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Review

Analysis of macrolide antibiotics by liquid chromatography

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Abstract

A compilation of the most representative single and multianalyte HPLC methods for the analysis of macrolide antibiotics published during the last two decades is presented in this paper. Its scope is the coverage of two main areas which require the determination of macrolide antibiotics at a low concentration level, i.e. pharmacokinetic studies and residue analysis. Both of these areas involve the treatment of biological and foodstuff matrices, respectively. A detailed explanation of the different sample preparation procedures as well as the experimental conditions and the main analytical features are provided for each referred method in order to allow the reader to select the most suitable conditions for their particular purpose.
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Keywords: Macrolide antibiotics; HPLC; Biological samples; Foodstuff

Contents

1. Introduction ... 377
2. Biological matrices ... 377
   2.1. Single analyte HPLC methods ... 377
      2.1.1. Spectrophotometric (UV, DAD) and fluorescence based methods ... 377
      2.1.2. Electrochemical methods (amperometric and coulometric) ... 379
      2.1.3. Mass spectrometry methods ... 381
   2.2. Multiresidue methods ... 381
      2.2.1. Electrochemical methods (amperometric and coulometric) ... 381
      2.2.2. Mass spectrometry methods ... 385
3. Food matrices ... 385
   3.1. Single analyte methods ... 388
      3.1.1. Spectrophotometric methods (UV, DAD) and fluorescence based methods ... 388
      3.1.2. Electrochemical methods (amperometric and coulometric) ... 389
      3.1.3. Mass spectrometry methods ... 389
   3.2. Multiresidue methods ... 390
      3.2.1. Spectrophotometric (UV, DAD) and fluorescence based methods ... 390
      3.2.2. Mass spectrometry methods ... 392
4. Conclusion ... 397
   Acknowledgement ... 397
   References ... 397

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1. Introduction

Macrolide antibiotics constitute a very important class of antibacterial compounds highly active against Gram-positive and Gram-negative cocci. Moreover, they are the most efficient medicine against diseases produced by *Mycoplasma species* [1].

Erythromycin, isolated from *Streptomyces erythraeus* in 1952, is considered as the most representative member of macrolides and the first used in medicine. Since then the interest for this family of antibiotics has increased as they constitute wide spectrum antibiotics that have been demonstrated to be also active against non-classical pathogens such as *Helicobacter pylori* (*HP*) and therapeutically efficient in *HP*-induced peptic ulcer disease. Moreover, this family of antibiotics arose as an interesting alternative for patients showing penicillin sensitivity or allergy. Therefore, they are administered for the treatment of a wide range of infections not only in human but also in animal medicine.

The macrolide antibiotics commonly used for these purposes are characterised by a common chemical structure including a macrocyclic lactone ring usually bearing from 12 to 16 atoms with one or more deoxy neutral and/or aminosugars linked via glycosidic bonds. Traditionally, the analyses of macrolides were carried out by microbiological assays [2] but these methods often lack the specificity and/or the sensitivity required for analytical purposes. Chemical analyses such as high performance liquid chromatography (HPLC) have therefore become the preferred technique for attempting to determine this family of antibiotics at low concentration levels.

Several reviews have already been published on the general analysis of antibiotics. However, these papers covered a large number of antibiotic families [3–7] and/or several chemical and microbiological techniques [8]. The aim of the present review is to focus on a specific family, namely macrolide antibiotics, in specific matrices when using liquid chromatography as an analysis technique and consequently to provide a detailed overview of the different experimental conditions used by different research teams.

This paper, therefore, compiles a selection of liquid chromatography methods published during the past decades, classifying them by detection mode. The first part of this paper is focussed on analytical methods developed for carrying out pharmacokinetic studies whereas the second part deals with analytical methods developed for residue analysis in foodstuffs. In both cases a clear distinction between single and multianalyte methods is presented.

2. Biological matrices

Overall, the development of analytical methods for the determination of macrolides in biological fluids is meant to perform pharmacokinetic studies as well as for therapeutic drug monitoring and assessment. These methods can allow the determination of the concentration of the target macrolide and/or their metabolites in different body fluids after its oral or intravenous administration. Therefore, the target matrices in these studies are, in most cases, urine and blood. In this frame most of the methods found in the literature are described for the determination of a single parent antibiotic.

2.1. Single analyte HPLC methods

2.1.1. Spectrophotometric (UV, DAD) and fluorescence based methods

Some members of this family of compounds have been determined in biological samples by using spectrophotometric detectors. In an early work, Lin et al. stated the lack of sensitivity often showed by a microbiological assay for detecting the levels of rosamicin in plasma and serum and proposed an alternative HPLC-UV method for determining rosamicin in human serum after oral administration of the antibiotic. The sample preparation involved a two-step liquid–liquid extraction (LLE) under alkaline conditions using diethyl ether, without further clean-up. The recovery of the target macrolide in serum samples was estimated to be around 86%. Separation was carried out in isocratic mode at ambient temperature on a C18 column. The detection was performed at 254 nm and the quantification was carried out using desepoxyrosamicin as the internal standard. The sensitivity of the method was estimated to be about 10 ng ml^{-1} and attributed to the presence of a strong chromophore group, a α,β-unsaturated ketone, in the rosamicin molecule. The method was applied to monitor the serum samples of volunteers after oral administration of two 250 mg doses of the antibiotic and the results obtained were compared with the ones obtained using a microbiological method. At higher concentration levels the results obtained by using both methods were in agreement whereas discrepancies appeared for the low concentration range. These differences were interpreted by the author as being a consequence of the higher sensitivity of the HPLC-UV method compared to the traditional microbiological ones [9].

Some years later an automated method based on a column switching system was used by Räder et al. for the determination of josamycin in plasma. This system allowed the direct injection of the sample. In this case the clean-up was carried out on-line by loading the non pre-treated sample onto a C18 column and performing a washing step with water in order to elute the hydrophilic compounds with the josamycin being retained on the clean-up column from where it was removed by backflushing and injected onto a cyano analytical column. The separation was carried out in isocratic mode using a mobile phase consisting of a mixture of aqueous sodium acetate solution and acetonitrile. The use of two clean-up columns provided the possibility of backflushing the josamycin from one column while the other column was being reconditioned for the following sample. Josamycin was determined in human plasma with a stated recovery of 100%. The method described reached a detection limit of 25 ng ml^{-1} [10].

Later on Skinner et al. described a HPLC assay for the analysis of josamycin in human serum and urine using oleandomycin as the internal standard. The sample preparation involved an initial deproteinisation process with acetonitrile and a further clean-up through a C18 extraction column followed by a preconcentration step leading to recoveries higher than 70 and 76% in serum and urine, respectively. The chromatographic separation of josamycin was carried out on a C18 column at 35 °C in isocratic mode with a mixture of acetonitrile and phosphate buffer as mobile phase and detection at two different wavelengths for josamycin and the internal standard (231 and 204 nm, respectively). The method reached detection limit of 25 ng ml^{-1} for both matrices [11].

The use of this type of detector is also suitable for the determination of spiramycin. In 1985, Dow et al. reported for the first time a fully automated HPLC method for the determination of this macrolide in plasma. After dilution with acetonitrile the sample was directly injected onto a clean-up precolumn. Following the clean-up procedure the sample was backflushed into the chromatographic column where the separation was performed in isocratic mode using a mixture of acetonitrile and diluted perchloric acid as mobile phase. The authors stated recoveries ranging between 71 and 96% in spiked samples over the concentration range tested. They performed a direct determination of spiramycin 1 based on peak heights and using spiramycin 2 as internal standard in human plasma. The measurements were carried out using a UV detector at 230 nm. The proposed method allowed the quantification of 50 ng ml^{-1} with a coefficient of variation of 8.4%. In contradiction to other works dealing with column-switching techniques [10], the system proposed by Dow et al. was such that both the clean-up column and the analytical column were washed and re-equilibrated in parallel avoiding the need of a second clean-up column. The proposed method was applied to human plasma after intravenous administration of 500 mg of spiramycin.

However, among the macrolide antibiotics a relatively wide number of them lack a suitable chromophore group which makes their specific and sensitive detection by UV detection difficult. Hence, macrolides such as erythromycin and its semisynthetic derivatives such as clarithromycin or roxithromycin do not appear to be suitable for residue determination by using UV detection. Nevertheless, it is possible to find among the literature some papers in which UV detection was successfully applied for the determination of these macrolides in biological samples. For instance ion-pair chromatography coupled to a UV detector set at 210 nm was demonstrated to be suitable for the determination of clarithromycin in aqueous solutions and gastric juice. The use of ion-pair agents as well as increased the temperature improved the efficiency, displayed by peak sharpening. Therefore, a mobile phase composed of a mixture of acetonitrile and aqueous 0.05 M phosphate buffer pH 4.6 containing 5 mM OCTS (1-octanesulphonic acid) (50:50) at 50 °C gave the best performance related to peak symmetry, detection limits and interfering peaks from the matrices. Several sample preparation procedures were tested including different LLEs and one filtration method. The latter was selected by the authors as it was the simplest one and gave as good results as the ones obtained with more complex methods. Detection limits of 400 and 780 ng ml^{-1} were reached for clarithromycin in aqueous solutions and in gastric juices, respectively. Estimated recoveries above 98% were obtained in gastric juices. These performances were considered adequate by the authors to carry out stability studies and finally the method was applied to perform the stability studies of the drug in aqueous solutions and in gastric juice.

Concerning roxithromycin, spectrophotometric detectors appear to be sensitive enough to attempt the determination of the macrolide for bioequivalence studies. Indeed, Macket et al. proposed a method based on LLE with a hexane-isoamylalcohol mixture and without further clean-up. Total recoveries of $90 \pm 3\%$ were obtained and the authors stated the need of using an internal standard in order to obtain satisfactory precision. The separation was performed in isocratic mode on a C18 column using a methanol phosphate buffer mixture as mobile phase at 60 °C and monitored at 220 nm. The quantification was based on peak heights using clarithromycin as internal standard. The quantification limit was estimated to be 500 ng ml^{-1} and considered by the authors to be low enough for performing pharmacokinetic studies. Precision values below 8% and accuracy above 91% were stated. The method was successfully applied in a bioequivalence study of roxithromycin in plasma samples [14].

Azithromycin is another macrolide with this lack of UV absorbance properties. Wilms et al. recently overcame this problem by using precolumn derivatisation. The method published by this research group allows the quantification of azithromycin in biological samples (blood, plasma and neutrophils) using clarithromycin as internal standard and after a derivatisation process with FMOC-Cl (9-fluorenylmetoxycarbonyl-chloride). The detection of the fluorofore derivatives was performed by using a fluorometric detector set at 267 nm (excitation wavelength) and 317 nm (emission wavelength) reaching detection limits of 42 and 119 ng ml^{-1} in plasma and blood, respectively. The biological samples were submitted to a LLE at alkaline pH with diethyl ether. Once separated the organic layer was evaporated and resuspended in acetonitrile before carrying out the derivatisation procedure by using the fluorophore agent. The analytical separation was then performed on a reversed phase column using an isocratic elution with a mixture of acetonitrile and phosphate buffer at slightly alkaline pH. The analytical method has been applied to carry out a pharmacokinetic study of azithromycin in a cystic fibrosis patient. The obtained recoveries at one concentration level were stated as lying between 99.4 and 103.7% depending on the selected matrix. Intra and inter assay variation were estimated at two different levels of concentration with coefficients of variation below 1.2 and 2.8%, respectively [15].

2.1.2. Electrochemical methods (amperometric and coulometric)

In order to overcome the lack of sensitivity showed by some macrolides when using spectrophotometric detectors, and as most of them have a suitable electroactive group, electrochemical detectors arose as an alternative detection mode to perform the determination of this family of antibiotics. A reversed-phase method using a C18 column coupled to an amperometric detector set at 1100 V versus Ag/AgCl reference electrode was applied by Hanada et al. for performing the pharmacokinetic study of erythromycin in rats fluids and tissues. The separation was carried out in isocratic mode with a mixture of acetonitrile and acetate buffer as mobile phase. Oleandomycin was used in this work as internal standard and the quantification of the target macrolide was based on peak height. Before injection the samples were submitted to LLE with *tert*-butyl methyl ether under alkaline conditions. Limits of detection of 100 ng ml^{-1} and 500 ng g^{-1} were reached in plasma and liver, respectively. The observed coefficients of variation within and between days were less than 8% and in accuracy was less than 10%. The small samples volume needed for this method were highlighted by the authors, which constituted an advantage whenever the studies should be carried out with small animals [16].

An extraction procedure based on the same reagent was previously reported by Nilsson et al. for the determination of erythromycin A in plasma. In this case, the recovery of the macrolide was estimated as 84%. An amperometric detector set at +1.1 V versus Ag/AgCl reference electrode was also used in this work but conversely to the Hanada et al. protocol, polymeric type packing material suitable for chromatography at high pH was selected, which allows separation at alkaline pH and 25 °C using a mobile phase composed of acetonitrile and phosphate buffer adjusted to pH 9.5. The authors stated a relatively stable day-to-day response of the glassy carbon electrode under the conditions of this work. Experimental parameters such as the pH, the working potential and the temperature were studied. The quantification was performed using oleandomycin as internal standard. Two different calibration curves were used to determine low or high concentrations of erythromycin and the quantification limit considered as the lowest injection point in plasma samples was 150 ng ml^{-1}. Inter and intra-day accuracy and precision in plasma were estimated through control samples at three concentration levels and within a 2 month period leading to precision values below 8.7% [17].

The determination of erythromycin A using oleandomycin as internal standard in plasma and whole blood was also proposed by Kato et al. but using a lower potential. In this case, the samples were injected after a LLE procedure with diethyl ether on a different polymer column (octadecil-bonded polymer gel) (Asahipak octadecyl polymer (ODP) column 150 mm × 4.6 mm) which also allowed the use of an alkaline mobile phase. After elution in isocratic mode with a mixture of acetonitrile and a phosphate buffer adjusted to pH 10.5, the drug was monitored with an amperometric detector using a glassy carbon electrode set at 0.72 V. The quantification was also based on peak height. The detection limit was 100 ng ml^{-1} in both matrices, with coefficients of variation of 4.4% in plasma and 5.3% in whole blood and recoveries were over 80.1% in plasma and over 75.2% in whole blood [18].

Another type of electrochemical detector, the so called coulometric detector, has also been successfully used for the determination of such macrolides. In 1984, Duthu selected a coulometric detector to perform the determination of erythromycin in human serum. In most cases, the coulometric detector comprises of a guard cell aimed to electrolyse the components of the mobile phase followed by a dual electrode, which in the case of macrolides should be operated in oxidative screen mode. In this work, the potentials were set at +0.7 and +0.8 V, respectively while the guard cell was set at +1.0 V. The extraction process (LLE) was performed with methyl *tert*-buthyl ether at alkaline pH. A diphenyl column provided the best results as regards prevention of peak tailing, and was therefore selected to carry out the separation in isocratic mode using an acetonitrile, sodium perclorate, ammonium acetate and methanol mixture adjusted at an apparent pH of 7.0 as mobile phase. The averaged recovery calculated with human serum fortified over the dynamic range (from 50 to 1500 ng ml^{-1}) was 84%. Finally, the method was applied to human serum samples from males medicated with erythromycin. A quantification level of 50 ng ml^{-1} was reached. The lack of electrochemical response observed for two *N*-demethyl derivatives of erythromycin suggested the importance of the tertiary amine of the desosaminyl sugar for the electrochemical detection to the author [19].

Another macrolide, azithromycin, was determined in serum using both types of electrochemical detectors (coulometric and amperometric). For the coulometric assay, the separation was performed in isocratic mode using a mobile phase composed of a mixture ammonium acetate/sodium perchlorate/acetonitrile/methanol adjusted to neutral apparent pH (6.8–7.0) on an alkylphenyl column, setting the guard cell at 1.0 V and the screening electrodes at +0.7 and 0.8 V, respectively. The 9-a-*N*-*n*-propyl analogue was used as an internal standard. Three linear ranges of concentration were established. The amperometric assay was also performed on isocratic mode on a polymer coated alumina column using a mobile phase composed in this case of a mixture of potassium phosphate acetonitrile and adjusted to alkaline pH (apparent pH 11.0) and monitored by a glassy carbon electrode set at an oxidation potential of +0.8 V versus Ag/AgCl reference electrode. Two different linear ranges of concentration were established and another analogue of azithromycin, the 9-a-*N*-propargyl, was used as an internal standard. In both methods the alkalinised samples were submitted to a LLE procedure with methyl-*tert*-buthyl ether followed by a washing step with hexane in the amperometric method whereas in the coulometric assay this step was replaced by a back extraction with citric acid and a re-extraction with ether again. Mean recoveries of 88% at 100 ng ml^{-1} and 86% at 500 ng ml^{-1} in rat

and human serum, respectively, were stated by the authors. Under the conditions stated in this work the same quantification limits were reached for both means of detection, i.e. $10\,ng\,ml^{-1}$. However, the intra-day assays performed in different concentration ranges revealed slightly lower precision values for the coulometric detector (4.3%) compared to 7.8% obtained with the amperometric detector. It has also to be noted that the use of the amperometric detector with the chromatographic column normally used in the coulometric assay lead to the passivation of the glassy carbon electrode. The amperometric method was also applied to some rat tissues (liver and kidney). In these cases, the tissues were first minced and homogenised with acetonitrile and centrifuged. An aliquot of the supernatant was evaporated until dryness at 50 °C and then resuspended in a solution of potassium carbonate before being submitted to a second extraction with methyl-*tert*-buthyl ether. After centrifugation, the ether layer was evaporated until dryness, at 37 °C and the residue was reconstituted in acetonitrile–water. The obtained solution was finally washed with hexane before injection onto the HPLC system. A limit of quantification of $100\,ng\,g^{-1}$ was stated for these samples with recoveries of 85 and 84% in liver and kidney, respectively [20].

Another team, Riedel et al., compared two different methods (HPLC and microbiological) for the determination of azithromycin in human serum. The methods were applied to a wide number of samples obtained in a clinical study resulting in similar values of sensitivity and precision. The HPLC method was performed in isocratic mode using a C18 column and a mobile phase at neutral pH constituted by a mixture of phosphate buffer, tetrabutylammonium perchlorate and acetonitrile. A coulometric detection system composing of a guard cell set at 1.0 V and two screening electrodes set at +0.8 and 0.9 V was selected in this work. The samples were extracted using the Shepard et al. [20] procedure described above and the quantification limit, defined as the lower calibration point, was stated to be $8\,ng\,ml^{-1}$. The microbiological assay was carried out using an agar cylinder diffusion technique with *Sarcina lutea*. Both methods appear to be suitable for the determination of azithromycin in serum from patients after therapeutic doses of the drug [21].

In 1995 Hedemmo et al. published a liquid chromatography method for the individual determination of roxithromycin and clarithromycin in human plasma using an automated solid phase extraction (SPE) with CN cartridges on the Prospekt unit which comprises of a microprocessor, a cartridge transport system, three six port valves and a solvent delivery unit. In this system, the whole SPE process (including conditioning, washing and elution steps) is automatically performed before switching the valve and backflushing the compounds and injecting them onto the analytical column. A C18 column was used in isocratic mode and the elution was carried out at 55 °C with an acetonitrile and phosphate buffer adjusted to pH 7 mixture as mobile phase. Finally, the signals of the macrolides were monitored with an electrochemical (coulometric) detector with the upstream electrode set at 0.61 V and the downstream electrode set at 0.85 V. Clarithromycin was used as an internal standard when assaying roxithromcyin and vice-versa. The authors noted the importance of using an internal standard with similar behaviour in order to compensate the loss of sensitivity showed by the detector. The recovery of the SPE method was $89.4 \pm 5.5\%$ (at a concentration level of $7.0\,\mu M$) and $99.0 \pm 5.3\%$ (at a concentration level of $5.0\,\mu M$) for roxithromycin and clarithromycin, respectively. For both analytes the limit of quantification was $0.5\,\mu M$ and the between-day standard deviation estimated for concentration around $3\,\mu M$ was for both macrolides below 5.8%. The obtained results were compared with an extensively used LLE procedure at alkaline pH using a mixture of hexane and isobutanol as extraction solvents and erythromycin as internal standard with both extraction procedures being in excellent agreement [22].

The same approach for the individual determination of clarithromycin and roxithromycin in human plasma using the other as internal standard was adopted some years later by Pappa-Lousi et al. The plasma samples were submitted to a liquid–liquid extraction with a mixture of phosphate buffer at pH 7, methanol and acetonitrile before performing a clean-up of the extract on a SPE cartridge (Oasis HLB). The authors stated that this sample procedure lead to better results than the LLE at alkaline pH using *tert*-methyl-butyl-ether. Likewise the other paper, reversed-phase liquid chromatography was selected and the separation was performed on a C18 column but in this case an amperometric detector set at 1.0 V versus Ag/AgCl reference electrode was selected for the monitoring of the macrolides. It was noted that the use of an internal standard was mandatory due to the loss of sensitivity attributed by the authors to an electrode passivation process. A detailed study of the influence of the nature of the organic components of the mobile phase and the effect of temperature on the electrochemical response as well as on the peak shape was carried out in this work. This study concludes that a mixture of methanol and phosphate buffer (pH 7) and 40 °C were the best conditions to perform the determination of the target macrolides. Detection limits were stated to be $100\,ng\,ml^{-1}$ for clarithromycin and for roxithromycin. Unexpectedly, a different criteria was chosen for calculating the detection limits in each macrolide (signal-to-noise ratio of 3.4 for clarithromycin and 2.5 for roxithromycin). The spiked plasma samples were submitted to a SPE procedure and the obtained recoveries were $93.4 \pm 3.8\%$ for clarithromycin and $87.7 \pm 3.2\%$ for roxithromycin at a concentration level of $1\,\mu g\,ml^{-1}$ [23].

A different kind of stationary phase was proposed by Kees et al. to carry out the individual determination of clarithromycin, roxithromycin and azithromycin in biological fluids such as plasma, serum, saliva or gastric mucosa. In this case, the separation was carried out in isocratic mode on a cyanopropyl silica column and using a mixture of phosphate buffer, acetonitrile and methanol adjusted at an apparent pH between 6.5 and 7.5 depending on the target macrolide.

Before their injection in the chromatographic system the samples were submitted to a LLE at alkaline pH, using *tert*-methyl-butyl ether as the extraction solvent. The organic layer was then evaporated and resuspended into a methanol–water mixture. The author stated recoveries between 80 and 90%. The macrolides were monitored with a coulometric detector set at +1.0 V (guard cell), +0.5 (screening cell) and +0.8 V (analytical cell). The use of an internal standard was noted to be mandatory. The method allowed the quantification of 30, 50 and 10 ng ml^{-1} of clarithromycin, roxithromcyin an azithromycin, respectively, in spiked plasma. Slight modifications of the mobile phase composition were done depending on the assayed macrolide. The loss of sensitivity of the detector was overcome by increasing the set potentials. Slight modifications on the percentage of acetonitrile and on the pH of the mobile phase allowed the use of the same column up to 800 injections before exchange [24].

2.1.3. Mass spectrometry methods

A sensitive method for the determination of clarithromycin in plasma using HPLC coupled to MS–MS detection has been recently described by van Roogen et al. for the determination of Clarithromycin using roxithromycin as internal standard. A tertiary acidic mobile phase composed of acetonitrile, methanol and acetic acid was selected for performing the separation at 60 °C on a microbore C18 column. The alkalinized samples were submitted to a LLE procedure with a hexane–ethylacetate mixture and without further clean-up before injection. The mean recoveries established at 8.78, 964 and 6737 ng ml^{-1} were 80.5, 90.8 and 90.5%, respectively, which were in agreement with the recovery values stated by Kees et al. [24] for the electrochemical detector. The detection of clarithromycin was carried out on a triple quadrupole mass spectrometer with an electrospray ionisation interface. The electrospray ionisation was performed in positive mode at a temperature of 400 °C and setting the nebulizing gas (nitrogen), curtain gas and turbospray at 70, 50 and 70 l h^{-1}, respectively. Declustering, focussing, entrace and exit potentials of 61, 230, −8 and 8 V, respectively, provided the optimal response for clarithromycin. The MS–MS was operated in multi reaction monitoring (MRM) mode following the transitions of the monoprotonated molecular ion for clarithromycin (m/z 748.5 > 158.2) and for roxithromycin (m/z 837.6 > 679.5). As expected this kind of detector allowed much lower quantification limits than the other methods, namely 2.95 ng ml^{-1}. This quantification limit was sufficient for the selected application but still high compared to usual limits reached with this technique. This difference was attributed by the authors to a carry-over process which could be substantially reduced by introducing an extensive needle wash leading therefore to a much lower quantification limit (0.4 ng ml^{-1}). The introduction of this step would however imply an unnecessary increment of the analysis time. Without the cleaning step it is possible to analyse more than 200 samples per day. The wide linear range enables the application of the method for the determination of clarithromycin up to 36 h after the administration of the drug. The method was applied to perform the pharmacokinetic profiles obtained after a single dose of 500 mg of three different formulations of the macrolide in human plasma [25].

The main experimental conditions of the methods reviewed in this section are compiled in Table 1.

2.2. Multiresidue methods

Besides the individual methods, which in general are characterised by showing a higher sensitivity some authors also attempted the challenge of developing multiresidue methods which compensate for a potentially lower sensitivity with the possibility of determining more than one target analyte in a single run.

2.2.1. Electrochemical methods (amperometric and coulometric)

In an early paper amperometric detection coupled to HPLC was presented by Croteau et al. for the determination of erythromycin and its esters in several biological fluids such as saliva, plasma and urine. The separation was carried out in isocratic mode on a C18 column using an acetate buffer, acetonitrile and methanol mixture adjusted at pH 7.0 and monitored by an amperometric cell set at +0.9 V versus an Ag/AgCl reference electrode. The samples were submitted to a sample preparation involving an extraction with diethyl ether followed by evaporation of the organic layer and subsequent resuspension of the residue. The quantification of the target analytes was performed using roxithromycin as internal standard. The recoveries were estimated in spiked plasma at a concentration of 10 μg ml^{-1} leading to absolute recoveries of 55, 77 and 74% for erythromycin base, estolate and ethylsuccinate, respectively. The linear range in plasma was from 0.25 to 10.00 μg ml^{-1} for erythromycin base and estolate and from 0.25 to 3.00 μg ml^{-1} for erythromycin ethylsuccinate whereas in urine all of them showed a linear range from 0.50 to 15.00 μg ml^{-1}. For all macrolides tested the limit of detection was 10 ng. The loss of sensitivity exhibited by this type of detector was overcome by the authors by performing a daily flush of the working electrode with concentrated acids followed by a period of stabilisation [26].

Following the same trend, a multiresidue method, able to discriminate between five macrolides (josamycin, kitasamycin, rosamicin, roxithromycin and oleandomycin) in a single run, on a C18 reversed phase column and using an amperometric detector was fully developed by optimising typical experimental parameters. The optimised method was then applied for the determination of rosamicin and roxithromycin in spiked human urine using oleandomycin as internal standard. The macrolides were separated in isocratic mode using a mixture of phosphate buffer adjusted at pH 6.7, water and acetonitrile and the macrolides were monitored in oxidative mode on a glassy carbon electrode set at

Table 1
Biological matrices single analyte methods

Compound	Matrix	Sample treatment	HPLC column/temperature/mobile phase (v/v)	Detection	LOD/LOQ (units)	Reference
Rosamicin	Serum	LLE – diethyl ether	Partisil 10-ODS 250 mm × 4.6 mm/ambient/acetate buffer 10 mM pH 4:ACN (75:25)	UV λ = 254 nm	10/– (ng ml^{-1})	[9]
Josamycin	Plasma	Clean-up column – Nucleosil C18 5 mm × 4.6 mm, 30 μm	Hypersil CPS 250 mm × 4.6 mm, 5 μm/–/sodium acetate 10 mM:ACN (54:46)	UV λ = 230 nm	25/– (ng ml^{-1})	[10]
	Serum Urine	LLE – ACN SPE – Bond-Elut C_{18}	Homemade C_{18} 250 mm × 3.9 mm, 10 μm/35 °C/ ACN:15 mM phosphate buffer pH 6 (5:2)	UV λ = 231 nm	25/– (ng ml^{-1}) 25/– (ng ml^{-1})	[11]
Spiramycin	Plasma	Clean-up column – Perisorb RP-18 50 mm × 7 mm, 30–40 μm	Nucleosil 5 C_8 150 mm × 4.6 mm/–/ACN:0.2% perchloric acid (26:74)	UV λ = 230 nm	–/50 (ng ml^{-1})	[12]
Clarithromycin	Gastric juice	Dilution with water (1:5) + filtration (0.45 μm)	Hypersil ODS 150 mm × 4.6 mm, 5 μm/50 °C/ACN: 50 mM phosphate buffer (pH 4.6) + 5 mM OCTS (1-octanesulphonic acid) (50:50)	UV λ = 210 nm	780/– (ng ml^{-1})	[13]
	Plasma	Centrifugation + dilution with phosphate buffer 0.1 M pH 10.5:ACN (90:10) automated SPE – Baker CN	Hypersil BDS C_{18} 100 mm × 4.6 mm, 3 μm/55 °C/phosphate buffer (4.5 mM NaH_2PO_4 + 0.068 M Na_2HPO_4):ACN (46:54) pHap 7.0	Coulometric screen I +0.65 V, screen II +0.85 V	–/0.5 (μM)	[22]
	Plasma	LLE – ACN, SPE – Oasis HLB	Inertsil ODS-3 250 mm × 4 mm, 5 μm/40 °C/phosphate buffer pH 7 (I = 20 mM):MeOH (20:80)	Amperometric +1.0 V	100/– (ng ml^{-1})	[23]
	Plasma	LLE – *tert*-methyl-butylether	Cyanopropyl silica Zorbax SB CN 150 mm × 4.6 mm, 5 μm/30 °C/50 mM phosphate buffer:ACN:MeOH (450:300:50) pHap 7.5	Coulometric guard +1.0 V, screen I +0.5 V, screen II +0.8 V	–/30 (ng ml^{-1})	[24]
	Plasma	LLE – hexane:ethyl acetate (1:1)	Discovery C_{18} 150 mm × 2.1 mm, 5 μm/60 °C/ACN:MeOH:0.1% acetic acid (25:25:50)	ESI–MS/MS MRM mode m/z 748.5 > 158.2	–/2.95 (ng ml^{-1})	[25]
Roxithromycin	Plasma	LLE – hexane:isoamylalcohol (98:2)	Nucleosil 100-3 C_{18} 150 mm × 4.6 mm, 3 μm/60 °C/MeOH:phosphate buffer 15 mM pH 6 (70:30)	UV λ = 220 nm	–/500 (ng ml^{-1})	[14]
	Plasma	Centrifugation + dilution with phosphate buffer 0.1 M pH 10.5:ACN (90:10) automated SPE – Baker CN	Hypersil BDS C_{18} 100 mm × 4.6 mm, 3 μm/55 °C/phosphate buffer (4.5 mM NaH_2PO_4 + 0.068 M Na_2HPO_4):ACN (46:54) pHap 7.0	Coulometric screen I +0.65 V, screen II +0.85 V	–/0.5 (μM)	[22]
	Plasma	LLE – ACN, SPE – Oasis HLB	Inertsil ODS-3 250 mm × 4 mm, 5 μm/40 °C/phosphate buffer pH 7 (I = 20 mM):MeOH (20:80)	Amperometric +1.0 V	100/– (ng ml^{-1})	[23]
	Plasma	LLE – *tert*-methyl-butylether	Cyanopropyl silica Zorbax SB CN 150 mm × 4.6 mm, 5 mm/30 °C/50 mM phosphate buffer:ACN:MeOH (450:350:50) pHap 7.0	Coulometric guard +1.0 V, screen I +0.5 V, screen II +0.8 V	–/50 (ng ml^{-1})	[24]
Erythromycin	Plasma Liver	LLE – *tert*-methyl-butylether	Cosmosil 5-C_{18} 150 mm × 4.6 mm, 5 μm/ambient/ACN:0.1 acetate buffer pH 6.6 (50:50)	Amperometric +1.1 V	100/– (ng ml^{-1}) 500/– (ng ml^{-1})	[16]
	Plasma	LLE – *tert*-methyl-butylether	PRP-1 150 mm × 4.1 mm, 5 μm/25 °C/ACN:50 mM potassium phosphate (pH 9.5) (40:60)	Amperometric +1.1 V	15/– (ng)	[17]
	Plasma Blood	LLE – diethyl ether	Asahipak octadecyl polymer (ODP) 150 mm × 4.6 mm, 5 μm/ Ambient/ACN:50 mM phosphate buffer pH 10.5 (37:63)	Amperometric +0.72 V	100/– (ng ml^{-1}) 100/– (ng ml^{-1})	[18]
	Serum	LLE – *tert*-methyl-butylether	Sepralyte diphenyl 250 mm × 4.6 mm, 5 μm/–/ACN:20 mM sodium perchlorate:20 mM ammonium acetate:MeOH (50:32:8:10)	Coulometric guard +1.0 V, screen I +0.7 V, screen II +0.8 V	–/50 (ng ml^{-1})	[19]

Table 1 (*Continued*)

Compound	Matrix	Sample treatment	HPLC column/temperature/mobile phase (v/v)	Detection	LOD/LOQ (units)	Reference
Azithromycin	Serum	LLE – *tert*-methyl-butylether + Back extraction 15 mM citric acid (pH 3.1) low cc + LLE – *tert*-methyl-butylether + clean-up hexane	Chromegabond γRP-1 alumina 150 mm × 4.6 mm, 5 μm/–/ACN:50 mM phosphate buffer (70:30) pHap 11.0	Amperometric +0.8 V	–/10 (ng ml^{-1})	[20]
	Brain Muscle Liver Kidney	Homogenized with ACN + evaporated + reconstituted LLE – *tert*-methyl-butylether + clean-up hexane			–/100 (ng g^{-1}) –/100 (ng g^{-1}) –/100 (ng g^{-1}) –/100 (ng g^{-1})	
	Serum	LLE – *tert*-methyl-butylether + clean-up hexane	Chromegabond alkylphenyl 50 mm × 4.6 mm, 5 μm/–/20 mM ammonium acetate:20 mM sodium perchlorate:ACN:MeOH (22:23:45:10) pHap 6.8–7.2	Coulometric guard +1.0 V, screen I +0.7 V, screen II +0.8 V	–/10 (ng ml^{-1})	[20]
	Serum	Same described in [20] but fully automated	Nucleosil C_{18} 125 mm × 4.6 mm, 5 μm/–/40 mM disodium hydrogen phosphate:50 mM tetrabutylammonium perchlorate:ACN (50:50:33) pHap 7.0	Coulometric guard +1.0 V, screen I +0.7 V, screen II +0.8 V	–/8 (ng ml^{-1})	[21]
	Plasma Blood Neuthrophils	LLE – diethyl ether Precolumn derivatisation with FCOM-Cl	Symmetry C_{18} 100 mm × 4.6 mm, 3.5 μm/28 °C/ACN:20 mM phosphate buffer pH 7.7 (76:24)	Fluorescence λ_{exc} = 267 nm λ_{emi} = 317 nm	–/42 (ng ml^{-1}) –/119 (ng ml^{-1}) –/72 (ng ml^{-1})	[15]
	Plasma	LLE – *tert*-methyl-butylether	Cyanopropyl silica Zorbax SB CN 150 mm × 4.6 mm, 5 μm/30 °C/50 mM phosphate buffer:ACN:MeOH (450:500:50) pHap 6.8	Coulometric guard +1.0 V, screen I +0.5 V, screen II +0.8 V	–/10 (ng ml^{-1})	[24]

1.19 V versus an Ag/AgCl/KCl (saturated) reference electrode. The urine samples were submitted to a fast sample preparation procedure consisting of a deproteinisation step of the alkalinised urine with acetonitrile. This simple procedure lead to samples being sufficiently clean to allow the determination of the macrolides in human urine in patients under therapy, at the expected levels with mean recovery values of $90.5 \pm 5.5\%$ and $102.6 \pm 3.6\%$ for roxithromycin and rosamicin, respectively. The stated detection limits were around 3.1 and 6.0 $\mu g\,ml^{-1}$ for rosamicin and roxithromycin, respectively, which, for the latter was below the 0.002% of the daily recommended therapeutic dosage [27].

In a previous paper amperometric detection was also selected by Taninaka et al. for attempting the simultaneous determination of erythromycin, roxithromycin and azithromycin in rat plasma. After an isocratic separation on a reversed-phase column using a mobile phase composed of phosphate buffer at pH 7.2 and acetonitrile, the macrolides were monitored on an amperometric detector set at 950 mV versus an Ag/AgCl reference electrode. The quantification of the macrolides, based on peak height, was performed using clarithromycin as internal standard. Furthermore, the authors stated the feasibility of using the method for the determination of clarithromycin also using roxithromycin as internal standard. Each macrolide was extracted from plasma samples under alkaline conditions with *tert*-buthyl methyl ether with recoveries of 107, 106, 112 and 94% for erythromycin, clarithromycin, roxithromycin and azithromycin, respectively. The addition of EDTA or tetraethylammonium to mobile phase leads to an increase of the background noise. This effect has been previously observed by Kees et al. [24] with the addition of ammonium acetate or tetrabuthylammonium to the mobile phase. The lower quantification levels tested were 30 $ng\,ml^{-1}$ for clarithromycin and azithromycin and 100 $ng\,ml^{-1}$ for erythromycin and roxithromycin which made the method suitable for performing pharmacokinetic studies within the clinical range (0.5–2.5 $\mu g\,ml^{-1}$). The pharmacokinetic study was performed in plasma after the intravenous administration of the individual drugs [28].

In another group, a method for the simultaneous determination of roxithromycin and oleandomycin using roxithromycin as internal standard in spiked human urine samples was applied to perform a comparison between the two main electrochemical detectors used for analytical purposes. In order to make a reliable comparison, the same analytical methodology was applied with both detectors, which comprised of a sample preparation involving a deproteinisation step in alkaline pH with acetonitrile followed by an evaporation step. Subsequently, the reconstituted samples were separated in isocratic mode on a reversed-phase analytical column using a phosphate buffer adjusted to pH 6.7, water and acetonitrile mixture as mobile phase. After the separation the macrolides were monitored either with an amperometric detector (glassy carbon) set at 1.19 V versus an Ag/AgCl reference electrode or with a coulometric detector (porous graphite) set at 0.6 and 0.75 V versus a αH_2/Pd reference electrode. Under these experimental conditions the amperometric detector lead to detection limits of 660 and 620 $ng\,ml^{-1}$ for oleandomycin and roxithromycin, respectively, while the detection limit obtained for both macrolides was 220 $ng\,ml^{-1}$ when using the coulometric detector. The comparison was based on the results obtained from the calibration curves, the intra-day and the inter-day assays carried out in spiked urine [29].

The use of electrochemical detectors, which effectively bypass some of the difficulties arising for using spectrophotometric detectors, was previously explored by other authors. Chen et al. reported a simple and sensitive method that allows the quantification of erythromycin and its potential metabolites or degradation products. The proposed method was applied to human spiked plasma and urine samples as well as to dog plasma and urine collected after the oral administration of 500 mg dose of erythromycin A. However, quantitative results were only given for Erythromycin A because of a lack of sufficient samples according to the authors. A LLE procedure using diethylether was applied for dog plasma whereas a simple deproteinization step carried out with acetonitrile was shown to be enough for determining the macrolides in dog urine samples mainly due to the higher concentrations of the drugs present in urine. A buffered mobile phase was used for the separation of the analytes in isocratic mode on a reversed phase column at ambient temperature. The importance of the appropriate pH for the resolution of the peaks as well as the critical role played by the alkalization of the plasma samples in order to obtain a high extraction efficiency were stressed by the authors. The detection was performed on a dual electrode coulometric detector in oxidative screen mode set at +0.7 and +0.9 V, respectively, and a detection limit for erythromycin A of 30 $ng\,ml^{-1}$ was stated when using 0.2 ml of plasma. This limit of detection was susceptible to be improved by increasing the initial volume of the plasma sample. The use of an internal standard (erythromycin B) leads to a reduction of the coefficient of variation of the respective response factors in spiked human plasma. No information was provided by the authors regarding to the detection and/or quantification limits for urine samples [30].

Coulometric detection was also chosen by Chu et al. for the determination of clarithromycin and its active metabolite (14(*R*)-hydroxyclarithromycin) in human plasma and urine using erythromycin A 9-*O*-methyloxime as internal standard. Alkalinized plasma and urine samples were submitted to LLE procedure with a mixture of ethyl acetate–hexane which lead to estimated extraction recoveries higher than 85%. The samples were analyzed in isocratic mode by using a mobile phase comprised by acetonitrile, methanol and water on a C8 column and monitored on a double electrode coulometric detector set at 0.5 and 0.78 V, respectively. Estimated quantification limits were 30 and 500 $ng\,ml^{-1}$ for plasma and urine samples respectively. Routine analysis of quality control samples in plasma and urine result in experimental values ranging mostly between 90 and 110% of the nominal values with inter-day precision below 7.6% [31]. Later

on the same analytical system was chosen by Wibawa et al. to develop and validate a method for the determination of clarithromycin, its metabolite 14-hydroxy-clarithromycin and its decladinose acid degradation product in rat plasma, gastric juice and gastric tissue. In this case, the separation was performed on a Kromasil ODS 5 μm (75 mm × 4.6 mm ID) at 30 °C using a mixture of acetonitrile and aqueous phosphate buffer (pH 7.0, 0.086 M) 45:55 (v/v) at 1.0 ml min^{-1}. The samples were submitted to LLE by using a *n*-hexane-2-butanol (4:1) mixture without further clean-up. The mean recoveries, depending on the matrix, ranged between 93.5 and 113% for clarithromycin, between 77.8 and 100% for the 14-hydroxy-clarithromycin and between 81.5 and 105.1% for the decladinose acid. In the same way, the calculated quantification limits were between 0.06 and 0.15 μg ml^{-1} for gastric juice, between 0.08 and 0.16 μg ml^{-1} for plasma and between 0.31 and 0.51 μg g^{-1} for gastric tissue. The signal of the declanidose acid was confirmed by the authors using LC–MS [32]. In another study, a three-electrode coulometric detector set at a guard potential of +0.95 V and at screening potentials of +0.65 and +0.85 V, was selected for the determination of erythromycin and 2-acetylerythromycin in human plasma using oleandomycin as internal standard. After an extraction process in alkaline medium with *tert*-buthyl methyl ether the method reached absolute extraction recoveries between 82 and 72% for the target macrolides. The separation was performed in isocratic mode on a C18 column (Ultrasphere C18 250 mm × 4.6 mm 5 μm) with a mobile phase composed of acetonitrile, methanol and acetate buffer adjusted to a final pH of 6.3. The authors stated detection limits of 250 ng ml^{-1} for both analytes [33].

A new generation of coulometric detector coupled to a high performance liquid chromatograph was used for developing a multiresidue method allowing for the first time the separation in a single run of ten commercially available macrolides (erythromycin, dirithromycin, tylosin, tilmicosin, spiramycin, josamycin kitasamicin, rosamicin, roxithromycin and oleandomycin). The compounds were monitored on a four channel coulometric assay set at 650, 750, 800 and 850 mV versus a αH_2/Pd reference electrode. A C8 reversed phase column was used and the separation was performed for the first time in gradient mode. The method was applied for the determination of nine macrolides in spiked human urine using roxithromycin as internal standard. The samples were submitted to a sample preparation procedure comprising a LLE using *tert*-methyl-butyl-ether followed by the evaporation of the organic layer till dryness and subsequent resuspension in the mobile phase. The detection limits for all the drugs were below 60 ng ml^{-1} [34].

2.2.2. *Mass spectrometry methods*

Mass spectrometry has emerged as the most advisable technique for regulatory purposes not only because it constitutes the most powerful detection mode in the analysis of compounds at residue level but also because it gives the possibility of being used as a confirmatory technique as it provides unequivocal structural information. One LC–MS method has been developed and validated for the determination of three major components of bitespiramycin and their major active metabolites in rat plasma. The proposed method was shown to be successful in applications supporting preclinical studies of the novel antibiotic. The separation was performed on a C18 reversed phase column at 25 °C. The critical role played by a selected proportion of the constituents of the mobile phase, namely acetonitrile, ammonium acetate and acetic acid in order to obtain a good chromatographic peak shape and resolution was demonstrated. An electrospray interface operated in positive mode was selected and mass spectrometer conditions were optimised in order to obtain maximum sensitivity of the doubly charged ions $[M + 2H]^{2+}$, which improved the specificity of the LC–MS method. The ion source was heated to 170 °C setting the capillary to 6 V and applying an ion-spray voltage of 4.5 kV. The sheath and auxiliary gas was nitrogen at respective flows of 0.75 and 0.15 l min^{-1} whereas helium was selected as collision gas at a flow rate of 0.2 l min^{-1}. The quantification was performed using selected reaction monitoring (SRM) mode. The base peaks of product ions were observed at *m/z* 699.2, 741.2 and 755.2. The samples were submitted to a LLE in alkaline medium with a mixture of ethyl acetate and propanol. The organic phase was then evaporated and reconstituted in mobile phase without a further clean-up procedure. This procedure lead to recovery rates of 61.1, 63.7 and 69.8% for ISV-SPM I, II and III, and 58.2, 64.1 and 70.8% for SPM I, II and III. The method was applied for the determination of the target macrolides in rat plasma after peroral/intravenous administration of bitespiramycin using roxithromycin and azithromcyin as IS for the ISV-SPMs and the SPMs forms, respectively. The obtained quantification levels were 4 ng ml^{-1} for ISV-SPMI and SPM I, 12 ng ml^{-1} for ISV-SPM II and SPM II and 18 ng ml^{-1} for ISV-SPMIII and SPM III. [35].

The main experimental conditions of the multianalyte methods detailled in this section are gathered in Table 2.

3. Food matrices

The abuse and misuse of antibiotics in human and veterinarian practices for many years has lead to a significant increase of antimicrobial resistance and therefore poses a risk for human health. In parallel, the quality of food products and drinking water has become a major concern of consumers, legislators and producers in the European Union in view of protecting consumer health. Hence the control of the origin and quality of food demands the development of reference analytical methods to enforce the relevant Commission policies. The Council Regulation 2377/90 EC [36] established maximum residue limits (MRLs) for residues of veterinary drugs in animal tissues entering the human food chain to safeguard human health. This regulation describes lists of compounds that have a fixed MRL (Annex I), or which need no MRL (Annex II) or which have a provisional

Table 2
Biological matrices multianalyte methods

Compound	Matrix	Sample Treatment	HPLC column/temperature/mobile phase (v/v)	Detection	LOD/LOQ (units)	Reference
Erythromycin base	Urine	LLE – diethyl ether	Nova-pack C_{18}/–/56 mM sodium acetate buffer:ACN:MeOH (56:50:4) pHap 7.0	Amperometric +0.9 V	10/– ng	[26]
	Plasma				10/– ng	
Erythromycin estolate						
Erythromycin ethylsuccinate						
Rosamicin	Urine	LLE – ACN	Symmetry 300 C_{18} 150 mm × 2.1 mm, 3.5 μm//25 mM phosphate buffer pH 6.7:water:ACN (625:100:275)	Amperometric +1.19 V	3.1/– (μg ml^{-1})	[27]
Roxithromycin					6.0/– (μg ml^{-1})	
Erythromyin	Plasma	LLE – *tert*-methyl-butylether	YMC-Pack ODS-AP 250 mm × 6.0 mm, 5 μm/30 °C/ACN: 50mM phosphate buffer (pH 7.2) (43:57)	Amperometric +0.95 V	–/100 (ng ml^{-1})	[28]
Azithromycin					–/300 (ng ml^{-1})	
Roxithromycin					–/100 (ng ml^{-1})	
Clarithromycin					–/300 (ng ml^{-1})	
Erythromycin A	Plasma	LLE – diethyl ether	μBondapack C_{18} 300 mm × 3.9 mm, 10 μm/ambient/ACN: MeOH:0.2 M sodium acetate (pH 6.7) (40:5:55)	Coulometric screen I +0.7 V, screen II +0.9 V	–/30 (ng ml^{-1})	[30]
	Urine	LLE – ACN			–/–	
Metabolites	Plasma				–/–	
	Urine				–/–	
Roxithromycin	Urine	LLE – ACN	Symmetry 300 C_{18} 150 mm × 2.1 mm, 3.5 μm//25 mM phosphate buffer pH 6.7:water:ACN (625:100:275)	Amperometric +1.19 V	6.2/– (μg ml^{-1})	[29]
				Coulometric screen I + 0.6 V[a], screen II +0.75 V[a]	2.2/– (μg ml^{-1})	
Oleandomycin				Amperometric +1.19 V	6.6/– (μg ml^{-1})	
				Coulometric screen I + 0.6 V[a], screen II + 0.75V[a]	2.2/– (μg ml^{-1})	
Clarithromycin	Plasma	LLE – ethyl acetate:hexane (1:1)	Nucleosil C_8 250 mm × 4.6 mm, 5 μm/–/ACN:MeOH:40 mM phosphate buffer pH 6.8 (39:9:52)	Coulometric screen I + 0.5 V, screen II +0.78 V	–/30 (ng ml^{-1})	[31]
	Urine				–/500 (ng ml^{-1})	
14 (*R*)-hydroxyclari-thromycin	Plasma				–/30 (ng ml^{-1})	
	Urine				–/500 (ng ml^{-1})	
Clarithromycin	Plasma	LLE – *n*-hexane:2-butanol (4:1)	Kromasil ODS 75 × 4.6 mm, 5 μm/30 °C/ACN:86 mM phosphate buffer pH 7 (45:55)	Coulometric guard +1.0 V, screen I +0.6 V, screen II +0.85 V	–/0.10 (μg ml^{-1})	[32]
	Gastric juice				–/0.15 (μg ml^{-1})	
	Gastric tissue				–/0.51 (μg ml^{-1})	
14-OH-clarithromycin	Plasma				–/0.08 (μg ml^{-1})	
	Gastric juice				–/0.08 (μg ml^{-1})	
	Gastric tissue				–/0.39 (μg ml^{-1})	
Decladinose clarithromycin	Plasma				–/0.16 (μg ml^{-1})	
	Gastric juice				–/0.06 (μg ml^{-1})	
	Gastric tissue				–/0.31 (μg ml^{-1})	
Erythromycin	Plasma	LLE – *tert*-methyl-butylether	Ultrasphere C_{18} 250 mm × 4.6 mm, 5 μm/ambient/ACN:MeOH:0.1 M sodium acetate buffer (pH 5.0) (42:10:48) pHap 6.3	Coulometric guard +0.95 V, screen I +0.65 V, screen II +0.85 V	0.25/– (μg ml^{-1})	[33]
2′-Acetyl-erythromycin					0.25/– (μg ml^{-1})	

Table 2 (*Continued*)

Compound	Matrix	Sample Treatment	HPLC column/temperature/mobile phase (v/v)	Detection	LOD/LOQ (units)	Reference
Spiramycin 2	Urine	LLE – *tert*-methyl-butylether	Symmetryshield RP8 150 mm × 3.9 mm, 5 μm/50 °C/A:1 M phosphate buffer (pH 6.7):water:ACN (12.5:687:150) B:1 M phosphate buffer (pH 6.7):water:ACN (12.5:237.5:600)	Coulometric screen I +0.65 V[a], screen II +0.75 V[a], screen III +0.80 V[a], screen IV +0.85 V[a]	10/40 (ng ml^{-1})	[34]
Spiramycin 3					10/50 (ng ml^{-1})	
Dirithromycin					30/100 (ng ml^{-1})	
Erythromycin					30/90 (ng ml^{-1})	
Josamycin					40/140 (ng ml^{-1})	
Kitasamycin					40/130 (ng ml^{-1})	
Oleandomcyin					20/60 (ng ml^{-1})	
Rosamicin					30/100 (ng ml^{-1})	
Tilmicosin					60/210 (ng ml^{-1})	
Tylosin					30/90 (ng ml^{-1})	
Spiramycin I	Plasma	LLE – *tert*-methyl-butylether: propanol (95:5)	Kromasil C_{18} 150 mm × 4.6 mm, 5 μm/25 °C/ACN:10 mM ammonium acetate:acetic acid (35:65:0.5)	LS (ESI) MS/MS *m/z* 422.3 > 350.1, 699.2	–/4 (ng ml^{-1})	[35]
Spiramycin II				LC (ESI) MS/MS *m/z* 443.2 > 371.2, 741.2	–/12 (ng ml^{-1})	
Spiramycin III				LC (ESI) MS/MS *m/z* 450.2 > 378.2, 755.2	–/18 (ng ml^{-1})	
Isovaleryspiram-ycin I			Kromasil C_{18} 150 mm × 4.6 mm, 5 μm/25 °C/ACN:10 mM ammonium acetate:acetic acid (45:55:0.5)	LC (ESI) MS/MS *m/z* 464.3 > 350.2, 699.2	–/4 (ng ml^{-1})	
Isovaleryspiram-ycin II				LC (ESI) MS/MS *m/z* 485.4 > 371.3, 741.2	–/12 (ng ml^{-1})	
Isovaleryspiram-ycin III				LC (ESI) MS/MS *m/z* 492.3 > 378.2, 755.2	–/18 (ng ml^{-1})	

[a] vs. H_2/Pd reference electrode.

MRL (Annex III). The use of compounds listed in Annex IV of this regulation is prohibited in livestock population.

Morevover, the monitoring of veterinary drug residues became an obligation for the member states and is governed by national surveillance schemes, established under Council Directive 96/23/EC [37]. Criteria defining the performance from both screening and confirmatory analytical methods have been established in Commission Decision 93/256/EEC [38] and by the Commission Decision 2002/657/EEC [39] implementing the Council Directive 96/23/EC [37].

As a result a large number of methods were published relating to different analytical methodologies able to detect and quantify macrolide residues at MRL level at least in target tissues such as kidney, liver, muscle and some target foodstuffs such as eggs or milk. This section will therefore focus on these matrices. In general multiresidue methods are preferred for regulatory purposes. However, methods developed for the determination of a single macrolide can also be found in the literature. This section will therefore tackle on one side, single analyte methods and on other side multiresidue methods.

3.1. Single analyte methods

3.1.1. Spectrophotometric methods (UV, DAD) and fluorescence based methods

The determination of the macrolide, Josamycin, at MRL levels was attempted by Leroy et al. using pre-column derivatization and spectrofluorometric detection. The developed method was assayed in four different porcine tissues namely muscle, liver, kidney and fat. In all tissues, the sample preparation involved (for all tissues) a homogenisation and a LLE with a mixture of acetonitrile and phosphate buffer adjusted to pH 6.0, followed by a clean-up of the extract using isooctane. The aqueous phase was then submitted to the derivatisation process involving a heating of the mixture for 2 h at 90 °C. The derivatisation reagent was cyclohexa-1,3-dione (CHD) in ammonia medium. After derivatisation the fluorescent derivative was submitted to chromatography and separated on a reversed phase column using a mixture of acetonitrile, methanol and phosphate buffer at pH 6.0 and detected with a fluorescence detector set at an excitation wavelength of 375 nm and an emission wavelength of 450 nm. The linearity of the method was tested from 0.1 to 3.2 $\mu g\ g^{-1}$ and detection limits about 25 $ng\ g^{-1}$ were reached for each tissue. From the author's point of view the method appears suitable for other macrolides which also possess the aldehyde group in their structure enabling the derivatisation process [40].

The determination of other macrolide, erythromycin A, was carried out in several tissues and milk from different animal species by Dreassi et al. The different samples were submitted to a two-step extraction with chloroform at alkaline pH, followed by the evaporation of the pooled chloroformic extracts and resuspension of the residue in acetonitrile. After filtration, the resuspended extracts were injected into the system without further clean-up. The authors carried out the monitoring of the macrolide using two different kinds of detectors, electrochemical and spectrophotometric. In both methods (using the electrochemical or the spectrophotometric detector) the separation was performed in isocratic mode on the same reversed phase column. The mobile phase was an acetonitrile–ammonium buffer mixture. The percentage of organic modifier, acetonitrile, was higher in the method with the electrochemical detector. Since erythromycin lacks a suitable chromophore group, an additional step in the sample preparation was needed in order to form a chromophore group in the molecule. The authors reported that an alkali treatment of erythromycin leads to form a α–β-unsaturated ketone, a strong chromophore group naturally present in the molecule of other macrolides such as rosamicin [9]. This reaction was, therefore, carried out resuspending the evaporated chloroformic residues in a solution 1 M of NaOH and keeping them at 40 °C for 1 h. After this time the solutions were neutralized with acetic acid and filtered before they were injected into the system. The quantification of the target macrolide was performed by using oleandomycin as internal standard for the electrochemical method. However, oleandomycin gave more than one product after the alkali treatment clarithromycin was chosen for the spectrophotometric method as internal standard. The macrolide was monitored with the electrochemical detector (amperometric) on a glassy carbon electrode set at 1100 mV versus an Ag/AgCl reference electrode, while the UV detection was carried out, once the chromophore group was formed, at 236 nm. Both methods reached the same quantification limits ranging from 10 (in milk) to 250 (in plasma) $ng\ ml^{-1}$ or $ng\ g^{-1}$ depending on the matrix and to similar values of accuracy and precision. These quantification limits are between 3.2 and four-fold lower than the maximum residue limits established for erythromycin in the target matrices. From the point of view of the authors the UV method is preferable to the electrochemical one mainly because the procedure used to restore the sensitivity of the electrode was time consuming [41].

Spectrophotometric detection was also shown to be suitable for the determination of tylosin. Indeed De Liguoro et al. presented a method for the determination of tylosin residues in different pig tissues at MRL level. The macrolide was extracted from the different tissues with a water-methanol mixture and the obtained extract was submitted to a clean-up step using cation-exchange cartridges before injection in the HPLC system. The separation was performed by reversed-phase chromatography using a gradient elution of a 0.01 M potassium phosphate and 0.02 M tetrabutylammonium bromide at pH 2.0 mixture (mobile phase A) and a methanol and acetonitrile mixture as mobile phase B and monitored at 280 nm. Recoveries were estimated in each four tissues fortified at three different concentration levels (50, 100 and 200 $\mu g\ kg^{-1}$) through an intra-day assay (in sextuplicate) and in inter-day assay (in quintuplicate). The mean recoveries obtained from the inter-day assay ranged from 69.9 to 85.3% depending on the matrix. Intra-day and inter-day precision was found below 9.1 and 10.1%, respectively [42]. A slight

modification of this method described by Liguoro et al. was made later by the same research group, in order to investigate the depletion of tylosin in turkey tissues. After 3 days of oral administration of the macrolide in the drinking water the animals were sacrified at different withdrawal times and the edible tissues were analysed. The results obtained in this work suggested that tylosin D was the major active metabolite of tylosin A in turkey tissues [43].

In 2002, Prats et al. published a HPLC method capable of detecting and quantifying tylosin residues in several tissues from different animal species also using a spectrophotometric detector. The separation was performed on a C18 column using an isocratic elution of mixture of acetonitrile and phosphate buffer at pH 2.4. The detector, set at 280 nm, was demonstrated to be sufficiently sensitive and selective enough to carry out the quantification of tylosin at residue levels after sample preparation. Albendazole was used as an internal standard with the aim of correcting losses of tylosin during the extraction procedure from muscle, kidney and liver tissues. The developed method was applied to all target tissues, i.e. muscle, liver, kidney and skin+fat from calf, pig and poultry. The extraction of the macrolide was performed with different organic solvents depending on the tissue. A similar procedure was applied for the extraction of the drug from muscle liver and kidney. In this case, after homogenisation with the internal standard the samples were submitted to an extraction process with chloroform at alkaline pH. After shaking and centrifugation the organic layer was collected and evaporated until dryness and finally resuspended in a water–acetonitrile mixture. This mixture was further cleaned up by liquid–liquid partition with dichloromethane. The dichloromethane layer was collected and again evaporated until dryness before being redissolved in mobile phase. For the extraction of tylosin in the other tissues no internal standard was added and a single extraction with ethyl acetate, without further clean-up, was carried out. The average recovery defined as the quantity of tylosin recovered after submitting samples to the extraction procedure, was estimated for each matrix with spiked samples at four concentration levels being 94% for skin/fat, 90% for muscle, 72% for kidney and 55% for liver. It was stated by the authors that the extraction procedure showed saturation at concentrations higher than 10 times the MRL. The minimum recovery of 54% was therefore obtained in liver spiked at 1000 $\mu g\,kg^{-1}$. The limits of quantification were 50 $ng\,g^{-1}$ for all the tissues and precision and accuracy were considered adequate by the authors. In addition, incurred samples from calves treated with the therapeutic dose (20 $mg\,kg^{-1}\,day^{-1}$ for 5 days) analyzed at two different withdrawal periods (7 and 14 days) showed values under MRL for all the target tissues after 14 days [44].

3.1.2. Electrochemical methods (amperometric and coulometric)

Billedeau et al. recently published a HPLC method for the determination of Erythromycin A in edible salmon tissue by electrochemical detection with confirmation by electrospray ionisation mass spectrometry. When the electrochemical detector was used the separation was carried out in isocratic mode on a C18 column and using an acetonitrile and 10 mM ammonium formate (45:55) mixture as mobile phase. In the case of the MS detector a different C18 was used and the separation was performed in gradient elution using acetonitrile–water mixtures from (20:80) up to (80:20) in 45 min and with a constant concentration of 3 mM ammonium formate. The sample preparation initially involved a two-step extraction in ammonium formate followed by a clean-up of the extract using hydrophilic–lipophilic balanced (HLB) polymeric-based C18 SPE cartridges, finalising with a liquid–liquid partioning process using dichloromethane. The resulting organic layer was collected, evaporated until dryness and the residue was resuspended in mobile phase before injection into the system. The analysis was first performed using an electrochemical detector (coulometric) working in oxidation mode and setting the potentials at +0.75 and +0.9 V. The guard cell was set at +1.0 V. For confirmatory purposes an LC–ESI–MS system was used. The mass spectrometer was then operated in positive-ion electrospray mode in full scan. The protonated molecular ion was selected for ion monitoring of the erythromycin A (m/z 734.9). The samples were monitored in parallel in a UV detector at 230 nm to check potential interfering peaks from the matrix. The elution mode used for the LC-ECD method allowed the analysis to be performed in only 15 min while the gradient program used in the LC–MS method considerably increased the time of the analysis (55 min). With the electrochemical detector, detection and quantification limits of 5 and 16 $ng\,g^{-1}$ were reached. Standard solutions of erythromycin at different concentrations submitted to the clean-up procedure leading to average recoveries of 69.1%. Moreover, the intra-day recovery was estimated in fortified salmon at different concentrations resulting in a mean recovery of 63.8%. The inter-day mean recovery, estimated at 400 $ng\,ml^{-1}$, was 64.0%. The authors stated an increase of the recovery obtained using LC–MS that they attributed to a matrix effect which strongly affected the MS detector. Despite the higher detection capability of the mass detector, the authors proposed the LC-ED method as a low-cost alternative procedure for the determination of erythromycin A in salmon tissues [45].

3.1.3. Mass spectrometry methods

Another method for the determination of erythromycin A in salmon tissues using an (ESI) ion-spray LC–MS–MS was previously published by Pleasance et al. The samples were submitted to a selective extraction and clean-up procedure using different solvents such as acetonitrile, hexane and dichloromethane. The separation was performed on a reversed phase column using a gradient elution with an acetonitrile and aqueous formic acid mixture as mobile phase. The detection was carried out on an LC–MS and on an LC–MS–MS system with collision induced dissociation (CID) and detection limits below 50 $ng\,ml^{-1}$ were reached. The main drawback of the method was found to be the need

for replacement of the guard column after only a few injections which suggested a poor clean-up of the samples [46].

Delepine et al. presented a particle beam liquid chromatography–mass spectrometry method for the determination of tylosin at maximum residue limit level in bovine muscle. The tissue was mixed with a buffer solution at pH 8.5 and the extraction was performed with chloroform. After centrifugation the organic layer was collected and submitted to further clean-up through SPE diol cartridges. The separation was carried out in isocratic mode on a RP-18 endcapped column using a ternary mobile phase consisting of a mixture of methanol, acetonitrile and 2% aqueous formic acid at acidic pH. Conversely to most of the published mass spectrometric methods, in this case the detection was done in negative ion chemical ionisation mode using methane as reagent gas. For confirmatory purposes and according to the EU criteria, four ions (m/z 917, 916, 898 and 580) were selected. The limit of detection was 10 ng g^{-1} which is ten times below its MRL. The authors selected the negative ion chemical ionisation in order to decrease the fragmentation compared to electron impact [47].

The same research group previously developed a similar method also using a particle beam interface for the determination of spiramycin in bovine muscle. The extraction was carried out in chloroform and further clean-up was performed with diol SPE cartridges. An isocratic elution was selected for the separation of the target macrolide on a C18 column. Five fragment ions (m/z 304, 330, 475, 683 and 684) were selected for monitoring, which complies with the specificity requirements for regulatory confirmation methods defined at the time (EEC 89/610). The method was validated from half to twice the MRL of spiramycin in bovine muscle (50 ng g^{-1}). In this case the method reached a detection limit of 20 ng g^{-1} [48].

Table 3 summarizes the main experimental conditions of the single analyte methods described in this section.

3.2. Multiresidue methods

3.2.1. Spectrophotometric (UV, DAD) and fluorescence based methods

Spectrophotometric detection has been proved to be suitable for the determination of tylosin and tilmicosin residues in bovine and porcine kidney and muscle as presented by Chan et al. The tissues were submitted to a two-step extraction. The first extraction was performed with acetonitrile whereas the second extraction was performed with a phosphate buffer at pH 2.5 and acetonitrile mixture (1:2) in order to improve the recovery of tylosin especially from kidney. The pooled extracts were then diluted with water before a clean-up procedure carried out on a SPE C18 cartridge. The separation was carried out in isocratic mode on a C18 column using a ternary mobile phase and monitoring the target analytes at 287 nm. Under these conditions averaged recoveries of around 80 and 93% with detection limits of 20 and 10 ng g^{-1} were obtained for tylosin and tilmicosin respectively [49]. Another HPLC method using a UV detector with wavelength programmation was proposed by Horie et al. for the determination of five macrolides, josamycin, kitasamycin, mirosamicin, spiramycin and tylosin in animal tissues such as muscle, liver and kidney from different animals such as chicken, swine and cattle. The samples were submitted to a LLE using a mixture of 0.3% metaphosphoric acid and methanol followed by a SPE clean-up on Bond Elut SCX cartridges. The authors noted that this clean-up procedure was suitable for all the matrices tested whereas the liquid–liquid partition, used previously by the same research group, has been proved to be not suitable for kidney or liver due to the extensive emulsion formed. The separation of the macrolides was performed on a C18 column using a gradient elution with a mixture of phosphate buffer and acetonitrile. The macrolide determination was monitored at two different wavelengths, namely 232 nm for josamycin, kitasamycin mirosamicin and spiramycin and 287 nm for tylosin. Recoveries ranged from 70.8 and 90.4% and the detection limits reached were estimated to be 50 ng g^{-1} for each macrolide [50].

A sample procedure based on the method published by Horie et al. [50] was applied some years later by Leal et al. for the determination of macrolide antibiotics in spiked chicken muscle. The authors achieved the separation of seven macrolides on a C18 reversed phase column using a binary gradient elution of phosphate buffer (mobile phase A) and phosphate buffer and acetonitrile mixture (mobile phase B). The method was also based on UV detection at different wavelengths which has been proved to be suitable for the determination of five from the seven tested macrolides in chicken poultry spiked below their MRLs. The authors tested two different detection systems based on UV absorption, i.e. wavelength-programming and multi-wavelength detection, the latter being the most suitable one. The detection of the seven macrolides was therefore achieved with a diode array detector (DAD) system set at 210, 232 and 287 nm. Detection limits between 10 and 50 ng ml^{-1} were stated for spiramycin, tilmicosin, tylosin, kitasamycin and josamycin while for oleandomycin and erythromycin were 400 and 900 ng ml^{-1}, respectively. As expected it has been proved that the proposed method was not sensitive enough for determining oleandomycin and erythromycin at the MRL values because of the lack of suitable chromophore groups in their chemical structures. The method proposed was therefore only applied for the determination of spiramycin, tilmicosin, tylosin, kitasamycin and josamycin in spiked chicken muscle and showed average recoveries between 60 and 80% at three spiking levels between half and twice of each MRL [51].

Fluorometric detection was chosen by Edder et al. for the determination of erythromycin and oleandomycin in several animal tissues and foodstuffs such as meat, liver, kidney, milk and eggs. The proposed method involved an extraction with acetonitrile and further defatting with hexane followed by a clean-up step on cation exchange cartridges. The obtained eluate was submitted to a precolumn derivatisation using a fluorophore reagent, 9-fluoromethylchloroformate (FMOC)

Table 3
Food matrices single analyte methods

Compound	Matrix	Sample treatment	HPLC column/temperature/mobile phase (v/v)	Detection	LOD/LOQ (units)	Reference
Josamycin	Muscle Liver Kidney Fat	LLE – ACN:10 mM phosphate buffer (pH 6.0) (35:65) Clean-up – isooctane Precolumn derivatisation with CHD	LiChrospher 100 RP-18 125 mm × 4 mm, 5 μm/45 °C/ ACN:MeOH:10 mM phosphate buffer pH 6.0 (45:5:55)	Fluorescence λ_{exc} = 375 nm λ_{emi} = 450 nm	25/– (ng g^{-1})	[40]
Erythromycin	Plasma Tissues Milk Fat	LLE – chloroform LLE – 1st chloroform + 2nd chloroform LLE – 1st cyclohexane + 2nd chloroform	Lichrospher 60 RP-SelectB 125 mm × 4 mm, 5 μm/–/ ACN:50 mM ammonium acetate buffer (32:68)	Amperometric +1.1 V	–/0.25 (μg ml^{-1}) –/0.010 (μg g^{-1}) –/0.125 (μg ml^{-1}) –/0.125 (μg ml^{-1})	[41]
	Plasma	LLE – chloroform, precolumn derivatisation with 1 M NaOH	Lichrospher 60 RP-SelectB 125 mm × 4 mm, 5 μm/–/ ACN:50 mM ammonium acetate buffer (16:84)	UV λ = 236 nm	–/0.125 (μg ml^{-1})	
	Tissues	LLE – 1st chloroform + 2nd chloroform, precolumn derivatisation with 1 M NaOH			–/0.125 (μg ml^{-1})	
	Milk Fat	LLE – 1st cyclohexane + 2nd chloroform Precolumn derivatisation with 1 M NaOH			–/0.010 (μg g^{-1}) –/0.125 (μg ml^{-1})	
	Salmon tissue	LLE1 – 1st 10 mM ammonium formate + 2nd 10 mM ammonium formate, SPE – Oasis HLB, LLE2 – 1st $MeCl_2$ + 2nd $MeCl_2$ + 3rd $MeCl_2$	Ansys Polaris C18-A 250 mm × 4.6 mm, 5 μm/–/ ACN:10mM ammonium formate (45:55)	Coulometric guard +1.0 V, screen I +0.75 V, screen II +0.90 V	5/16 (μg kg^{-1})	[45]
			Phenomenex Prodigy ODS (3) 250 mm × 2.0 mm, 5 μm/–/ A:ACN B water (constant 3 mM ammonium formate)	LC (ESI) MS *m/z* 734.9, 576.6, 558.6, 158.2	–/–	
	Salmon tissue	LLE1 – 1st ACN + 2nd ACN; clean-up 1 – hexane, LLE2 – 1st 1N NaOH + $MeCl_2$ + 1% disodium hydrogen phosphate + 2nd $MeCl_2$; clean 2–10% NaCl	Zorbax Rx-C8 250 mm × 4.6 mm/–/A:water B ACN (constant 0.2% formic acid)	LC (ISP) MS *m/z* 734	SIM 10/– (μg kg^{-1}) TIC 50/– (μg kg^{-1})	[46]
				LC (ISP) MS/MS *m/z* 734 > 158, 558, 576	–/–	
Tylosin	Fat	LLE – water Clean-up SPE – benzenesulfonic SCX	Prodigy ODS 250 mm × 4.6 mm, 5 μm/–/10 mM KH_2PO_4 + 20 mM tetrabutylammonium bromide (pH 2.0):MeOH + ACN (7 + 3) (1:1)	UV λ = 280 nm	5/– (μg kg^{-1})	[42]
	Kidney Liver Muscle	LLE – water:MeOH (15:3) Clean-up SPE – benzenesulfonic SCX			16/– (μg kg^{-1}) 11/– (μg kg^{-1}) 15/– (μg kg^{-1})	
	Muscle	LLE 1 – chloroform:70 mM phosphate buffer (pH 8.5) (10:1) LLE 2 – 1st dichlorometane + 2nd dichlorometane	ODS2 20 mm × 4.6 mm, 5 μm/ambient/ACN:40 mM phosphate buffer pH 2.4 (33:66)	UV λ = 280 nm	–/50 (μg kg^{-1})	[44]
	Liver	LLE 1 – chloroform:7 N NaOH (10:1) LLE 2 – 1st dichlorometane + 2nd dichlorometane			–/50 (μg kg^{-1})	
	Kidney	LLE 1 – chloroform:50% NaOH (10:1) LLE 2 – 1st dichlorometane + 2nd dichlorometane			–/50 (μg kg^{-1})	
	Skin + fat Fat	LLE1 – ethyl acetate LLE2 – MeOH			–/50 (μg kg^{-1})	
	Muscle	LLE – $CHCl_3$ + phosphate buffer pH 8.5 Clean-up SPE – bond-elut Diol	RP-18 125 × 4.0 mm, 5 μm/–/MeOH:ACN:2% aqueous formic acid (45:20:35)	LC (PBI) MS *m/z* 916, 917, 898. 580, 562, 520	10/– (μg kg^{-1})	[47]
Spiramycin	Muscle	LLE – phosphate buffer pH 8.5 + $CHCl_3$; clean-up SPE – bond-elut Diol	LiChrospher RP18 125 mm × 4.0 mm, 5 μm/–/2% formic acid/50 mM ammonium acetate (v/v):MeOH:ACN (50:35:15)	LC (PBI) MS *m/z* 684 *m/z* 475 *m/z* 683 *m/z* 330 *m/z* 304	 19/– (μg kg^{-1}) 19/– (μg kg^{-1}) 3/– (μg kg^{-1}) 17/– (μg kg^{-1}) 11/– (μg kg^{-1})	[48]

before separation by HPLC using a C18 reversed phase column in gradient elution using a mixture of phosphate buffer and acetonitrile (mobile phase A) and acetonitrile (mobile phase B) and detection with a fluorometric detector set at 260 nm as excitation wavelength and 305 nm as emission wavelength. The conditions of the derivatisation process as well as the chromatographic separation were optimised during the method development. Roxithromycin was used as internal standard, which allowed the authors to correct for the recovery and obtain satisfactory results in terms of repeatability, linearity, detection and quantification limits. It was noticed by the authors that when the samples were frozen and defrosted many times the recoveries obtained were very low. Mean recoveries for erythromycin of 53 and 99.3% were obtained with external and internal calibration respectively. Depending on the selected matrix detection limits ranged from 50 to 100 $\mu g\,kg^{-1}$ were reached for oleandomycin and between 25 and 50 $\mu g\,kg^{-1}$ were reached for erythromycin. The higher detection limit obtained for oleandomycin was attributed to the elution of endogenous peaks near the macrolide peak [52].

3.2.2. Mass spectrometry methods

After several methods in which the suitability of a particle beam interface for the confirmation of spiramycin and tylosin individually in bovine tissues was proven [47,48] the same authors attempted for the first time the development of a multiresidue method which would allow the simultaneous determination of five macrolides (spiramycin, tylosin, tilmicosin, erythromycin and josamycin) in bovine muscle spiked at 50 $\mu g\,kg^{-1}$. As in previous works, a particle beam interface was used and for each macrolide four ions were selected for confirmatory purposes. The extraction of the macrolides was performed at alkaline pH using chloroform as the extracting agent. After centrifugation, the organic layer was collected and filtered through glass wool and submitted to a further clean-up on diol SPE cartridges. The main differences stated by the author between the single analyte methods previously published and the multiresidue one were the use of a ternary gradient with a mixture of 0.1% trifluoroacetic acid (TFA) in water, methanol and acetonitrile as mobile phase which allowed the separation of the five macrolides and the replacement of formic acid by TFA, which on one hand increased the sensitivity and on the other hand eliminated the memory effect observed for tilmicosin on the chromatographic column. The monitoring of the macrolides was performed both in negative and in positive chemical ionization modes [53].

The development of a multiresidue method for the determination of the same macrolides this time by high-performance liquid chromatography coupled to mass spectrometry with electrospray in the positive ion mode was presented by Dubois et al. [54]. The proposed method was applied for the determination of the five macrolides using roxithromycin as internal standard in a wide range of different food matrices, including several tissues, milk and eggs. A Biomatrix column was placed before the analytical column in order to eliminate the remaining macromolecules from the samples. This additional column has been proven to increase the lifetime of the analytical column. In addition, the number of injections before the cleaning of the instrument was also increased by use of a switching valve, which limited the flow through the mass spectrometer to the elution period. The separation was performed on a C18 reversed phase column with a two-linear-step gradient elution of ammonium acetate and acetonitrile. The optimisation of the MS–MS conditions was done for each macrolide and four product ions from the same precursor ion were selected. Each macrolide was then monitored for quantification purposes in multiple reaction monitoring (MRM) mode. In this work, the authors apply a novel extraction process replacing the organic solvents widely used for the extraction of this family of antibiotics by Tris buffer at pH 10.5 followed by protein precipitation with sodium tungstate and a further SPE clean-up. With this sample preparation procedure the authors stated their achievement of cleaner final solutions than the ones obtained using organic solvents for the extraction and/or clean-up by liquid–liquid partioning especially for some matrices such as liver or kidney. However, the recovery rates were dramatically low for erythromycin in eggs and milk (below 45%). Nevertheless, even with this low recovery rate the authors reached good values in terms of reproducibility and accuracy. The higher detection limit found was 37 $ng\,g^{-1}$ and corresponds to spiramycin in milk, being however far below its corresponding MRL in this matrix. The method was in-house validated through intra and inter-day assays in samples spiked at MRL/2, MRL and 2 × MRL levels. The within laboratory reproducibility and accuracy obtained were compliant to the values stated in the Commission Decision 93/256/EEC [38].

Another confirmatory method for tylosin, tilmicosin and erythromycin, with a micro LC–MS–MS, using in this case atmospheric pressure ionisation and an ionspray interface, was developed by Draisci et al. [55]. The method was applied for the determination of the three macrolides, mentioned above, in bovine kidney, muscle and liver using roxithromycin as internal standard. The authors performed the extraction of spiked and incurred samples following the sample preparation procedure previously published by Delepine et al. [53] and consisting of an extraction of the alkalinised samples with chloroform. After centrifugation of the sample, the organic layer was collected, filtered and submitted to a further clean-up procedure on diol SPE cartridges. The separation of the macrolides was performed under isocratic conditions on a microbore reversed-phase column using a ternary mobile phase composed of acetonitrile, methanol and 1% trifluoroacetic acid. The drugs were monitored on the MS–MS spectrometer operated in the positive ion mode. The molecular related ions were selected as precursor ions for collision induced dissociation and two product ions were selected for each macrolide to carry out the selected reaction monitoring. The proposed method allowed quantification levels of between 30 and 40 $ng\,g^{-1}$ for tylosin, 20 and

150 ng g^{-1} for tilmicosin and between 50 and 80 ng g^{-1} for erythromycin to be reached, depending on the tissue. Precision values estimated by analysing six replicates at four levels of concentration on 3 different days were below 14.9%. The analytical performance of the method also complied to the criteria given in the Commission Decision 93/256/EEC [38].

Another liquid chromatography–mass spectrometry method has been proposed by Codony et al. for the determination of spiramycin, tylosin, oleandomycin, erythromycin, tilmicosin, kitasamycin, josamycin in poultry muscle using roxithromycin as internal standard. Conversely to the method proposed earlier by the same research group, the LC–MS detector reached detection limits sufficiently low to allow the determination of all the macrolides at MRL levels. The samples were submitted to a sample treatment identical to the one used in their previous work, i.e. an extraction using a mixture of metaphosphoric acid and methanol followed by a clean-up on a cationic SPE cartridge of the partially evaporated extract. The separation was performed on a C18 column applying a gradient elution with a mobile phase consisting of a mixture of 0.02% aqueous trifluoroacetic acid and acetonitrile as organic modifier. The mass spectrometer, equipped with an electrospray interface, was operated in positive mode and each compound was monitored selected ion monitoring (SIM) mode for quantification purposes. The quantification was performed using roxithromycin as internal standard. After the optimisation of both chromatographic and detection parameters, the method was applied to the determination of spiramycin, tilmicosin, oleandomycin, erythromycin, tylosin, kitasamycin and josamycin in spiked chicken muscle. The authors noted a substantial enhancement of the signal attributed to matrix effect. To minimise this effect, the method of matrix matched standards was selected to perform the calibration curves and detection limits of 35 ng ml^{-1} for spiramycin, 8 ng ml^{-1} for tilmicosin and below 4 ng ml^{-1} for the rest of the tested macrolides were obtained. The recoveries were tested at MRL/2, MRL and $2 \times$ MRL values and ranged from 56 to 93% depending on the macrolide. The main drawback of this method concerned the number of diagnosis ions required for confirmatory purposes according to the European legislation [37]. Indeed the method complied to the European requirements only for three of the target macrolides [56].

A liquid chromatography multiresidue method with electrospray ionisation mass spectrometry has been developed by Horie et al. for the determination of erythromycin, oleandomycin, kitasamycin, josamycin, mirosamicin, spiramycin, tilmicosin and tylosin in a single run at a quantification level of 10 ng g^{-1} in spiked meat and fish. The separation of the drugs was carried out at 40 °C on a C18 column with gradient elution using a mixture of aqueous acetic acid and acetonitrile as mobile phase. A sample preparation used in previous works and involving an initial extraction and deproteinisation procedure with a mixture of metaphosphoric acid and methanol followed by a partial evaporation of the extract and a final clean-up step on Oasis SPE HLB cartridges was selected. In this work the percentage of metaphosphoric acid was optimized in order to reduce the degradation of the macrolides in acidic media while keeping the efficiency of the extraction process. In addition, and despite the excellent clean-up obtained with the ion exchange cartridges used in their previous work, the authors change the type of the SPE cartridges in order to simplify the manipulation. The quantification of the drugs was performed using both protonated molecular and fragment ions. The detection limit stated in the method was 10 ng g^{-1} for all the target macrolides. The intra-assay was carried out on cattle, swine and chicken muscle and liver, yellowtail and red sea bream whereas the inter-assay was carried out in cattle and swine muscle and liver spiked at 200 ng g^{-1}. The estimated overall mean recovery at this concentration level (200 ng g^{-1}) was above 70% with standard deviations lower than 10% for each sample. For spiramycin the authors decided to also monitor its main metabolite (neospiramycin) as it displays a high antimicrobial activity [57].

The determination of five macrolide antibiotics (spiramycin, tilmicosin, oleandomycin, erythromycin and tylosin) using roxithromycin as internal standard have been determined for the first time in honey by J. Wang. This author developed a liquid chromatography method with mass spectrometry detection using an electrospray interface and operated in positive ion mode. The detector was also set in single ion recording mode in order to develop both LC–ESI–MS/MS and LC–ESI–MS methods. The separation of the macrolides was carried out on a C18 column using a gradient elution with a mixture of acetonitrile, 1% formic acid and water as mobile phase. The honey samples were extracted with phosphate buffer adjusted to pH 8.0. After centrifugation the supernatant was submitted to SPE on Oasis HLB cartridges. The eluate was then evaporated until dryness, and the residue was resuspended in an ammonium acetate/acetonitrile mixture and filtered before injection into the system. Both methods (MS and MS/MS) have been validated using singly charged ions for monitoring in order to increase their simplicity and specificity. The matrix-matched approach was selected to perform the calibration curves. Both methods were tested through inter and intra assays. For the LC–ESI–MS method the obtained recoveries, for samples spiked from 20 to 40 ng g^{-1}, were between 97.8 and 109.3% with R.S.D. below 12% and detection limits below 1 ng g^{-1}. As regards to the LC–ESI–MS/MS the inter and intra assays were performed with samples spiked from 6 to 80 ng g^{-1} and recovery rates ranged from 98.3 to 114.6% with R.S.D. below 13%. In this latter case the detection limits were between 0.01 and 0.07 ng g^{-1}. The author therefore concluded that both LC–ESI–MS and LC–ESI–MS–MS methods were suitable for the determination of the target macrolides in honey for regulatory purposes [58].

The main experimental conditions of the methods described in this section are gathered in Table 4.

Table 4
Food matrices multianalyte methods

Compound	Matrix	Sample treatment	HPLC column/temperature/mobile phase (v/v)	Detection	LOD/LOQ (units)	Reference
Tylosin	Muscle	LLE 1 – ACN	Inertsil C_{18} 250 × 4.6 mm, 5 μm/–/0.1M ammonium	UV λ = 287 nm	20/– (μg kg^{-1})	[49]
Tilmicosin	Kidney	Lle2 – 0.1 M phosphate buffer pH	formate (pH 5.0 with trifluoroacetic		10/– (μg kg^{-1})	
		2.5 + ACN Clean-up SPE – Bondelut C_{18}	acid-TFA):ACN:MeOH (60:30:10)			
Spiramycin	Muscle	LLE – 0.3% metaphosphoric acid:MeOH	Puresil 5C_{18} 150 mm × 4.6 mm, 5 μm/35 °C/A:25 mM	UV λ = 232 nm	50/– (μg kg^{-1})	[50]
Kitasamycin	Liver	(7:3)	phosphate buffer (pH 2.5) B:ACN		50/– (μg kg^{-1})	
Mirosamicin	Kidney	Clean-up SPE – BondElut SCX			50/– (μg kg^{-1})	
Josamycin				UV λ = 287 nm	50/– (μg kg^{-1})	
Tylosin					50/– (μg kg^{-1})	
Spiramycin	Muscle	LLE – 0.3% metaphosphoric acid:MeOH	Hypurity Elite C_{18} 250 mm × 4.6 mm, 5 μm/–/A:25 mM	UV λ = 232 nm	20/50 (μg l^{-1})	[51]
Kitasamycin		(7:3)	phosphate buffer (pH 2.5) B:25 mM phosphate buffer (pH		50/110 (μg l^{-1})	
Josamycin		Clean-up – SPE BondElut SCX	2.5):ACN (60:40)		20/50 (μg l^{-1})	
Tilmicosin				UV λ = 287 nm	10/30 (μg l^{-1})	
Tylosin					30/80 (μg l^{-1})	
Oleandomycin				UV λ = 210 nm	450/1130 (μg l^{-1})	
Erythromycin					930/2950 (μg l^{-1})	
Erythromycin	Meat	LLE – CAN	Inertsil 150-5 ODS-2 125 mm × 4 mm/ambient/A:30 mM	Fluorescence	50/100 (μg kg^{-1})	[52]
Oleandomycin	Fish	Defatted – hexane	phosphate buffer pH 7.0:ACN (36:64) B:ACN	$λ_{exc}$ = 260 nm		
	Egg	Clean-up SPE – IST sulphonic acid		$λ_{emi}$ = 305 nm		
Erythromycin	Liver	Precolumn derivatisation with FMOC			50/100 (μg kg^{-1})	
Oleandomycin	Kidney				100/200 (μg kg^{-1})	
Erythromycin		Defatted – centrifugation			25/50 (μg kg^{-1})	
		LLE – 0.1 M citrate buffer pH6.0 + hexane				
Oleandomycin	Milk	Clean-up SPE – IST sulphonic acid			50/100 (μg kg^{-1})	
		precolumn derivatisation with FMOC				
Spiramycin	Muscle	LLE – phosphate buffer pH 8.0 + $CHCl_3$;	LiChrospher RP18 125 mm × 4 mm, 5 μm/–/A:0.1%	LC (PBI) MS NCI *m/z* 684, 683, 665, 475, PCI *m/z* 844, 843, 825, 684	–/50 (μg kg^{-1})	[53]
		clean-up SPE – BondElut Diol	trifluoroacetic acid (TFA)B:MeOH C:ACN			
Tilmicosin				LC (PBI) MS NCI *m/z* 869, 868, 678, 677, PCI *m/z* 870, 869, 679, 678		
Tylosin				LC (PBI) MS NCI *m/z* 916, 915, 580, 562, PCI *m/z* 917, 916, 773, 772		
Erythromycin				LC (PBI) MS NCI *m/z* 619, 618, 443, 442, PCI *m/z* 735, 734, 716, 576		
Josamycin				LC (PBI) MS NCI *m/z* 828, 827, 736, 735, PCI *m/z* 829, 828, 769, 768		
Tylosin	Muscle	LLE – 1st + 2nd Tris buffer pH 10.5;	Purospher C_{18} 125 mm × 3 mm, 5 μm/30 °C/ A:ACN	LC (ESI) MS/MS	–/50 (μg kg^{-1})	[54]
Tilmicosin		deproteinisation – acetic acid + sodium	B:0.1 ml l^{-1} ammonium acetate	tylosin:916.3 > 772.2,	–/25 (μg kg^{-1})	
Spiramycin		tungstate buffer;		318.4, 407.4, 598.4	26/150 (μg kg^{-1})	
Josamycin		clean-up SPE – HLB Oasis			–/100 (μg kg^{-1})	
Erythromycin					–/200 (μg kg^{-1})	

Table 4 (*Continued*)

Compound	Matrix	Sample treatment	HPLC column/temperature/mobile phase (v/v)	Detection	LOD/LOQ (units)	Reference
Tylosin	Kidney			Tilmicosin: 869.6 > 696.6,	–/50 (μg kg^{-1})	
Tilmicosin				522.2, 678.9, 505.7	–/500 (μg kg^{-1})	
Spiramycin					–/150 (μg kg^{-1})	
Josamycin					–/200 (μg kg^{-1})	
Erythromycin					–/200 (μg kg^{-1})	
Tylosin	Liver			Spiramycin:	–/50 (μg kg^{-1})	
Tilmicosin				843.4 > 540.2, 318.2,	–/500 (μg kg^{-1})	
Spiramycin				700.2, 522.5	–/300 (μg kg^{-1})	
Josamycin					–/100 (μg kg^{-1})	
Erythromycin					–/200 (μg kg^{-1})	
Tylosin	Egg			Josamycin: 828.7 > 174,	–/50 (μg kg^{-1})	
Tilmicosin				109, 600.4, 228.8	–/25 (μg kg^{-1})	
Spiramycin					–/100 (μg kg^{-1})	
Josamycin					–/100 (μg kg^{-1})	
Erythromycin					–/100 (μg kg^{-1})	
Tylosin	Milk	Defatted – centrifugation; LLE –		Erythromycin:	–/25 (μg kg^{-1})	
Tilmicosin		1st + 2nd Tris buffer pH 10.5,		734.3 > 576.3, 558.3,	–/25 (μg kg^{-1})	
Spiramycin		deproteinisation – 3M sodium acetate		540.3, 522.3	37/100 (μg kg^{-1})	
Josamycin		buffer + sodium tungstate buffer, clean-up			–/100 (μg kg^{-1})	
Erythromycin		SPE – HLB Oasis			–/20 (μg kg^{-1})	
Tylosin	Muscle	LLE – chloroform + phosphate buffer pH ~8.0	LC-ABZ Supelcosil microbore 300 mm × 1 mm, 5 μm/ ambient/ACN:MeOH:1% trifluoroacetic acid (60:20:20)	LC (API) MS/MS (API) *m/z* 916 > 772, 174	–/30 (μg kg^{-1})	[55]
	Liver Kidney	Clean-up SPE – Bond Elut Diol			–/40 (μg kg^{-1})	
Tilmicosin	Muscle			LC (API) MS/MS (API)	–/20 (μg kg^{-1})	
	Liver Kidney			*m/z* 435 > 174, 99	–/150 (μg kg^{-1})	
Erythromcyin	Muscle			LC (API) MS/MS	–/50 (μg kg^{-1})	
	Liver Kidney			*m/z*734 > 576, 158	–/80 (μg kg^{-1})	
Spiramycin	Muscle	LLE – 0.3% metaphosphoric acid:MeOH (7:3) Clean-up SPE – BondElut SCX	Hypurity Elite C_{18} 250 mm × 4.6 mm, 5 μm/–/A:0.02% trifluoroacetic acid B:0.02% trifluoroacetic acid:ACN (60:40)	LC (ESI) MS *m/z* 842 > 843, 540, 422	35/– (μg l^{-1})	[56]
Tylosin				LC (ESI) MS *m/z* 915 > 916	<4/– (μg l^{-1})	
Oleandomycin				LC (ESI) MS *m/z* 687 > 688, 544,	<4/– (μg l^{-1})	
Erythromycin				LC (ESI) MS *m/z* 733 > 734, 576, 558	<4/– (μg l^{-1})	
Tilmicosin				LC (ESI) MS *m/z* 868> 869, 696, 435	8/– (μg l^{-1})	
Kitasamycin				LC (ESI) MS *m/z* 771> 772	<4/– (μg l^{-1})	
Josamycin				LC (ESI) MS *m/z* 827> 828	<4/– (μg l^{-1})	

Table 4 (*Continued*)

Compound	Matrix	Sample treatment	HPLC column/temperature/mobile phase (v/v)	Detection	LOD/LOQ (units)	Reference
Spiramycin	Honey	LLE – 0.1 M phosphate buffer pH 8.0 Clean-up SPE – HLB Oasis	YMC ODS-AQ S-3 50 mm × 2 mm/–/A:ACN B:1% formic acid C:water	LC (ESI) MS *m/z* 843 LC (ESI) MS/MS *m/z* 843 > 174, 318, 540	<1/– (μg kg^{-1}) 0.02/0.8 (μg kg^{-1})	[58]
Tilmicosin				LC (ESI) MS *m/z* 869 LC–MS–MS (ESI) *m/z* 869 > 156, 174	<1/– (μg kg^{-1}) 0.07/0.9 (μg kg^{-1})	
Oleandomycin				LC (ESI) MS *m/z* 688 LC (ESI) MS/MS *m/z* 688 > 158, 544	<1/– (μg kg^{-1}) 0.02/0.7 (μg kg^{-1})	
Erythromycin				LC (ESI) MS *m/z* 734 LC (ESI) MS/MS *m/z* 734 > 158, 558, 576	<1/– (μg kg^{-1}) 0.02/0.5 (μg kg^{-1})	
Tylosin				LC (ESI) MS *m/z* 916 LC (ESI) MS/MS *m/z* 916 > 145, 174, 772	<1/– (μg kg^{-1}) 0.01/0.4 (μg kg^{-1})	
Erythromycin	Muscle Liver Fish	LLE – 0.2% metaphosphoric acid:MeOH (6:4) Clean-up SPE – HLB Oasis	TSKgel Super ODS 100 mm × 2 mm, 5 μm/40 °C/A:0.2% acetic acid B:ACN containing 0.2% acetic acid	LC (ESI) MS *m/z* 733.9, 734.5, 716.4, 576.3,	10/– (μg g^{-1})	[57]
Oleandomycin				LC (ESI) MS *m/z* 688.9, 688.4, 670.4, 544.3		
Kitasamycin				LC (ESI) MS *m/z* 771.9, 772.5, 702.5, 558.3		
Josamycin				LC (ESI) MS *m/z* 828.0, 828.5, 860.4, 786		
Mirosamicin				LC (ESI) MS *m/z* 727.9, 728.4, 554.3		
Spiramycin				LC (ESI) MS *m/z* 843.1, 422.3, 843.5, 699.5, 540.3		
Neospiramycin				LC (ESI) MS *m/z* 698.8, 350.2, 721.5, 699.5, 540.3		
Tilmicosin				LC (ESI) MS *m/z* 869.2, 435.3, 869.5, 695.5		
Tylosin				LC (ESI) MS *m/z* 916.1, 916.5, 742.3, 582.3		

4. Conclusion

The previous sections detailed a wide number of HPLC methods applied for the determination of macrolide antibiotics in biological and food matrices. In general reversed phase is the preferred elution mode for this family of antibiotics using, in most cases, C18 or C8 columns. Liquid–liquid extraction with organic solvents, mainly with *tert*-methylbuthylether has been demonstrated to provide clean extracts from biological samples, but it is also possible to perform a simpler sample preparation by dilution or filtration of the samples whenever the target concentrations are not too low, as it can be the case for urine samples for example. On the other hand when the analysis should be performed at the lowest values, i.e. residue analyses mainly in food matrices, further clean-up with solid phase extraction cartridges has been proven to be the most suitable approach. Despite the undoubted advantages of using mass spectrometry detection, macrolides can also be analyzed at low concentration levels with other kinds of detectors, with or without prior derivatisation of the analytes, which may constitute powerful and cost-effective alternatives, in particular when only screening and/or post-screening are needed.

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