medicated feed or any drug. Failure to observe prescribed withdrawal periods for permitted drugs can cause higher than allowable concentrations.

Another common route of veterinary antibiotics into the human food supply stems from the practice of administering illegal and unlicensed antibiotics as a prophylaxis against disease organisms in food-producing animals. Producers find it easy to use, because the drugs are readily obtainable and cheap. Thus, they serve as cost-effective insurance against possible loss from illness. Recently HACCP has been introduced to promote food safety from farm to table by reducing hazardous biological, chemical, and physical factors.[2]

Legislation

From a legislative point of view, the use of antibiotics is only justified for therapy or metaphylaxis (no symptoms have yet appeared in infected animals), if it has been proved by appropriate and objective diagnostic measures that the animals are infected by a pathogen sensitive to the antibiotic that is to be administered. Prophylaxis is only admissible in substantiated exceptional cases (e.g., immunosuppression, perioperative). The diagnosis may generally be based on the identification of the pathogen and the antibiogram. This microbiological diagnosis is always necessary when switching the therapy to another antibiotic, in the case of use of not fixed antibiotic combinations, when the antibiotic is not used in compliance with the label instructions (other dosage or animal species than designated), and regularly in the case of repeated or long-term use in larger animal herds.

These results suggest compulsory guidelines for prudent use of antibiotics in animals as an important tool of risk management to reduce the consumption of antibiotics and the consequential development of resistance.[25]

As the use of veterinary drugs is very important in agricultural production, and animal origin food free circulation becomes difficult due to the different maximum limits set by each European Union (EU) country, the EU council has set maximum residue limits of these antibiotics in order to protect the public health and to assist the commerce of this kind of foods.

All active substances used in veterinary medicinal products for food-producing animals require the establishment of a maximum residue limit (MRL) under Council Regulation of the European Communities (EEC) 2377/90. MRLs are based on the concept of ADI, but are matched against drug residue depletion in the target species, so that values can be established for tissues and animal products.[26]

According to 657/2002/EC, even for substances for which no permitted limit has been established, and, in particular, for those substances whose use is not authorised or is specifically prohibited in the Community, it is necessary to provide for the progressive establishment of minimum required performance limits (MRPL) of an analytical method in order to ensure harmonised implementation of Directive 96/23/EC.[27]

According to council regulation (EEC) No 2377/90 of 26 June 1990 as amended by regulations (EEC) Nos. 675/92, 762/92, 3093/92, 895/93, 2901/93, 3425/93, 3426/93, 955/94, 1430/94, 2701/94, 2703/94, 3059/94, 1102/95, 1441/95, 1442/95, 1798/95, 2796/95, 2804/95, 281/96, 282/96, 1140/96, 1147/96, 1311/96, 1312/96, 1433/96, 1742/96, 1798/96, 2010/96, 2017/96, 2034/96, 17/97, 211/97, 270/97, 434/97, 716/97, 748/97, 749/97, 1836/97, 1837/97, 1838/97, 1850/97, 121/98, 426/98, 613/98, 1000/98, 1076/98, 1191/98, 1568/98, 1569/98, 1570/98, 1916/98, 1917/98, 1958/98, 2560/98, 2686/98, 2692/98, 2728/98, 508/1999, 804/1999, 953/1999, 954/1999, 997/1999, 998/1999, 1308/1999, 1931/1999, 1942/1999, 1943/1999, 2385/1999, 2393/1999, 2593/1999, 2728/1999, 2757/1999, 2758/1999, 1286/2000, 1295/2000, 1960/2000, 2338/2000, 2391/2000, 2535/2000, 2908/2000, 749/2001, 750/2001, 807/2001, 1274/2001, 1322/2001, 1478/2001, 1553/2001, 1680/2001, 1815/2001, 1879/2001, 2162/2001, 2584/2001, 77/2002, 868/2002, 869/2002, 1181/2002, 1530/2002, 1752/2002, 1937/2002, 61/2003, 544/2003, 665/2003, 739/2003, 806/2003, 1029/2003, 1490/2003, 1873/2003, 2011/2003, 2145/2003, 324/2003, 546/2004, 1101/2004, 1646/2004, 1851/2004, 1875/2004, 2232/2004, 65/2005, and 712/2005, the maximum residue limits of penicillins in foodstuffs of animal origin are shown in Table 2.

Regulation 2377/90/EC for amoxicillin, ampicillin, penicillin G, cloxacillin, dicloxacillin, oxacillin, and phenethicillin (only for cattles) has been amended by regulation (EEC) No 508/1999. For nafcillin, phenethicillin (only for pigs), and penicillin V, the regulation has been amended by regulation (EEC) No. 546/2004, No. 2757/1999, and by regulation (EEC) No. 1286/2000, respectively. These values refer to intra-mammary use only.

Lower tolerance levels of antibiotics in food have been stipulated by the US Food and Drug Administration (USFDA). These are 10 ppb for amoxicillin in cattle, ampicillin in cattle, and swine; cloxacillin in cattle milk, muscle, liver, kidney, and fat; 10 ppb for penicillin G in turkey muscle, liver, kidney, and fat; 50 ppb for penicillin G in cattle muscle, liver, kidney, and fat; 5 ppb in milk; and 0 ppb for penicillin G in chickens, swine, and sheep muscle, liver, kidney, and fat.[13]

ANALYTICAL METHODOLOGY

Various techniques have been used for the determination of penicillins in animal derived food products. HPLC methods prevail in the analysis of

Table 2. European union maximum residue limits for penicillins in edible animal tissues

Penicillin	Animal species	MRL
Amoxicillin Ampicillin Penicillin G*,** (benzylpenicillin)	Every species that produces food *Cattles, **Pigs	Muscles: 50 μg/kg, fat: 50 μg/kg, liver: 50 μg/kg, kidney: 50 μg/kg, milk: 4 μg/kg (−**)
Cloxacillin Dicloxacillin Oxacillin Nafcillin*	Every species that produces food *Ruminant	Muscles: 300 μg/kg, fat: 300 μg/kg, liver: 300 μg/kg, kidney: 300 μg/kg, milk: 30 μg/kg
Penicillin V (phenoxymethylpenicillin)	Pigs	Muscles: 25 μg/kg, liver: 25 μg/kg, kidney: 25 μg/kg

food matrices; however, other non-chromatographic techniques have been proposed as well. These techniques include flow injection analysis,[28] capillary electrophoresis,[29–31] and micellar electrokinetic capillary chromatography.[32] Biosensors have also been used for the determination of β-lactams in milk using a surface plasmon resonance-based biosensor[33,34] or a lactate oxidase-based biosensor.[35] ELISA was used to investigate penicillin G, ampicillin, and cloxacillin-binding to proteins and the Charm II test to determine penicillin G semi-quantitatively.[36] Finally, the stability of ampicillin residues in muscle tissue during their freezing storage has been examined using, apart from HPLC-UV and LC-MS, a quantitative microbiological assay.[37]

As already mentioned, HPLC is the technique of choice for residue analysis in animal derived food products. These are further discussed in the next paragraph, while information from all reported methods are summarized in Table 3.

HPLC Conditions

A LiChrosorb RP-18, 1 cm × 4.0 mm, 5 μm, pre-column and a LiChrosorb RP-18, 15 cm × 4 mm, 5 μm, analytical column, operated at 40°C were used for the determination of PENG in cattle liver, kidney, and muscle. The mobile phase for the pre-column was MeOH-H_2O–0.2 M phosphate buffer (pH 5.0) (7:12:1), while for the analytical column, MeOH-H_2O–0.2 M phosphate buffer (pH 5.0) (1:18:1) was used.[38]

Table 3. Overview of HPLC methods for the determination of penicillins in animal derived food samples

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
PENG[38]	Cattle liver, kidney, muscle	Add. H_2O (40 mL), 5% Na_2WO_4 sol. (20 mL), 0.33 N H_2SO_4 (20 mL) homog. sample (ca.10 g), centr., supern. to a basic Al_2O_3 col., wash. with water. Add. 20% NaCl sol. (20 mL). SPE on Sep-Pak C_{18}, wash. with 2% NaCl sol. (10 mL) & MeOH-H_2O-20%NaCl sol. (3:15:2, 10 mL). El.with H_2O (19 mL).	Pre-col.: LiChrosorb RP-18 1 cm x 4.0 mm × 5 μm Anal.:LiChrosorb RP-18 RP 15 cm × 4 mm × 5 μm, 40°C. Guard: 5 cm × 2.1 mm Permaphase ETH. Pre-col. MP. MP. 1: MeOH- H_2O-0.2 M phos. buf. (pH 5.0) (7:12:1), FR. 1:1,0 MP. 2: MeOH- H_2O-0.2 M phos. buf. (pH 5.0) (1:18:1), FR. 2:1.5 pre-col. rins.sol.: MeOH- H_2O, 20% NaCl sol. (9:27:4)	Liver: 75.0 Kidney: 92.6 Muscle: 91.0	UV: 210 nm, LOD: 0.05 μg/g LR: 0.10–0.50 μg/mL
PENG, PENV, AMP[39]	Milk	Sep-Pak C_{18} cartridge precond. with MeOH (20 mL), H_2O (20 mL) & 2% NaCl sol. (2 mL), milk sample filt. Through glass-wool plug. Sep-Pak C_{18} cartridge, H_2O (10 mL), wash. with H_2O (5 mL), MeOH (10 mL)- H_2O-20% NaCl sol. (1:8:1) with 20 mM 18-crown-6-ether, el.with 15% (v/v) MeOH (10 mL)	Guard: Permaphase ETH, 5 cm × 2.1 mm, Anal.: LiChrosorb RP-18, 15 cm × 4.3 mm, 5 μm, FR: 1.0, 45°C. MP: MeOH- H_2O-0.2 M phos. buf. (pH 4.0) (5:13:2) with 10 mM sodium alkylsulphonate	PENG: 98.4–101.1 PENV: 95.9–97.7 AMP: 87.0–88.0	UV: 210 nm, LOD: 0.03 μg/g

PENG[40]	Bovine milk	Milk (0.5 mL) dil. with ACN-MeOH-H_2O (40:20:40) (0.5 mL), centr.	LC-UV: Brownlee Microbore Phenyl Spheri-5 220 mm × 2, 1 mm, 5 μm, 40°C, MP: 25% (v/v) ACN in H_2O with 0.0025 M SDS, −0.5% (v/v) 85% H_3PO_4 & 0.5% (v/v) TEA, FR: 0.3–0.5. LC-MS: Brownlee Phenyl Spheri-5, 220 mm × 4.6 mm, 5 μm, col. 40°C, MP: IPA-0.2 M CH_3COONH_4-CH_3-COOH (12.5:85.5:2), FR: 1	81.9	UV: 210 nm, LOD (ng): 0.3 LR: 10–500 LC-MS LOD: 3 (100ppb,for the $[M+H]^+$ ion at m/z 335) LR: 20–500
CLO, AMP/HETA, AMO[41]	Bovine milk	Dil. of milk sample (0.5 mL) with ACN-MeOH-H_2O (40:20:40, v/v), centr. ultrafilt.	Brownlee Microbore Phenyl Spheri-5, 220 mm × 2.1 mm, 5 μm. LC-UV-PDA: FR: 0.2,0.45, MP: AMO:1.5% IPA, 5% CH_3COOH in 0.2 M CH_3COONH_4, 93.5% H_2O AMP/HETA: 10% IPA, 2% CH_3 COOH in 0.2 M CH_3COONH_4, 88% H_2O, CLO: 15% IPA, 2% CH_3COOH in 0.2 M CH_3COONH_4, 83% H_2O Thermospray LC-MS FR.: 0.8–1.2, MP.: AMO: 15% ACN, 5% MeOH, 2 mM SOS, 2 mM SDS, 0.4 % TEA, 0.4% H_3PO_4(85%), 79.2 % H_2O. AMP/HETA:20% ACN, 5 mM SDS, 0.4% H_3PO_4 (85%), 0.4% TEA, 79.2%, H_2O. CLO:27.5% ACN, 2.5 mM SDS, 1.5 mM SOS, 0.1% H_3PO_4 (85%), 72.4% H_2O	AMP: 66.0–87.2 CLO: 79.2–95.9 AMO: 77.0–81.5	UV-PDA: (220 nm), confirmation by thermo-spray LC-MS LC-UV-PDA: LOD (ppb) CLO: 50, AMP: 75, AMO: 100 LR: 5–500 ng Thermospray LC-MS LOD(ppb): CLO: 100, AMP: 200, AMO: 200 (at 210 nm)

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
PENG[42]	Milk	Extr.: ACN add. (40 mL) to milk (20 mL), standing (5 min), filt., CH_2Cl_2 (30 mL), C_6H_{14} or light petroleum (30 mL) add. to the filtrate. Org. layer wash. with H_2O (5 mL, water layer evap.(in 40–50°C, to 1–2 mL), sample sol. (4 mL) filt. Cleanup: Equilibr. with 0.01 M phos. buf. (pH 7.0), FR.: 1, MP. (grad.): buf. (A)-ACN (B), (100:0) (0–3 min)–(40:60) (25–30 min)–(100:0) (31 min), fraction conc. ($\leq$0.5 mL), vol. adj. (to 0.5 mL)	PLRP-S, FR: 1, MP: 0.01 M phos. buf. (pH 1.96)-ACN (66:34).	92 ± 9	UV or DAD: 200 or 210 nm, LOD: 2ppb
PENG, PENV, CLO[43]	Beef & pork tissues	Tissue (15 g) blend. with H_2O (45 mL), ACN (40 mL) add., filt., 0.2 M H_3PO_4 (10 mL) add. to the filtr. (30 mL), extr. with CH_2Cl_2, ACN & C_6H_{14} add. to the combined CH_2Cl_2 layers, wash. with H_2O (4 mL $\times$ 2), extr. with 0.01 M phos. buf. (pH 7) (1 mL $\times$ 4), conc. to 1 mL.	PSDVB col., 150 $\times$ 4.6 mm, 5 μm (100 Å) (Polymer Labs. PLRP-S), FR.: 1, MP. (is.): 0.01 M phos. buf. (pH 7)-ACN 85:15 (PEN G), 82:18 (PEN V), 78:22 (CLO).	PENG: 71–101 PEN V:, 67–118 CLO: 69–107	UV: 210 nm, LOD: $\leq$10 ng/g

AMO[44]	Broiler tissues (kidney, liver, muscle, fat & skin + fat)	Minced tissue (1 g): Spiking with I.S. (AMP: 500 ng/g), 0.01 M KH_2PO_4 (7 mL) add. Fat, skin + fat analysis: 0.01 M KH_2PO_4 (4 mL) add., mix. 0.01 M KH_2PO_4 (3 mL) add., extr., centr. of the extr., ultrafilt. of the supern. (1 mL), centr., 100 mM PFPA sol. (50 μL) add. Purif.: C_{18} SPE col., cond. with MeOH (5 mL), HPLC water (5 mL), 5 mM PFPA sol. (3 mL), wash. after loading with water (3 mL), el. with 2% NH_3 sol. (3 mL) in MeOH, evap., reconst. in HPLC water (500 μL).	PLRP-S polymeric col., 150 mm × 2.1 mm, 100Å, same type pre-col., 5 mm × 3.0 mm, RT. MP. (grad.): 0.1% HCOOH in H_2O (A), ACN (B), 0–7 min: 90%A–10% B, 7.1–15 min: 50%A–50%B, 15.1–21 min: 90%A–10%B, FR.: 0.2, auto-sampler Temp.: 5°C, IS: AMP (1 g)		MS/MS LOD (ng/g): Kidney: 57.2, liver: 56.1, muscle: 57.4, fat: 60.0, skin + fat: 60.7 LR: 0–500
PENG[45]	Chinook salmon	Extr.: ACN (15 mL) & I.S., 0.5 mL) add. to finely chopped salmon muscle tissue (5 g), homog. (×3), centr. (at 4°C), supern. storing in the dark at 4°C, ACN (15 mL) add., homog., supern. evap. under N_2 in water bath (40°C), ACN (2 mL) add. to the residue, 0.02 M phos. buf. buf. (pH 6.75)	Ultrasphere OS (C_8), 25 cm × 4.6 mm; 5 μm MP.(isocr.): ACN-0.02 M phos. buf. (pH 6.75) (43:200), FR.: 1.0, IS: PENV, 1 mg/mL	63.9–65.4	UV: 214 nm LOD: 0.05 ppm LR: 0.05–3.0 ppm

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
		(20 mL) add. SPE: C_{18} col., cond. with 50% ACN 6 mL × 2. & phos. buf. (6 mL × 2), PENG & I.S. el.with ACN (4 mL × 2), evap. (at 40°C), reconst. with MP. (1 mL)			
PENG[46]	Chinook salmon	Add 16 mg/mL I.S.sol.(0.5 mL) &ACN (15 mL) to the tissue (5 g), homog. centr., extr. repeated with ACN (15 mL) add., supern. comb.& evap. under N_2 (40°C), reconst. with ACN (2 mL) & 0.02 phos. buf. (pH 6.75) (20 mL). SPE: C_{18} col., el. with ACN (4 mL × 2), evap. under N_2 water bath (40°C), reconst. with MP. (1 mL) (ACN:0.02 M phos. buf., pH 6.75; 43:200 v/v)	C_8 Beckman Ultraspere, 5 μm; 4.6 mm, 25 cm, C_8 guard col., MP: ACN:0.02 M phos. buf., pH 6.75; 200:52 v/v, FR.: 1, IS: PENV (16 μg/mL)		UV: 214 nm LOD: 0.05 μg/g LR: 0.05–3.0 μg/g

PENG, AMP, AMO, CLO[47]	Bovine milk	Dil. of 0.5 mL aliquot of milk with ACN-H_2O (1:1) (0, 5 mL), filt., centr., spiking	FI-ESP-MS: col.: 150 × 2 mm Ultremex 3, C_{18}, MP. Various MP. MeOH-H_2O (8:2), (1:1), (2:8), FR.: 0.012–0.024, HPLC–ESP–MS:40%(v/v) ACN: 1%(v/v) HOAC in water (pH 3.0), FR. into the 70:1 splitter: 0.3, FR. entering the ESP interface: 0.0043. Microbore HPLC-ESP-MS:30%(v/v) ACN: 1%(v/v) HOAC in water (pH 2.9), FR.: 0.004	100	E.S.P.-MS UV 230 nm LOD (pg/μL): PENG: 5, CLO, AMO, AMP: 10, LR:20–200 ppm
PENG[48]	Milk	Milk decreamed by centr. (5°C) and deprot. using H_2SO_4/Na_2WO_4, C_{18} SPE. Deriv. by imidazole and $HgCl_2$ sol.	Fractionation: Kromasil 5 C_8 25 cm × 4.6 mm × 5 μm, MP.:).01 M phos. buf. (pH 6.7)-ACN (80:20, v/v), FR.: 1.0. Anal.: Kromasil 5C_8 25 cm × 3.2 mm,5 μm, MP: 0.01 M H_3PO_4 acid (A), 0.01 M H_3PO_4-ACN (20:80, v/v) (B), grad. El.: 0 min:100% A, 5 min:100% A, 35 min:100% B, FR: 0.4	70–73	UV: 220, 320 nm LOQ: 2 μg/kg LR: 0.015–0.75 μg/mL
OXA, PENG, PENV, CLO, DICLO[49]	Bovine muscle	Bovine muscle (25 ± 0.2 g) spiking, extr. with ACN (25 mL), 0.5 M phos. buf. add. (pH 2.2) (5 mL), ACN add. (65 mL), centr., NaCl (7 g) & CH_2Cl_2 (50 mL) add. into the supern., separ. funnel, filt. of the org. layer & evap. (at 30°C), CH_2Cl_2	RP, LiChrospher 100 RP–18e 250 × 4 mm, 5 μm, MP.: (is.) ACN-0.2 M Phos. buf. (pH 3.0) (35:65, v/v), 2 mM Na_2EDTA, FR.: 1.		Electr. [at +0.65 V (vs. Ag-AgCl)] UV: 225 nm LOD (ng): PENG: 1.2, PENV: 1.4, OXA: 2.7,

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
		add. (3 mL), repeat add. Evap. of CH_2Cl_2, light petroleum add. (3 mL), evap. (ca0.5 mL), sonic., phos. buf. (pH 7), aq. layer phos. buf. (pH 7), centr. SPE: ACN-H_2O (50:50 v/v) cond., 0.02 M phos. buf. (pH 7) sample appl., ACN-0.2 M phos. buf. (pH 3) (10:90, v/v) wash.			CLO: 2.5, DICLO: 4.6
AMO, PENG, AMP, OXA, CLO, DICLO[50]	Cows' raw milk	Centr. (30 mL), dil. of defatted milk (10 mL) with H_2O (20 mL) add. IS sol. (200 μL) (PEN V), deprot. 0.17 M H_2SO_4 (6 mL), 5% Na_2WO_4 (5.6 mL), centr., filt. SPE: tC18 cond. with MeOH (20 mL), H_2O (20 mL), 2% NaCl sol. (10 mL), wash. sol.: H_2O (2 mL), el.with ACN (2 mL), phos. buf. (150 μL) (pH 9.0) add., evap. to 100 μL under N_2 at 50°C, phos. buf. (400 μL) (pH 9.0) deriv. reagent I (75 μL)	RP Waters NovaPak C_{18} 150 × 3.9 mm × 4 μm, 30°C, MP.: ACN (100 mL) to phos. buf. (1000 mL) (A), ACN (300 mL) to phos. buf. (1000 mL) (B), grad.: 0% B, 100% B over 30 min, isocr. 100% B 13 min, 100% A in 2 min, IS: PENV (60 ng/g)	AMO, PENG, AMP: 95–102, OXA, CLO: 92–98, DICLO: 87–94	UV: 323 nm LOD (μg/L): PENG: 1.3, AMO, OXA, CLO: 1.4, AMP: 1.5, DICLO: 2.7

		benzoic anhydride in CH_3CN add., separ. funnel after CH_2Cl_2 (20 mL), phos. buf. (5 mL) (pH 2.45), evap. of CH_2Cl_2 phase (35–40°C), redis. in phos. buf. (pH 9.0) (500 μL), deriv. reagent I (75 μL), deriv. reagent II react.with 1,2,4-triazole and $HgCl_2$ sol. (450 μL).			
CLO[51]	Milk & blood of dairy cows	0.1 M HCl & MeCN (10 mL) add. to blood serum (2.5 mL), milk (5 mL) (for pH adj. to 6.3 & deprot., I.S. (OXA, 250 ng) add. centr., aq. phase extr. with CH_2Cl_2 (2 × 5 mL), centr. org. phase evap. extr. (for milk samples) with $CHCl_3$ (2 × 5 mL), milk & blood extr. rediss. with MP(200 μL)	C_{18} 15 mm × 3.9 mm, Nova Pak, 4 μm, 60Å, MP: MeCN-0.02 M KH_2PO_4 (21:79, v/v) (pH 5), FR: 1.2, IS: OXA	Blood: 81–93 Milk: 75–84	UV: 225 nm, LR (ng/mL): Milk: 25–500 Blood: 50–1000

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
OXA, CLO, DICLO[52]	Cow's milk, meat, cheese	Milk: 5% Na_2WO_4 sol. (4 mL), 0.2 mol/L H_2SO_4 (4 mL) add. to homog. sample (5 g), vol. adj. with H_2O (to 30 mL), shaking, centr., 20% NaCl (10 mL), SPE. Meat: as above (×2) Cheese: extr. (×2) with 0.025 mol/L phos. buf. (pH 6.5, 25 mL) to homog. melted cheese (5 g), shaking, centr., filt., 20% NaCl sol. (10 mL), 5% Na_2WO_4 (5 mL) & 0.2 mol/L H_2SO_4 (5 mL) add., sonic. centr. SPE: cond. By MeOH (5 mL) & H_2O (5 mL), wash. with 2% NaCl sol. (5 mL), sample extr. Load., wash. with 2% NaCl sol. (10 mL), H_2O, OXA el. with 0.0025 mol/L phos. buf. (pH 6.5) & ACN (10:90) (1 mL). Deriv.: 2 mol/L imidazole & 1 mmol/L $HgCl_2$ sol., H_3PO_4 (for pH adj. to 8.5), filt. (1 mL) add. to the eluate, shaking. air thermostat at 65°C, 3 h, filt.	Nova-Pak C18 150 × 3.9 mm, 4 μm, 40°C, MP. (grad.): 0.1 mol/L sodium dihydrogensulphate, 0.05 mol/L $Na_2S_2O_3$ in water, H_3PO_4 (pH adj. 6.5) (A), ACN (B), lin. grad.: 0 min–75%A, 25%B, 6 min–55%A, 45%B, 6.1 min–75%A, 25%B, FR.: 1.	Milk OXA: 78–82 CLO: 85–86 DICL: 83–84 Tissue OXA: 87–89 CLO: 90–93 DICL: 87–91 Cheese OXA: 75–82 CLO: 78–85 DICL: 79–85	UV: 345 nm LOD (ng/g): OXA: 7, CLO: 5, DICLO: 5 (for milk samples) LR: 5–1500 ng/mL

AMO, PENG, AMP, OXA, CLO, NAF, DICLO[53]	Cattle & pig muscle, liver, kidney tissues	Extr. in aq. sol. by precip. of org. materials, H_2SO_4, Na_2WO_4, SPE extr. clean up on divinylbenzene-co-N-vinylpyrrolidone polymeric sorbent, LLE further clean up with diethyl ether, extract deriv. with benzoic anhydride & 1,2,4-triazole mercury (II) reagent	Waters Nova-Pak C_{18} RP 150 mm × 3.9 mm, 4 μm, 38–40°C, MP: ACN (100 mL) dil. with phos. buf. (to 1000 mL) (pH 6.5) (eluent A), ACN (240 mL) & MeOH (60 mL) dil. with phos. buf. (to 1000 mL) (pH 6.5) (eluent B), ACN (300 mL) & MeOH (200 mL) dil. with phos. buf. (to 1000 mL) (pH 6.5) (eluent C), grad.: 0 min 80%A–20%B, 100%B over 30 min, 100%C over 19 min (const. 1 min), 80%A–20%B over 2 min, FR: 1.0, IS: PEN V.	AMO: 66–77 PENG: 73–75 AMP: 81–82 OXA: 72–75 CLO: 74–75 NAF: 66–72 DICLO: 65 PENV: 86–90	UV: 323 nm LOD (μg/kg) AMO: 10.1–10.5 PENG: 9.5–9.9 AMP: 9.8–11.1 OXA: 9.2–11.0 CLO: 9.1–10.8 NAF: 8.9–10.1 DICLO: 18.3–20.9
AMP[54]	Muscle tissues of beef, pork, chicken, catfish	Preparation, extr. deprot. (5.0 g)Blend. homog. Equilibr. (30 min), homog. with 0.01 M (14 mL) Na_3PO_4 buf. (pH 4.5), TCA (75% w/v) H_2O sol. (1 mL) add., centr., supern. filt. Deriv. reaction TCA (20%, w/v) H_2O sol. (0.2 mL)& HCHO (7%, w/v) (0.2 mL) water bath, 100°C, 30 min), adj. to 2 mL with 20% ACN in H_2O, filt.	Prodigy 5 μm, ODS-3, 250 mm × 4.6 mm, MP.: ACN-;0.02 M KH_2PO_4 buf. (pH 3.5) (25:75, v/v), FR.: 1	Beef: 87.7–95.4 Pork: 88.5–92 Chicken: 88.7–94.9 Catfish: 89.9–95.2	Fl.: λ_{exc}. 346 nm: λ_{em}. 422 nm LOD: 0.6 ng/g LR:1–50 ng/g

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
PENG[55]	Ovine milk	ACN (20 mL) to milk (10 mL), add. CH_2Cl_2 (15 mL), org. layer wash. with H_2O (3 mL), aq. phases evap. 40–50°C water bath.	Supelcosil LC-18-DB, 5 μm; 4.6 × 150 mm, MP. (is.): phos. buf. (pH 7)-ACN (82:18, v/v), FR.: 1	78.6–85.8	UV: 214 nm LOD: 2.6–8.8 ng/mL LR: 1 mg/mL–10 ng/mL
OXA, CLO, DICLO[56]	Milk	Extr.: phos. buf. (30 mL) (pH 8) &1 mol/L H_2SO_4 sol. (1.65 mL) add. deprot., centr. (0–5°C), 5 mol/L NaOH sol. (600 μL) add., centr. (0–5°C). C_{18} cleanup: wash. with MeOH (10 mL), H_2O (10 mL), 2% NaCl sol. (5 mL), el.with H_2O-ACN (60:40) (1 mL), Precol. deriv.: Deriv. reagent (0.5 mL) 1,2,4-triazole and $HgCl_2$ sol. (pH 9.0, 65°C).	Symmetry Waters C_8 150 × 3.9 mm, 5 μm, MP.: ACN-MeOH-0.1 mol/L phos. buf. (pH 6.5) (37:5:58, v/v/v), FR.: 1	OXA: 79.3–83.7 CLO: 76.4–78.3 DICLO: 64.6–67.6	UV: 340 nm LOD μg/L: OXA: 2, CLO: 3, DICLO: 5 LR: 15–240
AMP, PENG, CLO, DICLO, NAF[57]	Milk	ACN (20 mL) add. in milk (10 mL), centr., supern. Conc.(to 2–3 mL, at 40°C), extract load into Baker-10 C_{18}, el.with MeOH (1 mL)	LC Kaseisorb ODS-300-5 250 mm × 4.6 mm x 5 μm, 40°C, MP.: ACN-MeOH-0.05 M KH_2PO_4 (20:10:80, v/v/v) with 5 mM sodium 1-decanesulfonate (pH 3.5 adj. H_3PO_4), FR.: 1.0.	79.8–89.4	Ion pair LC (UV: 210 nm) AMP, PENG: 0.03 μg/mL, CLO, DICLO, NAF: 0.05 μg/mL

PENG[58]	Cattle, pig, chicken tissues (muscle, kidney, liver)	Homog. with 0.1 M phos. buf. (pH 6.5), ultrafilt. (deprot.)	Mightysil RP-4GP (end-capped) 250 mm × 4.6, 5 μm, guard col. 4.6 × 5 mm (same packing material), MP: EtOH-0.1 M phos. buf. (pH 6.5) (1:4, v/v), FR: 1.0, RT	Muscle 79.9–90.3 Kidney 80.2–89.5 Liver 78.8–88.5	DAD: 211 nm LOD:0.04 ppm LR:1–50 ng
PENG[59]	Milk	Extr./deprot. EtOH (0.5 mL) add. to the sample (0.5 mL), reaction with 5 M 1,2,4- triazolemercury (II) chloride sol. (at 65°C for 10 min), ultra centr., ultrafilt.	Mightysil RP-4GP (5 μm), 4.6 × 250 mm, guard col.: 4.6 × 5 mm, MP: ACN-0.1 M phos. buf. (pH 6.5) (35:65, v/v),FR:1.0.RT	86.4–91.8	DAD: 325 nm LOD: 0.004 μg/mL LR: 0.2–20 ng
PENG[60]	Beef & pork tissues	Extr./deprot. I: H_2O (5 mL), 0.1 M Et_4NCl (2 mL), ACN (40 mL) add. to the cut tissue (5 g), for kidney: 0.2 M Et_4NCl (1 mL), 0.005 M KH_2PO_4 (1 mL), ACN (40 mL) add., supern. filt. II: H_2O (45 mL),blend., β-lactamase sol. (0.1 mL) add. (to 10 mL of homog. aliquot), 0.1 M Et_4NCl (2 mL), ACN (40 mL) add. (for extr./deprot., for kidney: 0.2 M Et_4NCl (1 mL), 0.005 M KH_2PO_4 (1 mL), ACN (40 mL) add., supern. filt.Evap.:0.01 M buf.	Cleanup system: LC-18, 4.6 × 150 mm, 5 μm, MP: 0.01 M KH_2PO_4 (initial), 60% ACN grad. (after 3 min at 40 min), initial cond. at 41 min, FR: 1.Anal.:Inertsil-ODS-2, 4,6 × 150 mm, 5 μm, MP: 0.0067 M KH_2PO_4, 0.0033 M H_3PO_4-ACN (68:32), FR: 1	Pork Muscle: 89 ± 4–14 ± 26 Liver: 82 ± 12–95 ± 11 Kidney 77 ± 9–104 ± 8 Calf Muscle 78 ± 7–88 ± 11 Liver 47 ± 7–77 ± 13	Cleanup: UV: 210 nm HPLC Anal.: UV: 215 nm LOD: 0.005 ppm (ng/g)

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
		(5:1 KH_2PO_4-Na_2HPO_4) (2 mL), tert-butyl alcohol (5 mL), H_2O (5 mL) add. to the filtrate, water bath (40–45°C) (evap. to 1–2 mL), vol. adj. to 4 mL, filt. Cleanup: 0.01 M Na_2HPO_4 (0.2 mL) add. to PENG fractions, conc.1 mL.		Kidney 66 ± 8–77 ± 9	
AMO, AMP, CLO, PENG[61]	Milk & tissues	Milk extr./deprot. Et_4NCl (2 mL), ACN (40 mL) add. to milk (10 mL), 0.01 M pH 6 buf. (5:1 KH_2PO_4–Na_2HPO_4), water bath (40–50°C), evap. (to 1–2 mL), H_2O add. (to final vol. of 4 mL), filt. Tissue-procedure I: H_2O (5 mL) & 0.1 M Et_4NCl (2 mL) (for liver & kidney), 0.2 M Et_4NCl (1 mL) & 0.005 M KH_2PO_4 (1 mL), ACN (40 mL) add. to cut tissue (5 g), mixture blend. filt., 0.01 M ph 6 buf.	Fractionation: Supelcosil LC-18, 150 × 4.6 mm, 5 μm, MP. (grad.): 0.01 M KH_2PO_4, t = 3 min: ACN grad. 0 min 60% ACN at 40 min, t = 41 min: initial cond., t = 55 min: ready to load. Anal. milk fractions. AMO: MP: 0.015 M H_3PO_4, 0.0075 M SDS-ACN (68:32), AMP. MP: 0.01 M H_3PO_4, 0.005 M KH_2PO_4, 0.005 M SDS-ACN (65:35).PENG: MP: 0.0033 M H_3PO_4, 0.0067 M KH_2PO_4 or 0.005 M H_3PO_4, 0.005 M KH_2PO_4 -ACN (72:28),		Anal.of milk fractions UV: 215 nm Anal. of tissue fractions AMO AMP After deriv. By HCHO·FL CLO, DICLO PENG UV 215 nm

		(2 mL), H_2O (5 mL), tert-butanol (5 mL) add. to filtrate [40 mL (20 mL for liver & kidney)], evap. as described for milk. Tissue-procedure II (β-lactamase) H_2O (45 mL) add. to tissue sample (15 g), blend., following procedure for milk (in 10 mL of homog.), evap. after tert-butanol & H_2O add. β-lactamase treatment: β-lactamase (0.1 mL) add. to milk or tissue homog. sample (10 mL) (Procedure II), mixt.incub. (1 h, at RT)	FR:1. PENV: MP.: 0.005 M H_3PO_4, 0.005 M KH_2PO_4 -ACN (67:33). CLOX, OXA, NAF, DICLO MP: 0.0020 M H_3PO_4, 0.0080 M KH_2PO_4-ACN (62:38) or 0.0025 M H_3PO_4, 0.0075 M KH_2PO_4-ACN (60:40), Anal. tissue fractions		
PENG, PENV, OXA, CLO, NAF, DICLO[62]	Pork muscle Beef muscle	Pork muscle: H_2O (30 mL) add. to (5 g) after slicing & homog., centr., supern. filt., residual reextr. with H_2O (20 mL), supern. Beef muscle: 2% NaCl aq. sol. (60 mL) add. to (5 g) after slicing & homog. supern. filt., residual reextr. with 2% NaCl aq. sol. (40 mL). Purif. of crude extr. using precleanup cartridge: Crude extr. ($\sim$70 mL for pork &	TSKgel ODS-80Ts, 5 μm, 150 $\times$ 4.6 mm; TOSOH, MP: ACN-0,02 M phos. buf. (pH 6.2) (43:57, v/v) with CTMAC, FR: 0.8.	Pork muscle PENG: 80–85, PENV: 89–90 CLO: 85– 93 OXA: 82–89 NAF:86–96	Pork muscle UV: 220 nm LOD: 0.02 mg/kg LR: 0.01–2 μg/mL (for PEN G, PEN V, DICLO), 0.005–1 μg/mL (for OXA, CLO, NAF) DICLO: 73–80 Beef muscle PENG: 77–92

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
		140 mL for beef) cleanup through a Bond Elut C_{18} cartridge, precond. with MeOH (5 mL) & H_2O (5 mL), wash. with H_2O (10 mL), 15% MeOH (15 mL) with 2% NaCl, H_2O (5 mL), air-drying in vacuum, el. with 55% MeOH (5 mL). Purif. of eluate from Bond Elut C_{18}: Clean up through a Sep-Pak Accell Plus QMA cartridge, precond. with MeOH (5 mL), H_2O (5 mL) & 55% MeOH (5 mL), wash. with 55% MeOH (3 mL) & H_2O (3 mL), evap. el. with HPLC MP. (2 mL)		PENV: 82–90 OXA: 74–86 CLO: 82–86 NAF: 85–90 DICLO: 71–83	

PENG, PENV, OXA, CLO, NAF, DICLO[63]	Bovine liver & kidney	Preparation of crude extracts of bovine liver & kidney. Bovine liver: 2% NaCl aq. sol. (50 mL), 5% Na_2WO_4 aq. sol. (5 mL), 0.17 M H_2SO_4 (5 mL) add. to a homog. sample (5 g), centr., residual plug reextr. with 2% NaCl aq. sol. (40 mL), supern. centr. Stand (15 min). Bovine kidney: 2% NaCl aq. sol. (55 mL), 5% Na_2WO_4 aq. sol. (2,5 mL), 0.17 M H_2SO_4 (2,5 mL) add. to a homog. sample (5 g), 2nd & 3rd extr. as above Purif. of crude extract using pre-cleanup cartridge (Bond Elut C_{18}): Crude extract (140 mL) cleanup as in ref 32. Purif. of eluate from Bond Elut C18 by cleanup cartridge: Clean up through a Sep-Pak Accell Plus QMA cartridge, as in ref. 32	TSKgel ODS-80Ts, 5 μm, 150 × 4.6 mm; TOSOH, 30°C, MP: ACN-0.02 M phos. buf. (pH 6.2) (43:57, v/v) with 12 mM CTMAC, FR: 0.8.	Liver: PENG: 82–86 PENV: 83–88 OXA: 91–96 CLO: 91–92 NAF: 84 DICL: 73–89 Kidney PENG: 82–83 PENV: 82–86 OXA: 92 CLO: 89–90 NAF: 80–89 DICL: 79–89	HPLC-UV: 220 nm LOD: 0.02 mg/kg LR: 0.01–2 μg/mL (for PENG, PENV, DICLO) & 0.005–1 μg/mL (OXA, CLO, NAF)

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
PENG, PENV, OXA, CLO, NAF, DICLO[64]	Bovine muscle, liver & kidney	Sample sol. prepared as described in 32, 33, except for using ACN-H_2O (2 mL, 30:70, v/v) with 50 mM DBAA as el. solvent from Sep-Pak Accell Plus QMA cartridge.	TSKgel ODS-80Ts, 5 μm, 150 × 4.6 mm, TOSOH, 30°C, MP. (grad.): ACN-H_2O (30:70, v/v) with 2 mM DBAA (A), ACN-H_2O (50:50, v/v) 2 mM DBAA (B), grad. Cond. t = 0–3 min: %B = 0, t = 3.1–8 min: %B = 100 (ramp lin.), t = 8.1–9 min: %B=0 (ramp back), t= 9–14 min: %B=0 (equilibrate the system), FR: 1.0.	>95	LC-ESI-MS-MS LOD: 0.02–0.03 mg/kg
DICLO[65]	Bovine & ovine milk	Sample (2 mL) purif. SPE-C_{18} (500 mg-Baker) cond. with MeOH (2 mL), H_2O (2 mL) & 0.2 M phos. buf. (1 mL), wash. with 0,2 M phos. buf. (2 mL) & H_2O (1 mL), DICLO el.with MeOH (2 mL), el. Evap., dissol. with H_2O.	Lichrosorb-RP 18, 5 μm; 150 × 4.6 mm), guard col. (Merck), MP: 0.05 M KH_2PO_4 (pH 7): 11 mM SHS (15:2, v/v), MeOH (50:50, v/v), FR:1.	Bovine milk 85.18 ± 0.63 Ovine milk 83.80 ± 0.062	HPLC-UV: 210 nm
AMP[66]	Muscle tissue	HPLC-UV: Extr. by phos. buf., clean up on C_{18} SPE cartridge, eluate deriv. with 1,2,4-triazole & mercuric chloride.	HPLC-UV: Lichrospher100-RP18e, Merck, C_8, 150 × 3.9 mm; 5 μm. guard col.: C_{18}, 4 × 4 mm; 5 μm.	HPLC-UV: 75 ± 7	UV: 325 nm MS:

		HPLC-ESI-MS: Tissue samples homog. with ammonium acetate buf. (pH 8.5, 0.1 M), clean-up using Bond-Elut C_{18} cartridges.	HPLC-ESI-MS: C_{18}, RP18e, Lichrospher, 125 × 4 mm, 5 μm, MP: MeOH-0.2% HCOOH in H_2O (45:55, v/v), grad.: from t=0 to t = 2 min, MeOH: 25%, from t=3 to 5 min MeOH = 80%, FR: 0.6, IS: cephalexin.	MS: 59 ± 15–76 ± 15	Mode: positive ion mode with electrospray interface, HPLC-ESI-MS:LOD: 16 μg/kg
PENG[67]	Veal calf liver tissues	Spiking of finely chopped liver tissue, add. of a 2 μg/mL PENV sol. ACN add., centr. (2-5°C), evap. of supern. to 500 μL under N_2, Deriv. by react.with 1,2,4-triazole and $HgCl_2$ sol. (60°C water bath). 2% NaCl add. (10 mL), filt. Tissue extracts cleanup on t-C18 Sep-Pak, Cond. with MeOH (5 mL), H_2O (5 mL), 2% NaCl (5 mL), el.with 1 mL el.sol. (60% ACN/35% H_2O/5% 0.2 M phos. buf.)	Inertsil C_8, 150 × 150 mm × 5 μm, RT, MP: ACN-0.05 M phos. buf. with 0.0157 M $Na_2S_2O_3$ (57:25 v/v), FR: 1.2 IS: PENV (60 ng/g)	65–85	UV: 325 nm LOD: 1.5 ng/g
AMO, AMP, CLO, PENG, PENV[68]	Milk	Extr.: ACN (5 mL) add., vortex, ACN (10 mL) add., centr., evap. of the extract under N_2 at 60°C, phos. buf. (3 mL) add., vortex.	Luna C_{18} (2) 25 cm × 4.6 mm, 5 μm, RT, MP: 1% CH_3COOH in H_2O (A) & 1% CH_3COOH in MeOH (B)	85–115	MS/MS

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
		SPE Cleanup: prewash. with MeOH (5 mL), ACN (10 mL), H_2O (5 mL), phos. buf. (3 mL), extract application, wash. with phos. buf. (3,5 mL), H_2O (2 mL), 3% ACN in H_2O, el.with 40% ACN in H_2O, evap. under N_2 at 60°C, CH_3COONH_4 buf. (0,5 mL), filt.	Screening HPLC grad. method: 80% A/20% B for 3 min, lin. grad. to 50%A/50% B at 8 min, lin. grad. to 10% A/90% B at 28 min, hold 5 min, FR: 0.5.Quant. HPLC: AMP, CLO, PEN G, PEN V (is.): 30% A/70% B, FR.: 0.5 for 15 min, AMO, (is cr.): 65% A/35% B, FR: 0.5		LOD ng/mL: AMO: 1, AMP: 0.2, CLO: 2, PENG:1, PENV: 2
PENG, AMP, OXA, AMO, DICLO, NAF[69]	Bovine milk	Extr. with 10% aq. sol. CH_3COOH (400 μL) add. in raw milk (5 mL), centr. at 4°C, supern. phase filt.	Merck-LiChrospher 100 RP18 250 mm × 4 mm, 5 μm, MP: H_2O and ACN acid. 0.1% HCOOH, FR.: 1, grad. el.: from 100% H_2O to 100% ACN in 6 min & from 100% ACN to 100% H_2O from 6 to 12 min.	PENG: 51–53 AMP: 74–78, OXA: 43–44, AMO: 51–53, NAF: 30–31, DICLO: 28–29	MS/MS LOD (μg/L):PENG: 1, AMP: 2, OXA: 5, AMO:,1, NAF: 5, DICLO: 4
PEN G, AMP, OX, AMO, NAF, DICLO[70]	Bovine milk	Milk sample (5 mL) mix with 10% CH_3COOH sol. (400 μL), centr. (10 min, at 4°C), upper fat layer filtr. (0.50 μm nylon filter 13 mm diameter)	Merck-LiChrospher 100 RP18, 250 mm × 4 mm,5 μm, MP(grad.):H_2O, ACN (acid. HCOOH), from 100% H_2O to 100% ACN in 6 min & from 100% MeCN to 100% H_2O from 6 to 12 min (2 min equilibr.). FR: 1, I.S.: NAF		ESI-MS/MS

PENG, AMO, AMP, OXA, CLO[71]	Meat	Extr. & deprot. with phos. buf. (pH 8.5) & H_2SO_4, cleanup on C_{18} SPE col. & precol. deriv., benzoic anhydride add. to the eluate (to acylate AMO and AMP) (at 50°C, 5 min)&1,2,4-triazole $HgCl_2$ sol. (pH 8.5) (mercaptide deriv. at 65°C, 30 min).	C_{18}, MP. (isocr.): ACN-phos. buf. (pH 6) 70		UV: 325 nm LOD: 5–11 μg/kg
AMO, AMP, OXA, CLO, DICLO, PENG, PENV, NAF[72]	Bovine muscle, kidney & milk	Fortified raw milk preparation & extr. Thawing of frozen samples at 25°C, spiking of the homog. raw milk samples (30 g). RT. keeping (10 min) vortex, centr., vol. adj. (to 30 mL) of defatted milk (10 g) with ACN, vortex, centr., saturated NaCl sol. (4 mL) add., ACN evap. (at 37°C), 0.05 M phos. buf. (pH 8.5) (15 mL) add., pH readj. to 8.5) with 0.2 M NaOH, SPE cleanup Fortified tissue preparation & extr. Homog. using knife mill, thawing of frozen samples at 25°C, spiking (4 g) RT. keeping (10 min) after vortex H_2O (2 mL) add., vol. adj. (to 20 mL) with	Phenomenex Synergi Polar-RP phenylether col., 250 mm × 2 mm; 4 μm, 80Å, guard col.: same material, FR: 0.2, MP. (grad.):0.005% HCOOH in H_2O (A), MeOH (B), t = 0–3 min: hold 100%A, t = 3–22 min: ramp lin. to 90% B, t= 22–37 min: hold 90% B, t = 37–52 min: 100%A.	Milk AMO: 57, AMP: 81, OXA: 115, CLO: 114, DICLO: 112, PENG: 95, PENV: 102, NAF: 38.1 Muscle AMO: 65, AMP: 88, OXA: 104, CLO: 94, DICLO: 94, PENG: 99, PENV: 99, NAF: 101 AMO: 46,	LC-MS-MS: first operating in positive (ESI (+)), then in negative LOD (ppb): Milk AMO: 5.6, AMP: 5.4, OXA: 34.9, CLO: 35.1, DICLO: 38.9, PENG: 5.4, NAF: 38.1, Muscle AMO: 57.7, AMP: 62.5, OXA: 367.6, CLO: 389.9, DICLO: 365.2 PENG:

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
		ACN, homog., centr., saturated NaCl sol. (4 mL) add. to the supern. (15 mL), evap., ACN evap. (at 37°C), 0.05 M phos. buf. (pH 8.5) (20 mL) add., vol. adj. (to 30.0 mL) with 0.05 M phos. buf. (pH 8.5), centr., pH readj. to 8.5 using 0.2 M NaOH, SPE cleanup		AMP: 78, OXA: 88, CLO: 90, DICLO: 76, PENG: 85, PENV: 85, NAF: 86	64.9, NAF: 407.5, Kidney AMO: 69.3, AMP: 65.6, OXA: 398.0, CLO: 358.8, DICLO: 428.1,PENG: 59.6, NAF: 424.
		Enzymatic digestion of PENG positive kidney sample 0.05 M phos. buf. (pH 8.5) & protease (10 mg) add. (to a vol. of 20 mL) to homog. kidney sample (3 g), vortex, incub. (2 h, at 55°C) centr., supern. (10 mL).SPE cleanup for milk & tissues: Cartridge precond. with MeOH (2 mL), H_2O (2 mL) & 0.05 M phos. buf. (pH 8.5) (2 mL), ACN evap., aq. sample extr. clean up (FR.: 0.5), col. wash with 0.05 M phos. buf. (pH 8.5) (3 mL) & H_2O (1 mL), El.with ACN- H_2O (1:1, v/v) (3 mL) (FR.: 1), evap. (at 40°C, to 1.5 mL) under N_2, vol. adj. (to 2.0 mL) with H_2O, centr. (at 10°C)			

AMO, AMP[73]	Bovine muscle, liver, kidney, milk	Milk (2 mL) spiking & I.S. (PENV), equilibr. (10 min at RT), mix.(in porcelain mortar) with sand (6 g) (10 min), packing into the extr. cell (stainless, 2 μm pore size & polyethylene, 20 μm pore size frits), heating in oven (at 65°C, 5 min), H_2O (3 mL, FR: 1) add.to the cell, pH adj. to 4.6 with 3 mol/L HCOOH, filtr. Tissue samples: Finely dicing, tissue sample (1 g) spiking, equilibr. (1 h at 4°C), mix. (in porcelain mortar) with sand (5 g) (<10 min), following procedure as above, exception: 150 ng of IS add. to the extract & pH adj. to 3, filtr.	Alltima (Alltech),: 250 mm × 4.6 mm,, 5 μm C_{18}, guard col.: 7.5 × 4.6 mm, 5 μm C_{18}, MP (grad.): MeOH (A), H_2O (B) with 10 mmol/L HCOOH: Milk & muscle extr. t_o, A = 25%, t_9, A = 60%, t_{10}, A = 80%, $t_{20,}$ A = 100%, $t_{25,}$ A= 100%, $t_{27,}$ A = 0%, t_{35}, A = 0%. Kidney & liver extr. t_o, A = 0%, t_5, A = 0%, t_6, A = 40%, $t_{15,}$ A = 60%, $t_{23,}$ A= 100%, $t_{28,}$ A = 100%, t_{30}, A = 0%, t_{38}, A = 0%. FR: into the ion source: 0.150 (4–20 min for milk, muscle, 10–23 min for kidney, liver), I.S: PENV.	Milk AMO: 94 AMP: 95 Kidney AMO: 88 AMP: 89 Muscle AMO: 93 AMP: 92 Liver AMO: 74 AMP: 74	LC-ESI-MS/MS: LOD (ppb) Milk AMO: 0.5 AMP: 0.1 Tissues AMO: 2.1 AMP: 0.5

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
AMP, CLO, PENV, PENG[74]	Milk, kidney, liver tissues	Kidney & liver tissues (5 g), Milk (10 mL): spiking, extr.:H_2O (5 mL) & 0.1 M Et_4NCl (2 mL), 0.005 M KH_2PO_4 (1 mL), CH_3CN (40 mL) add., 0,001 M pH 6 KH_2PO_4-Na_2HPO_4 buf. (2 mL), H_2O (5 mL), tert. butanol (5 mL) in the supern. Conc. under N_2 at 40°C to 5 mL, filt.	C_{18} Higgins 150 mm × 3.0 mm × 5 μm, MP.: 25 mM CH_3COOH in H_2O & CH_3OH (72:25), FR.: 0.150.	PENG: 73–85 (kidney), 70–80 (liver), PENV: 55–80 (kidney), 60–75 (liver), AMP: 50–68(kidney), 50–65 (liver), CLO: 50–63 (kidney), 47–58 (liver)	MS, LOD: PENG, PENV: 1 ng/kg (in kidney and liver), 0.7 μg/L (in milk), AMP: 1.4 μg/kg (in kidney and liver), 1.7 μg/L (in milk)
PENG, AMP, NAF[75]	Bovine, swine tissues	Mixed IS sol. to the aliquot (5 g), blend. with ultra pure H_2O (5 mL), centr., supern. (350 μL) ultrafilt. (pre-washed with 400 μL each of 1.0% Tween 20 & ultra pure H_2O), centr.	TSK-Guardgel ODS-80 Ts, 5 μm, 15 mm × 3.2 mm, MP.: 0.05% HCOOH (A), 0.05% HCOOH in MeOH (B), FR.: 0.2. IS: PENG-d5, AMP-d5, NAF-d6 for PENG, AMP & NAF,)	AMP: 96.1–106.6 NAF: 96.1–102.6 PENG: 94.4–100.4	ESI-MS/MS LOD: 0.002 ppm

PENG, AMO, AMP, OXA, CLO[76]	Poultry muscle	(2 g) homog. sample with 0.01 M phos. buf. (pH 6.5) (5 mL), proteins prec. with 30% TCA (1 mL) add., centr., filt., supern. (1 mL) deriv. with HCHO (50 μL) (45 min, 100°C), supern. extr. (×2) with diethyl ether, extracts evap. reconst. with MP.	MP.: phos. buf.-ACN (75:25, v/v)		Fl. λ_{exc} 346 nm λ_{em} 422 nm. LOD: 5 μg/kg
PENG[77]	Bovine plasma, kidney, urine	Kidney extr.: Centr. of samples (0.1 g), I.S. add. (100 μL–0.5 μg/mL), H_2O add. (to vol. 1000 μL, ACN add. (1 mL), centr., ACN evap. from supern. under N_2 (40°C) to 0.4–0.5 mL, dil. with H_2O (2 mL) add. SPE: cleanup through Bond-Elut C_{18} cond. with MeOH (2.5 mL) & H_2O(2.5 mL),El. with ACN (3 mL), H_2O Add., evap. under N_2 to <0.4 mL (40°C), vol. adj. to 1 mL, filt., centr. Urine extr.: I.S. add. (200 μL of 10 ppm PHE, equiv. to 4000 ng/mL), sample dil. (to 50 mL) with H_2O, shaking, filt.	Zorbax SB-C_{18} Rapid resol., 2.1 × 3.0 mm, with 3.5 μm silica, 30°C, inj. vol.: 25 μL kidney, 5 μL urine samples, MP. (grad.): 90% A (0.1% HCOOH in H_2O) + 10% ACN, 40 + 60 next 1.5 min, held 2.0 min, back to initial 1 min and held 4 min before next inj. FR: 0.3, IS: PHE (deuterated PENG, 100 μL of 0.5 μg/mL for kidney, 200 μL of 10 ppm for urine)	Almost 100	LC/MS/MS LOQ ng/mL: plasma:12, kidney: 10, urine: 63

(*continued*)

Table 3. Continued

Analytes	Sample type	Sample preparation	Chromatographic conditions	Recovery (%)	Detection-linear range-LOD
Degradation products of AMO: 4HDPG, PA, PCA, ADP, UDP[78]	Granular premixes	AMO premix dil. in petroleum ether, buf. sol. add. (pH 6 ± 0.05, 40 mL), buf. sol. pH 6 ± 0.05, storing at 25°C for 3 h, filt.	Nucleosil 120 C_{18} 250 mm × 46 mm., 10 μm, MP. (multistep grad.): A: buf. sol. (pH 3 ± 0.05), B: MeOH 0–15 min. 98:2 (A:B), 20–25 min 70:30, 28–30 min 98:2. FR: 1.75, 40°C.	4HDPG 101.78, PA: 100.11, PCA: 98.60, ADP: 97.64, UDP: 95.00	DAD: 230 nm LOD(μg/mL) 4 HDPG:1.39, PA: 2.50, PCA: 3.04, ADP: 3.00, UDP: 6.00
PENG, CLO, OXA, DICLO, AMP, NAF[80]	Milk & yoghurt	UV: Centr. & ultrafiltr. of milk samples, on-line SPE using restricted-access sorbent (alkyl-diol-silica, ADS). Deriv. LC-MS/MS: Milk sample spiking with penicillin (1 mg/mL), storing for 3 days at 5°C, sample dil. (1:1000), centr. (at 2000 × g), filtr. through 0.2 μm poly(vinylidene fluoride) (PVDF) filter.	C_{18}. No details in text.		UV: 300 nm

The same analytical column was used at 45°C to determine PENG, PENV, and AMP in milk using a Permaphase ETH, 5 cm × 2.1 mm guard column. The mobile phase consisted of MeOH-H_2O-0.2 M phosphate buffer (pH 4.0) (5:13:2) containing 10 mM sodium alkylsulphonate.[39]

A Brownlee Microbore Phenyl Spheri-5, 220 mm × 2.1 mm, 5 μm, analytical column operated at 40°C with 25% (v/v) ACN in H_2O containing 0.0025 M dodecanesulfonate, 0.5% (v/v) 85% H_3PO_4 and 0.5% (v/v) TEA as mobile phase and a Brownlee Phenyl Spheri-5, 220 mm × 4.6 mm, 5 μm, operated at 40°C with IPA-0.2 M CH_3COONH_4-CH_3COOH (12.5:85.5:2) as eluent were used for the determination of PENG in bovine milk.[40]

The same microbore column has been used for the determination of CLO, AMP/HETA, and AMO in bovine milk using a mobile phase of 1.5% IPA, 5% CH_3COOH in 0.2 M CH_3COONH_4, 93.5% H_2O for AMO, 10% IPA, 2% CH_3COOH in 0.2 M CH_3COONH_4, 88% H_2O for AMP/HETA, 15% IPA, 2% CH_3COOH in 0.2 M CH_3COONH_4, 83% H_2O for CLO.[41]

A PLRP-S (Polymer Labs, 150 × 4.6 mm, 5 μm (100 Å), column was used for the determination of PENG in milk with a mobile phase of 0.01 M phosphate buffer (pH 1.96)-ACN (66:34),[42] as well as for the determination of PENG, PENV, and CLO[43] in beef and pork tissues using 0.01 M phosphate buffer (pH 7)-ACN 85:15 for PENG, 82:18 for PENV and 78:22 for CLO.[44] The same column was used for AMO determination in broiler tissues (kidney livermuscle fat and skin and fat) with the same type of pre-column, 5 mm × 3.0 mm, operated at room temperature.[45] The mobile phase consisted of 0.1% HCOOH in H_2O (A) and ACN (B). Gradient elution was applied as follows: 0–7 min: 90%A–10%B, 7.1–15 min: 50%A–50%B, 15.1–21 min: 90%A–10%B. Ampicillin was used as internal standard.

An Ultrasphere OS (C_8), (Beckman 25 cm × 4.6 mm, 5 μm) analytical column was used for the determination of PENG in Chinook salmon using a mobile phase which consisted of ACN-0.02 M phosphate buffer (pH 6.75) (43:200), or ACN:0.02 M phosphate buffer, pH 6.75; 200:52 v/v. PENV was used as internal standard.[45,46]

PENG, AMP, AMO, and CLO were determined in bovine milk using an Ultremex 3, C_{18}, 150 × 2 mm, analytical column with a mobile phase consisting of 40% (v/v) ACN: 1% (v/v) HOAC in water (pH 3.0).[47]

A Kromasil 5 C_8 25 cm × 4.6 mm, 5 μm column was used to determine PENG in milk with a mobile phase consisting of 0.01 M H_3PO_4 acid (A) and 0.01 M H_3PO_4-ACN (20:80, v/v) (B). Gradient elution was performed: 0 min, 100% A; 5 min, 100% A; 35 min, 100% B.[48]

A LiChrospher 100 RP-18e, 250 × 4 mm, 5 μm, analytical column was used for the determination of OXA, PENG, PENV, CLO, and DICLO in bovine muscle with a mobile phase which consisted of ACN-0.2 M phosphate buffer (pH 3.0) (35:65, v/v) and 2 mM Na_2EDTA.[49]

A Waters NovaPak C_{18} analytical column 150 × 3.9 mm, 4 μm, operated at 30°C, was applied to the separation of AMO, PENG, AMP, OXA, CLO, and

DICLO in cows' raw milk.[50] The mobile phase consisted of ACN (100 mL) to phosphate buffer (1,000 mL) (A) and ACN (300 mL) to phosphate buffer (1,000 mL) (B). A gradient program was used as follows: 0%B, 100%B over 30 min, remaining isocratic 100%B for 13 min, and then returning to 100%A in 2 min. Penicillin (60 ng/g) was used as internal standard. The same column was used to determine CLO in milk and blood of dairy cows with a mixture of MeCN-0.02 M KH_2PO_4 (21:79, v/v) (pH 5) as mobile phase and OXA as internal standard,[51] as well as for the determination of OXA, CLO, and DICLO in cow's milk, meat, and cheese at 40°C with a mixture of 0.1 mol/L sodium dihydrogensulphate, 0.05 mol/L $Na_2S_2O_3$ in water, H_3PO_4 (pH 6.5) (A) and ACN (B).[52] A linear gradient was applied: 0 min–75%A, 25%B, 6 min–55%A, 45%B, 6.1 min–75%A and 25%B. The same analytical column 150 × 3.9 mm, 4 μm, was used for AMO, PENG, AMP, OXA, CLO, NAF, and DICLO[53] determination in cattle and pig muscle, liver, and kidney tissues at 38–40°C with a mobile phase consisting of ACN (100 mL) diluted with phosphate buffer (to 1,000 mL) (pH 6.5) (eluent A), ACN (240 mL) and MeOH (60 mL) diluted with phosphate buffer (to 1,000 mL) (pH 6.5) (eluent B) and ACN (300 mL) with MeOH (200 mL) diluted with phosphate buffer (to 1,000 mL) (pH 6.5) (eluent C). The gradient program was as follows: 0 min 80%A–20%B, 100%B over 30 min, 100%C over 19 min (remaining constant for 1 min), 80%A-20%B over 2 min. PENV was used as internal standard.

A Prodigy, ODS-3, 250 mm × 4.6 mm, 5 μm, analytical column was used for determination of AMP in muscle tissues of beef, pork, chicken, and catfish. The mobile phase consisted of ACN-0.02 M KH_2PO_4 buffer (pH 3.5) (25:75, v/v).[54]

A Supelcosil LC-18-DB, 150 × 4.6 mm, 5 μm, analytical column was used to determine PENG in ovine milk using with a mobile phase consisting of phosphate buffer (pH 7)-ACN (82:18, v/v), delivered isocratically.[55]

A Symmetry Waters C_8 150 × 3.9 mm, 5 μm, analytical column was used for the separation of OXA, CLO, and DICLO in milk using ACN-MeOH-0.1 mol/L phosphate buffer (pH 6.5) (37:5:58, v/v/v) as mobile phase.[56]

AMP, PENG, CLO, DICLO, and NAF were determined in milk using an LC Kaseisorb ODS-300-5 250 mm × 4.6 mm, 5 μm, analytical column operated at 40°C. The mobile phase consisted of: ACN-MeOH-0.05 M KH_2PO_4 (20:10:80, v/v/v) with 5 mM sodium 1-decanesulfonate (pH 3.5 adj. with H_3PO_4).[57]

A Mightysil RP-4GP (end-capped) 250 mm × 4.6 mm, 5 μm, analytical column operated at room temperature was used to determine PENG[58] in cattle, pig, and chicken tissues (muscle, kidney, liver) with a guard column 4.6 mm × 5 mm containing the same packing material. The mobile phase consisted of: EtOH-0.1 M phosphate buffer (pH 6.5) (1:4, v/v). The same column was used to determine PENG in milk with a mobile phase consisting of ACN-0.1 M phosphate buffer (pH 6.5) (35:65, v/v).[59]

PENG was determined in beef and pork tissues using an LC-18 column, 4.6 × 150 mm, 5 μm, for the cleanup system, with a mobile phase of 0.01 M KH_2PO_4 and 60% ACN delivered under a gradient program of 41 min. Analysis was performed using an Inertsil-ODS-2, 150 × 4.6 mm, 5 μm, column with a mobile phase consisting of: 0.0067 M KH_2PO_4, 0.0033 M H_3PO_4-ACN (68:32).[60]

A Supelcosil LC-18, 150 × 4.6 mm, 5 μm, analytical column was used for the determination of AMO, AMP, CLO, and PENG in milk and tissues. The mobile phase consisted of 0.01 M KH_2PO_4, and ACN under a gradient program ending at 60% ACN at 40 min, t = 41 min: return to starting conditions, t = 55 min: ready to load another sample.[61]

A TSKgel ODS-80Ts, 150 × 4.6 mm, 5 μm, analytical column operated at 30°C was used to separate PENG, PENV, OXA, CLO, NAF, and DICLO[62] in pork and beef muscle and bovine liver and kidney[63] with a mobile phase which consisted of ACN-0.02M phosphate buffer (pH 6.2) (43:57, v/v) with 12 mM cetyltrimethyl-ammonium chloride. PENG, PENV, OXA, CLO, NAF, and DICLO were separated in bovine muscle, liver, and kidney with a mobile phase consisting of ACN-H_2O (30:70, v/v) with 2 mM di-n-butylamine acetate (DBAA) (A) and ACN-H_2O (50:50, v/v) 2 mM DBAA (B). A gradient program was applied as follows: t = 0–3 min: %B = 0, t = 3.1–8 min: %B = 100 (ramp linearly), t = 8.1–9 min: %B = 0 (ramp back), t = 9–14 min: %B = 0 (hold to re-equilibrate the system).[64]

A Lichrosorb-RP 18, (150 × 4.6 mm, 5 μm) analytical column with a guard column (Merck) was used to determine DICLO in bovine and ovine milk using a mobile phase which consisted of 0.05 M KH_2PO_4 (pH 7): 11 mM SHS (15:2, v/v) and MeOH (50:50, v/v).[65]

A Lichrospher100-RP18e (Merck) C_8, 150 × 3.9 mm; 5 μm, analytical column was used for the determination of AMP in muscle tissue with a guard column: C_{18}, 4 × 4 mm; 5 μm and a Lichrospher RP 18e, 125 × 4 mm; 5 μm, column with a mobile phase: MeOH-0.2% HCOOH in H_2O (45:55, v/v), under a gradient program: from t = 0 to t = 2 min, MeOH: 25%, from t = 3 to 5 min MeOH = 80%, with cephalexin as internal standard.[66]

An Inertsil C_8, 150 × 4 mm, 5 μm, analytical column operated at room temperature was used to determine PENG in veal calf liver tissues. The mobile phase consisted of ACN-0.05 M phosphate buffer with 0.0157 M $Na_2S_2O_3$ (57:25 v/v). PENV was used as internal standard (60 ng/g).[67]

A Luna C_{18} 25 cm × 4.6 mm, 5 μm, analytical column operated at room temperature was used for the determination of AMO, AMP, CLO, PENG, and PENV in milk using a mixture of 1% CH_3COOH in H_2O (A) and 1% CH_3COOH in MeOH (B) as mobile phase under a gradient program as follows: 80%A/20%B for 3 min, changed linearly to 50%A/50%B at 8 min, linear gradient to 10%A/90%B at 28 min, hold for 5 min for screening and an isocratic for quantitation: 30%A/70%B, for 15 min, AMO, is cratically: 65%A/35%B.[68]

A Merck-LiChrospher 100 RP-18, 250 mm × 4 mm, 5 μm, analytical column was used for the separation of PENG, AMP, OXA, AMO, DICLO, and NAF, in bovine milk with a mobile phase of H_2O and ACN acidified with 0.1% HCOOH. A gradient program starting from 100% H_2O to 100% ACN in 6 min and from 100% ACN to 100% H_2O from 6 to 12 min was applied (2 min equilibration between each chromatographic run). NAF was used as internal standard.[69,70]

PENG, AMO, AMP, OXA, and CLO were determined in meat using a C_{18} analytical column. The mobile phase consisted of ACN-phosphate buffer (pH 6) delivered isocratically.[71]

A Phenomenex Synergi Polar-RP phenylether column, 250 mm × 2 mm, 4 μm, 80Å and a guard column containing the same material were used to determine AMO, AMP, OXA, CLO, DICLO, PENG, PENV, and NAF in bovine muscle, kidney, and milk. The mobile phase consisted of 0.005% HCOOH in H_2O (A), MeOH (B) delivered under a gradient program t = 0–3 min: hold 100%A, t = 3–22 min: ramp linearly to 90%B, t = 22–37 min: hold 90%B, t = 37–52 min: 100%A.[72]

An Alltima (Alltech), C_{18}, 250 mm × 4.6 mm, 5 μm, analytical column with a 7.5 × 4.6 mm, 5 μm C_{18}, guard column was used for the determination of AMO and AMP in bovine muscle, liver, kidney, and milk. A gradient program was applied using MeOH (A), H_2O (B) both containing 10 mmol/L HCOOH. The gradient for milk and muscle extracts was: t_o, A = 25%, t_9, A = 60%, t_{10}, A = 80%, t_{20}, A = 100%, t_{25}, A = 100%, t_{27}, A = 0%, t_{35}, A = 0% and for kidney and liver extracts: t_o, A = 0%, t_5, A = 0%, t_6, A = 40%, t_{15}, A = 60%, t_{23}, A = 100%, t_{28}, A = 100%, t_{30}, A = 0%, t_{38}, A = 0%. PENV was used as internal standard.[73]

AMP, CLO, PENV, and PENG were determined in milk, kidney, and liver tissues using a C_{18} Higgins, 150 mm × 3.0 mm, 5 μm, column. The mobile phase consisted of 25 mM CH_3COOH in H_2O and CH_3OH (72:25).[74]

PENG, AMP, and NAF were determined in bovine and swine tissues using a TSK-Guardgel ODS-80 Ts, 15 mm × 3.2 mm, 5 μm, column. The mobile phase consisted of 0.05% HCOOH in distilled H_2O (A), 0.05% HCOOH in MeOH (B). Internal standards used were PENG-d5, AMP-d5, and NAF-d6, for PENG, AMP, and NAF, respectively.[75]

PENG, AMO, AMP, OXA, and CLO were determined in poultry muscle with a mobile phase which consisted of phosphate buffer-ACN (75:25, v/v).[76]

A Zorbax SB-C_{18} 2.1 × 3.0 mm, 3.5 μm, column operated at 30°C was used to determine PENG in bovine plasma, kidney, and urine, with a mobile phase consisting of 90%A (0.1% HCOOH in H_2O) and 10% ACN, 40–60 over the next 1.5 min, held for 2.0 min, back to initial over 1 min and held 4 min before the next injection. PHEN was used as internal standard.[77]

A Nucleosil 120 C_{18} 250 mm × 4.6 mm, 10 μm, column operated at 40°C column was used for the determination of AMO and its degradation products in granular premixes. The mobile phase consisted of A: buffer

solution (pH 3 ± 0.05) and B: MeOH delivered with a gradient program: 0–15 min. 98:2 (A:B), 20–25 min 70:30, 28–30 min 98:2.[78]

A typical chromatogram of penicillins separation in calf tissue is presented in Figure 2.[79]

Detection Techniques

As far as detection techniques are concerned, among the papers found in literature using HPLC for the determination of penicillin residues in food matrices, almost half of them apply direct UV detection, either using a PDA or a multi-wavelength UV-Vis detector at 200–225 nm.[38–41,46–49,51,55,57,58,60–63,65,78]

A significant number of methods use pre-column derivatization techniques. Derivatization of the antibiotic material is frequently used either to add a fluorogenic or chromogenic moiety to the antibiotic compound to enhance detection, specificity, and sensitivity;[80] however, derivatization procedures suffer from several limitations, such as being problematic when making measurements in complicated food matrices.

Derivatization for fluorescence detection is performed by reaction with TCA (20%, w/v) aqueous solution and HCHO (7%, w/v) $\lambda_{exc} = 346$ nm: $\lambda_{em} = 422$ nm.[54,61,76]

Derivatization for UV detection mainly uses benzoic anhydride to acylate aminopenicilllins and 1,2,4-triazole/imidazole and mercury(II) chloride in

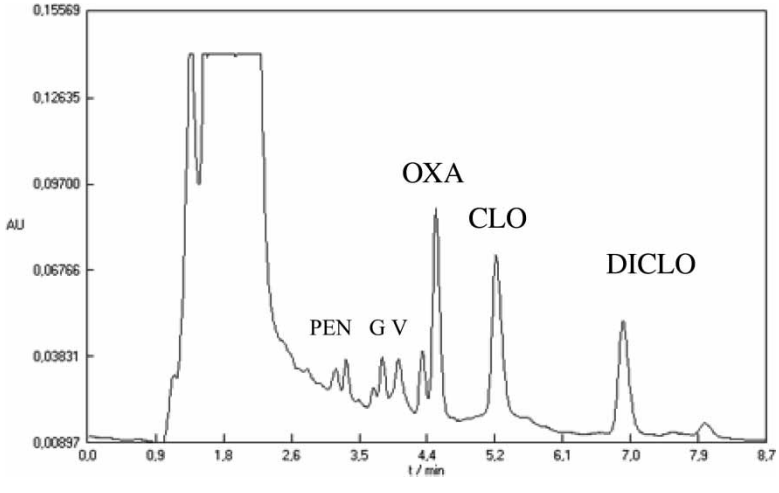

Figure 2. High performance liquid chromatogram of penicillins in calf tissue. Data from author's laboratory. Unpublished results.[79]

alkaline media (pH $\sim$ 9) at 60–65°C in order to form mercury mercaptide derivatives and, thus, achieve higher sensitivity. UV detection is performed at 320–345 nm.[48,50,52,53,56,59,66,67,71,81]

Electrochemical detection, to the best of our knowledge, was applied only in one paper, at +0.65 V vs. Ag-AgCl.[49]

Mass spectrometry is widely used for confirmation analysis. LC-MS techniques and LC-MS-MS have been applied by many researchers. These are further discussed in the next section.[40,41,44,47,64,68,69,72,74,75,77]

Liquid Chromatography Coupled with Tandem Mass Spectrometry

For unambiguous identification of antibiotic residues in animal food products, public agencies in many countries rely on detection by mass spectrometry. Liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) provides an analytical methodology for simultaneous confirmation and quantification of β-lactam drug residues. Several LC-MS methods were published in recent years for milk and animal derived edible tissues. The electrospray source appears to have become the most popular mode of ionization. However, the reported choice of ionization mode, either positive or negative, appears to be dependent upon the mobile phase composition.[15]

β-Lactam antibiotics could be detected as protonated molecular ions $[M+H]^+$ using the positive electrospray ionisation mode (ESI (+)). The negative ion spectra are lower in intensity, exhibiting an $[M-H]^-$ ion and producing less fragmentation at higher CAD voltages, as compared to positive ion spectra. The negative electrospray ionisation mode (ESI (−)) was found to be the most sensitive electrospray condition for the analysis of the monobasic penicillins (penicillin G, penicillin V, oxacillin, cloxacillin, dicloxacillin, and nafcillin).

The mechanism of fragmentation in the negative mode differs, in part, from the positive mode, which is the cleavage of the β-lactam ring. The latter seems to be the most important mechanism of fragmentation of penicillins that gives its origin to the fragment ion m/z 160. This can be considered as a group specific fragment ion, because the R- group in the penicillins in most cases is -OH. Its complementary $[C_6H_9HSO_2+ H]^+$ $[M+H\text{-}159]^+$ fragment is compound specific. Other ions are due to the loss of small lateral groups from the entire molecule or from the two above mentioned fragment ions. Fragments formed by the cleavage of the amide moiety are more specific for the different penicillins. Penicillin G formed a $[C_6H_5CH_2]^+$ ion at m/z 91, ampicillin exhibited a $[C_6H_5CHNH_2]^+$ ion at m/z 106 as the base peak, amoxicillin showed a loss of NH_3 at m/z 349, cloxacillin, which contains one chlorine, showed two fragments at m/z 321 and 178, assignable to its amide moiety. Several ions with lower m/z ratios had their origin from a successive cleavage of the amide groups.[70]

The positive ion fragmentation pattern and the major negative ion fragmentation pattern are presented in Figure 3. The molecular and fragment ions of the investigated analytes and other information on MS detection of penicillins are reported in Table 4.[69,70,82]

Sample Preparation

Various extraction and purification schemes have been applied to the determination of penicillins in food matrices, yielding various recovery rates. Detailed information is provided in Table 3.

PENG was extracted from cattle liver, kidney, and muscle after deproteinization by 5% Na_2WO_4 and 0.33 N H_2SO_4. After centrifugation, the supernatant was transferred onto a basic Al_2O_3 column, and washed with water. Sep-Pak C_{18} SPE cartridges were used for further clean up and analytes were eluted with H_2O, yielding 75.0–92.6% recoveries.[38]

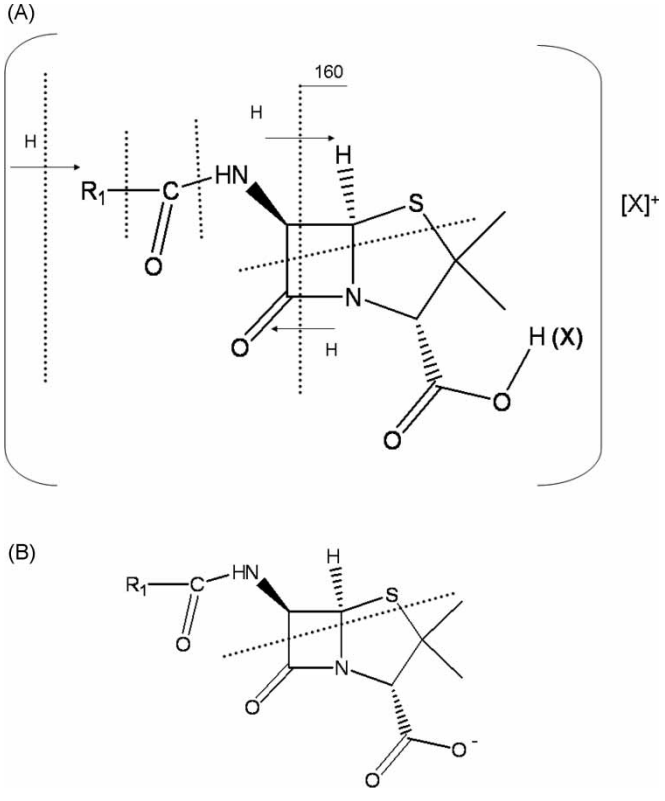

Figure 3. (A) Positive ion fragmentation pattern. (B) The major negative ion fragmentation pattern.[82]

Table 4. Mass spectrometric conditions for the determination of penicillins in food samples

Analyte and matrix	MS conditions
PEN G in Bovine milk[40]	Source Temp.: 270°C, vaporizer Temp.: 105°C, operating: in pulsed positive-ion/negative-ion detection mode under full scan conditions (for initial acquisition of the spectra for PEN G), for confirmation of PEN G in milk: positive ion mode, monitoring m/z: 309 & 335 each for 0.5 s.
CLO, AMP/HETA, AMO in Bovine milk[41]	Source Temp.: 320°C, vaporizer Temp.: 120°C, detection: positive/negative ion pulsd mode under full scan conditions (150–550 daltons in 2 s) (for initial acquisition of the spectra for PENs), for confirmation of CLO in milk: positive ion mode monitoring m/z: 160, 277, 410, confirmation of AMP in milk: positive ion mode monitoring m/z: 324, 350, confirmation of AMO in milk: positive ion mode monitoring m/z: 207, 366 (with each ion being monitored for 200 ms).
AMO, AMP in Broiler tissues (kidney, liver,muscle, fat &skin + fat)[44]	Positive ion MS/MS mode, tune parameters for AMO & AMP: capillary: 4.00 kV, cone: 25 V, source Temp.: 120°C, desolvation Temp.: 250°C, cone gas flow: ±40 L/h, desolvation gas flow: ±500 L/h, resolution (LM1, HM1, LM2, HM2): 15.0, ion energy: 1, 1.0, ion energy: 2, 2.0, entrance: −1, exit: 1, multiplier: 650 V, collision gas: argon (Pirani pressure: $\pm 2.3 \times 10^{-3}$ mbar), dwell time: 0.5 s, optimal settings for collision energy: 14 eV (for AMO), 17 eV (for AMP), monitoring mode: multiple reaction monitoring (MRM) mode, transitions: m/z = 365.9 > 208.1 (quantification trace) m/z = 365.9 > 160.2 (identification trace) (for AMO), m/z = 349.9 > 106.3 (quantification trace) (for AMP).
PEN G, AMP, AMO, CLO, in Bovine milk[47]	FI-ESP-MS: needle voltage: 3.4–3.8 kV, positive ion operation with skimmer (S) & Lens (L) voltages of $S_1 = 24$ V, $S_2 = 17$ V, $L_1 = -22$ V, $L_2 = -4$ V, $L_3 = -58$ V, potential difference (between skimmer & the end of the capillary): 40–240 V, (m/z): 50–500, step size: 0.1 units, rate: 0.33 scans/s. HP-ESP-MS: needle cylindrical electrode (V_1): 2.7 kV, end plate (V_2): 2.7 kV, capillary (V_3): 2.8 kV, positive ion operation: required reversed polarity& V_{1-3} operated about 1 kV higher than in negative ion operation, best results for negative ions with: $S_1 = -40$ V, $S_2 = -17$ V, $L_1 = 38$ V, $L_2 = 83$ V, $L_3 = 37$ V, positive ion operation: required reversed polarity & only slight adj. of L_1, L_2, L_3, CAD voltage: $-40 - (-)400$ V (40–400 V), (m/z): 40–500, step size: 0.1 units, rate: 0.5 scans/s, threshold value: 250, checking: by anal. adenosine-5′-monophosphate in IPA-H_2O (1:1) for negative ion & arginine/gramicidin S in MeOH- H_2O (1:1) for positive ion operation.
PEN G, PEN V, OXA, CLO, NAF, DICLO in Bovine muscle, liver & kidney[64]	LC-ESI-MS-MS: Desolvation gas (N_2) temp.: 400°C, FR.: 370 L/h, ion source Temp.: 100°C, ion mode: negative, rate of full scan data collection (150–500 m/z): 0.17 scans/s,collision gas: Ar collision gas pres.: 1.9×10^{-3} mbar, position of Z-spray probe optimization: using 10 μg/mL sol. of PEN G in A at FR.: 200.
AMP Muscle tissue[66]	Mode: positive ion mode with electrospray interface, capillary temp.: 200°C, spray voltage: 5 kV, sheath gas pressure: 80 psi, auxiliary gas: 2.5 psi, monitoring ions for AMP: m/z 350, 351, 372, 373, 382. For IS cephalexin: m/z 348.

AMO, AMP, CLO, PEN G, PEN V, in Milk[68]

Ion ESI mode: positive

Analyte	Precursor ion (amu)	Relative collision energy (%)	Isolation window (amu)	Scan range (amu)	Product ions used for quantitation (amu)
(13.5–16.5 min, tune ion m/z 350) AMP (22.0–33.0 min, tune ion m/z 457.9)	350.0	22.5	2.5	95–500	160.0, 190.9, 332.9
PENG	357.0	26	2.0	95–500	181.0, 198.0, 229.4
PENV	373.0	25	1.5	100–500	182.0, 214.0, 231.3
CLO	457.9	27	1.5	125–500	182.0, 299.0, 330.3

PEN G, AMP, OXA, AMO, DICLO, NAF, in Bovine milk[69]

Dwell time: 0.150 s (for each transition), nitrogen collision gas: 2, channel electron multiplier: 2300 V

Molecule	Transition	Declustering potential (V)	Focusing potential (V)	Entrance potential (V)	Interquad lens 1 (V)	Stubbies (V)	Collision cell entrance potential (V)	Collision energy (eV)	Collision cell exit potential (V)
PENG	335/160	32	310	−9	−10	−23	10	32	12
AMP	350/106	21	320	−8	−10	−18	12	25	10
OXA	402/160	20	300	−6	−7	−23	16	17	10
AMO	366/114	12	300	−6	−8	−17	15	28	10
NAF	414/199	16	300	−8	−10	−20	12	20	14
DICLO	470/160	17	310	−10	−11	−29	20	20	12

PEN G, AMP, OX, AMO, NAF, DICLO, in Bovine milk[70]

Electrospray ionization tandem mass spectrometry). Ion mode: negative (sensitivities 6 to 10 times lower than positive ion mode)

Molecule	Molecular ion $[M+H]^+$	Fragment ion	Fragment ion	Fragment ion	Fragment ion
PENG	335	160	289	176	
AMP	350	106	160	174	192
OXA	402	160	243		
AMO	366	114	160	208	349
NAF	415	199	300	171	256
DICLO	470	160	311		

(*continued*)

Table 4. Continued

Analyte and matrix	MS conditions
AMO, AMP, OXA, CLO, DICLO, PENG, PENV, NAF, in Bovine muscle, kidney and milk[72]	First operating in positive (ESI (+)), then in negative (ESI (−)) (for max.sensitivity), heated capillary Temp.: 330°C, needle voltage: 5 kV, sheat gas: N_2, N_2 pressure: 70 psi, collision gas: Ar, Ar pressure: 2.5 mTorr, half height mass peak width: 0.7 Da (for both quadrupoles (Q_1 & Q_2)
AMO, AMP in Bovine muscle, liver, kidney, milk[73]	Source: turbo ion spray (voltage: 5000 V) source (probe temp.: 300 °C), PI mode, drying (set at 8 L/min), curtain (setting: 20 psi), collision gas (pressure: 30 mTorr): N_2, nebulizer gas: high-purity air (setting: 30 psi), mass axis calibration: by infusion of propylene glycol solution (at 10 μL/min), (unit mass resolution established & maintained in each mass resolving quadrapole by keeping a full width at half-maximum of ∼0.7 amu), optimization: by standard solutions of 50 pg/mL, infusion: at 5 μL/min, quantitation: by selected reaction monitoring (SRM), 2 fragmentation reactions/analyte, providing inj. Vol.: <25 μL (for tissues), <50 μL (for milk).

Compound	SRM transition m/z	Declustering potential V	Collision potential V	Retention window min A[a]	Retention window min B[b]	Dwell time ms
AMO	366 → 114	20	−40	0–9	0–12	400
	366 → 208		−28			400
AMP	350 → 106	25	−35	9–14	12–18	400
	350 → 192		−25			400
PENV	351 → 160	25	−30	14–20	18–23	600

[a]Milk and muscle.
[b]Kidney and liver.

AMP, CLO, PEN V, PENG in Milk, kidney, liver tissues[74]	Positive ion electrospray mode

PENG, AMP, NAF in Bovine, swine tissues[75]

Desolvation gas: N_2, desolvation gas Temp.: 200°C, desolvation gas flow: 370 L/h, ion source Temp.: 100°C, ion mode: positive (for tetracyclines), negative (for PENs) (more abundant ions were observed for tetracyclines in positive mode & for PENs in negative mode), collision gas: Ar, collision gas pressure: 1.9×10^{-3}

Antibiotics	Precursor ion (m/z)	Cone voltage (V)	Collision energy (eV)	Monitor ion (m/z)	Retention time window (min)
PENG	333 $[M^+H]^-$	20	12	192 $[M\text{-}H\text{-}141]^-$	4.20–5.10
PENG-d5	338 $[M^+H]^-$	20	12	197 $[M\text{-}H\text{-}141]^-$	4.20–5.10
AMP	348 $[M^+H]^-$	20	13	207 $[M\text{-}H\text{-}141]^-$	3.30–4.10
AMP-d5	353 $[M^+H]^-$	20	13	212 $[M\text{-}H\text{-}141]^-$	3.30–4.10
NAF	413 $[M^+H]^-$	20	10	272 $[M\text{-}H\text{-}141]^-$	4.20–5.10
NAF-d6	419 $[M^+H]^-$	20	10	278 $[M\text{-}H\text{-}141]^-$	4.20–5.10

PEN G in Bovine plasma, kidney, urine[77]	Electrospray source: in positive ion mode, tune: optimized in positive MS/MS mode (for the 3 SRM transitions), PENG, m/z: 335 → 160 & m/z: 335 → 176, PHEN, m/z: 365 → 160, optimization: by infusing 1 μg/mL solution into mobile phase of 50:50 MeCN:0.1% aqueous HCOOH at flow rate of 0.3 mL/min, low mass resolution: set in MS1&MS2 (LM = 12), argon collision cell pressure: 3.2 m Torr (equilibration & stabilizing:2 injection of control samples at the beginning of each run).Tuning conditions: ESI capillary: 3 kV, cone: 15 V, extractor: 3 V, Rf lens: 0.4 V, source Temp.: 120°C, desolvation Temp.: 400°C, desolvation gas flow: 700 L/h, cone gas flow: 10 L/h, entrance lens-5, exit lens-1, collision energy: 14 V, acquisition dwell time: 100 ms/transition

PENG, PENV, and AMP were extracted from milk on Sep-Pak C_{18} SPE cartridges after elution with 15% (v/v) MeOH. High recoveries, within the range from 87.0 to 101.1%, were obtained.[39]

PENG was determined in bovine milk after deproteinization with ACN-MeOH-H_2O (40:20:40) and centrifugation, yielding 81.9% recovery.[40]

PENG was extracted from milk, after deproteinization, with ACN and LLE with CH_2Cl_2, C_6H_{14} or light petroleum. The organic layer was further cleaned up, on line, giving a recovery of 92 $\pm$ 9%.[42]

CLO, AMP/HETA, and AMO were extracted from bovine milk after dilution of milk samples with ACN-MeOH-H_2O (40:20:40, v/v), centrifugation, and ultrafiltration. Recoveries obtained were within the range 66.0–87.2% for AMP, 79.2–95.9% for CLO, and 77.0–81.5% for AMO.[41]

PENG, PENV, and CLO were extracted from beef and pork tissues after LLE with CH_2Cl_2, CAN, and hexane. The combined organic layers were washed with H_2O and extracted with 0.01 M phosphate buffer, providing 71–101% recoveries for PENG, 67–118% for PENV, and 69–107% for CLO.[43]

AMO was extracted from broiler tissues (kidney, liver, muscle, fat and skin, or fat) after treatment with 0.01M KH_2PO_4 and ultrafiltration. After addition of 100 mM pentafluoropropionic acid, further purification was performed by SPE on C_{18} cartridges and elution with 2% NH_3 solution in MeOH.[44]

PENG was extracted from Chinook salmon by ACN and purified by SPE using C_{18} column. Elution with ACN provided 63.9–65.4% recovery.[45]

PENG, AMP, AMO, and CLO were extracted from bovine milk after dilution with ACN-H_2O, filtration, and centrifugation with almost quantitative recovery.[47]

PENG was extracted from Chinook salmon by ACN and purified by SPE on a C_{18} column and elution with ACN.[46]

PENG was determined in milk after decreaming by centrifugation at 5°C and deproteinization using H_2SO_4/Na_2WO_4. Further clean up by SPE on C_{18} cartridges provided a 70–73% recovery.[48]

OXA, PENG, PENV, CLO, and DICLO were extracted from bovine muscle. After addition of ACN and 0.5 M phosphate buffer and centrifugation, LLE was performed with CH_2Cl_2 and light petroleum; the aqueous layer was further purified on SPE.[49]

AMO, PENG, AMP, OXA, CLO, and DICLO were extracted from cows' raw defatted milk after deproteinization with 0.17 M H_2SO_4 and 5% Na_2WO_4. After centrifugation, the supernatant was passed through C_{18} SPE cartridges and analytes were eluted by ACN. High recoveries were obtained: 95–102% for AMO, PENG, AMP, and OXA; 92–98% for CLO; and 87–94% for DICLO.[50]

CLO was extracted from milk after deproteinization with 0.1 M HCl and ACN. After centrifugation, LLE was performed with CH_2Cl_2 and $CHCl_3$. Recovery ranged from 75 to 84%.[51]

OXA, CLO, and DICLO were extracted from cow's milk, meat, and cheese. Milk samples, after deproteinization with 5% Na_2WO_4, 0.2 mol/L

H_2SO_4, were purified by SPE. Elution with 0.0025 mol/L phosphate buffer (pH 6.5) and ACN (10:90) yielded 75–91% recovery.[52]

AMO, PENG, AMP, OXA, CLO, NAF, and DICLO were determined in cattle and pig muscle, liver, and kidney tissues by extraction in aqueous solution, after precipitation of organic materials with H_2SO_4 and Na_2WO_4. SPE on divinylbenzene-co-N-vinylpyrrolidone polymeric sorbent was applied for clean up and LLE for further clean up with diethyl ether, providing 65–90% recovery.[53]

AMP was extracted from beef, pork, chicken, and catfish muscle tissues after deproteinization of the homogenized sample by 0.01 M Na_3PO_4 (buffer pH 4.5) and TCA (75% w/v) in water, providing recoveries 87.7–95.4%.[54]

PENG was extracted from ovine milk after deproteinization with ACN. LLE with CH_2Cl_2 provided 78.6–85.8% recovery.[55]

OXA, CLO, and DICLO were extracted from milk after deproteinization by 1 mol/L H_2SO_4. After centrifugation at 0–5°C, cleanup was performed on a C_{18} column. Elution with H_2O-ACN (60:40) yielded 79.3–83.7% recovery for OXA, 76.4–78.3% for CLO, and 64.6–67.6% for DICLO.[56]

AMP, PENG, CLO, DICLO, and NAF were extracted from milk after deproteinization with ACN. Clean up was performed by SPE on Baker-10 C_{18} cartridges, with which analytes are eluted with MeOH, providing 79.8–89.4% recovery.[57]

PENG was extracted from cattle, pig, and chicken tissues (muscle, kidney, and liver), after deproteinization, by ultrafiltration. The recovery obtained was 79.9–90.3% from muscle, 80.2–89.5% from kidney. and 78.8–88.5% from liver.[58]

PENG was extracted from milk after extraction and deproteinization by EtOH, and ultrafiltration. The recovery ranged from 86.4 to 91.8%.[59]

PENG was extracted from beef and pork tissues, after deproteinization with 0.1 M Et_4NCl and ACN or β-lactamase. Extraction was performed with tert-butyl alcohol and water, and further cleanup after addition of 0.01 M Na_2HPO_4 (0.2 mL) yielded 89–114% recovery from pork muscle, 82–95% from pork liver, 77–104% from pork kidney, 78–88% from calf muscle, 47–77% from calf liver, and 66–77% from calf kidney,[60]

AMO, AMP, CLO, and PENG were extracted from milk and tissues, after deproteinization, by Et_4NCl, CAN, as above.[61]

PENG, PENV, OXA, CLO, NAF, and DICLO were extracted from pork and beef muscle by H_2O or 2% NaCl, respectively. Purification of the crude extract was performed by SPE on Bond Elut C_{18} cartridges and elution with 55% MeOH. Eluate from the Bond Elut C_{18} was further purified by cleanup using a Sep-Pak Accell Plus QMA cartridge, where elution was performed with the HPLC mobile phase. The recovery was higher than 70% for all analytes in both matrices.[62]

PENG, PENV, OXA, CLO, NAF, and DICLO were extracted from bovine liver and kidney after deproteinization with 2% NaCl, 5% Na_2WO_4,

and 0.17 M H_2SO_4. Purification of the crude extract was performed using a pre-cleanup cartridge (Bond Elut C_{18}), where elution was performed by 55% MeOH. Further purification of eluate was performed with a Sep-Pak Accell Plus QMA cartridge and elution followed the procedure described as in Reference.[62] Recovery rates from 73–92% were obtained.[63]

PENG, PENV, OXA, CLO, NAF, and DICLO were extracted from bovine muscle, liver, and kidney, as described in References,[62,63] except for using ACN-H_2O (30:70, v/v) containing 50 mM DBAA as elution solvent from a Sep-Pak Accell Plus QMA cartridge. Recovery was higher than 95%.[64]

DICLO was extracted from bovine and ovine milk after purification on SPE-C_{18} (500 mg-Baker). Elution with MeOH yielded high recovery in both milk samples (83–85%).[65]

AMP was determined in muscle tissue after homogenization extraction with phosphate buffer or ammonium acetate buffer, when LC-MS/MS was applied for analysis. Clean up on a Bond-Elut C_{18} SPE cartridge yielded 75 $\pm$ 7% mean recovery.[66]

PENG was extracted from calf liver tissues after treatment with 2% NaCl. The tissue extracts' cleanups were performed on C_{18} Sep-Pak, where elution was performed by 60% ACN-35% H_2O-5% 0.2 M phosphate buffer, providing recovery rates of 65–85%.[67]

AMO, AMP, CLO, PENG, and PENV were extracted from milk after deproteinization by ACN. SPE clean up, where elution was performed with 40% ACN in H_2O, provided 85–115% recovery rates.[68]

PENG, AMP, OXA, AMO, DICLO, and NAF were extracted from bovine milk after addition of 10% CH_3COOH and centrifugation at 4°C. Filtration of upper fat layer (0.50 μm nylon filter, 13 mm diameter) provided moderate to low recovery rates 28–78%.[69,70]

PENG, AMO, AMP, OXA, and CLO were extracted from meat after deproteinization with phosphate buffer (pH 8.5) and H_2SO_4. Cleanup was performed on a C_{18} SPE column, providing 70% recovery.[71]

AMO, AMP, OXA, CLO, DICLO, PENG, PENV, and NAF were extracted from bovine muscle, kidney, and milk. Defatted milk was deproteinized by ACN and cleaned up by SPE. Tissue samples were treated with H_2O, CAN, and NaCl solution. Enzymatic digestion of PENG in a positive kidney sample was performed with 0.05 M phosphate buffer (pH 8.5) and incubation for 2 h, at 55°C. Further cleanup was performed by SPE. Elution with ACN-H_2O (1:1, v/v) yielded high recovery rates, except for NAF (38.1%) and AMO (57%), in milk and AMO (46%) in kidney.[72]

AMO and AMP were extracted from bovine muscle, liver, kidney, and milk. Milk samples or finely diced tissue samples were mixed with sand and packed into extraction cells, heated in an oven (at 65°C, 5 min) and, after addition of H_2O, adjusting the pH to 4.6 with 3 mol/L HCOOH, samples were filtered. Recovery obtained was almost 90% in kidney, 92–95% in milk and muscle, and 74% in liver.[73]

AMP, CLO, PENV, and PENG were extracted from milk, kidney, and liver tissues, after treatment with H_2O and 0.1 M Et_4NCl, 0.005 M KH_2PO_4, ACN, 0.001 M pH 6 KH_2PO_4-Na_2HPO_4 and tert-butanol (5 mL). After filtration, recovery rates obtained were: 73–85% for PENG in kidney and 70–80% in liver; 55–80% for PENV in kidney and 60–75% in liver; 50–68% for AMP in kidney and 50–65% in liver; and 50–63% for CLO in kidney and 47–58% in liver.[74]

PENG, AMP, and NAF were extracted from bovine and swine tissues with water; samples were cleaned up by ultrafiltration, providing 94.4–106.6% recovery.[75]

PENG, AMO, AMP, OXA and CLO were extracted from poultry muscle after protein precipitation with 30% TCA (1 mL) and LLE with diethyl ether.[76]

PENG was extracted from bovine plasma, kidney, and urine after treatment with ACN. Cleanup by SPE through Bond-Elut C_{18} with elution by ACN yielded almost quantitative recovery.[77]

Degradation products of AMO were extracted, with high recovery rates, from granular premixes after extraction with petroleum ether.[78]

PENG, CLO, OXA, DICLO, AMP, and NAF were determined in milk and yoghurt after centrifugation and ultrafiltration of the milk samples, and cleanup with on-line SPE using restricted-access sorbent (alkyl-diol-silica, ADS).[81]

CONCLUSIONS

Antibiotics are vital drugs in veterinary medicine. Some of them are used in human medicine as well. Therefore, an increasing concern has been expressed regarding the potential impact of drug residues in animal derived food on consumers' health. Studies have shown that antibiotics used in food-producing animals at low, subtherapeutic levels improve growth rate and efficiency of feed use, reduce mortality and morbidity, and improve reproductive performance. To ensure that residual drugs have no harmful effects on human health if ingested, tolerance limits representing the maximal level or concentration of antimicrobial residues permitted in animal tissues at the time of slaughter, have been established by regulatory agencies. For the monitoring of compliance with legal tolerances, rapid, accurate, and sensitive methods, free from interference, are required.

Penicillins still form the most important group of antibiotics in veterinary medicine. However unauthorised or even approved antibiotics or the failure to follow legislation directives for approved antibiotics could result in unsafe residues in food products of animal origin. Therefore, the monitoring of these compounds is obviously of great importance for human health protection.

Various methods of analysis of penicillins have been reported, with the majority of them dealing mainly with one or more compounds in a single matrix. Liquid chromatography has become the analytical method of choice for the identification and quantification of antibiotic residues in food. Recent advances in LC and LC-MS/MS analysis of penicillin residues in food products of animal origin have been presented in this review, focusing on detection, confirmation, and sample preparation.

ABBREVIATIONS

Acid.	Acidified
Add.	Addition
Adj.	Adjusted
ADP	Amoxycillin diketopiperazine
AMO	Amoxicillin
AMP	Ampicillin
Anal.	Analysis
6-APA	6-Aminopenicillanic acid
Aq.	Aqueous
Buff.	Buffer Solution
Centr.	Centrifugation
CLO	Cloxacillin
Col.	Column
Conc.	Concentrated/concentration
Cond.	Conditions/conditioned
CTMAC	Cetyltrimethyl-ammonium chloride
DAD	Diode array detector
DBBA	Di-n-butylamine acetate
Deprot.	Deproteinization
Deriv.	Derivatization
Det.	Detection
DICLO	Dicloxacillin
Dil.	Dilution
Diss.	Dissolution
Dist.	Distilled
El.	Eluent/elution
Electr.	Electrochemical
Em.	Emission
ESI	Electrospray Ionization
Equil.	Equilibration
EU	European Union
Evap.	Evaporation to dryness

Exc.	Excitation
Extr.	Extraction
Filt.	Filtration
FL	Fluorescence
FLU	Flucloxacillin
FR	Flow Rate (mL/min)
Grad. El.	Gradient Elution
HDPG	4-Hydroxy-D-phenylglycine
HETA	Hetacillin
Homog.	Homogenization/homogeneous
HSA	Heptane Sulphonic Acid
Inj.	Injection
IS	Internal Standard
Is. El.	Isocratic Elution
Lin. Gr.	Linear Gradient
LLE	Liquid Liquid Extraction
LR	Linear Range
MIP	Molecularly Imprinted Polymers
Mix.	Mixing
Mixt.	Mixture
MP	Mobile Phase
MRL	Maximum Residue Limit
NAF	Nafcillin
OXA	Oxacillin
PA	Penilloic acids
PCA	Penicilloic acids
PDA	Photodiode array detector
PENG	Penicillin G
PENV	Penicillin V
PHEN	Phenethicillin
PFPA	Pentafluoropropionic acid
Phos.	Phosphate
PSDVB	Polystyrene-divinylbenzene
Precip.	Precipitation
Reconst.	Reconstitution
SDS	Sodium dodecylsulphonate
SOS	Sodium octanesulphonate
SHS	Sodium 1-heptansulphonate
Sol.	Solution
Sonic.	Sonication
TCA	Trichloroacetic acid
TEA	Triethylamine
Ultrafilt.	Ultrafiltration
UDP	Unknown degradation products

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