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# Sample preparation strategy for the simultaneous determination of macrolide antibiotics in animal feedingstuffs by liquid chromatography with electrochemical detection (HPLC-ECD)

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**Abstract**

A novel and suitable clean-up method that allows, for the first time, the simultaneous determination of a rather large number of macrolide antibiotics (erythromycin, rosamicin, spiramycin, tylosin, kitasamycin and josamycin in feedingstuffs by high performance liquid chromatography with electrochemical detection (HPLC-ECD) is presented in this work. The effectiveness of the developed clean-up method allows the quantification of the target macrolides in poultry feed using standard calibration curves instead of matrix matched standards, which overcomes the general problem of finding representative blanks. Furthermore an additional back extraction included in the sample preparation procedure allows the determination of an additional macrolide (oleandomycin) with detection limits, expressed as apparent concentration in poultry feed, ranging from 0.04 to 0.22 mg $kg^{-1}$ and relative standard deviation values ranging from 3.6 to 10.1% depending on the target analyte. Moreover, this additional step has been proven to enlarge the scope of the method by the extension of its applicability, at the target level of concentration, to other animal feedingstuffs such as pig and cattle. The analysis of real feedingstuffs containing macrolides demonstrated the fitness for purpose of the whole analytical procedure as well as a good fitting between real and spiked samples. The proposed methods appeared therefore as a sound alternative in the frame of control (e.g. for post-screening purposes) and/or monitoring surveillance programmes at the target level of 1.0 mg $kg^{-1}$ established according to the reported lowest dosage of additive needed to lead a growth promoting effect.


*Keywords:* Macrolide antibiotics; Multianalyte method; Electrochemical detection; High performance liquid chromatography; Solid phase extraction; Feedingstuffs

## 1. Introduction

Macrolides have been used in the rearing of food-producing animals for treating diseases (therapeutically) or as feed additives. The veterinary drugs that can be authorised as a medicinal product intended for food producing animals within the European Union (EU) are regulated by Council Regulation 2377/90/EC and its amendments. Among them we can find several macrolides with definitive MRLs such as erythromycin, spiramycin or tylosin [1]. Nevertheless the presence of antibiotic residues in foodstuffs could cause toxic effects, directly in sensitive individuals such as allergic reactions and also indirectly because their widespread usage could be responsible for the promotion of resistant strains of bacteria [2]. In order to help to decrease resistance to antibiotics used in medical therapy the European Commission set out different actions in the veterinary field such as the phase-out of the remaining use of some antibiotics including some macrolides such as spiramycin and tylosin as feed additives [3].

Unfortunately the legislation is far from being worldwide harmonized, which could lead to the presence of EU non-authorised macrolides (i.e. kitasamycin or oleandomycin) in feedingstuffs coming from third countries. In order to be able to enforce this regulation as well as to control the illegal use of these compounds, different kind of analytical methods suitable for carrying out a full control strategy, which would include screening, post-screening and confirmatory steps, is required. Those methods should be sensible enough to detect and / or quantify macrolides in different feedingstuffs at the target level of 1.0 mg $kg^{-1}$. The

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selection of the target level was based on the reported lowest dosage of additive needed to lead a growth promoting effect [4].

For the determination of various residues and contaminants in specific food items Commission Decision 2002/657/EC introduced a validation guideline [5], which classifies the analytical methods in two main groups so called screening and confirmatory methods. Depending on the intended use of the method they should fulfil different performance requirements. Overall screening methods are designed to detect the presence of the target compound or a class of compounds at the level of interest. The main role of screening methods is to reduce the workload on the confirmatory methods and therefore ideally they should have the capability for a high throughput and are used to scrutinize large numbers of samples for potential non-compliant results. Moreover screening methods should show an excellent performance to avoid false *negative* results. Conversely to screening methods, confirmatory methods should provide full or complementary information to perform an unequivocal identification and/or quantification of the target compounds, showing a low rate of false *positive* results. In the field of the determination of antibiotics most of the screening methods are microbiological methods whereas confirmatory methods are frequently based on mass spectrometry. Beside those methods there is still a practical interest in developing alternative methods, so called post-screening methods, aimed to further reduce the number of samples submitted to confirmatory analysis. The performance characteristics of the post-screening methods would be between the requirements of the screening and the confirmatory methods.

Most of macrolide antibiotics show a suitable electroactive site, electrochemical detection hence emerged in the early 80's as one of the most appropriate direct detection mode to carry out their monitoring and could therefore also constitute a sound alternative for post-screening purposes within the frame of a control strategy [6]. However, traditional electrochemical detectors are hardly compatible with gradient elution which explains that most of the published works referred to single analyte methods [7–10]. Despite the intrinsic drawbacks of this kind of detectors its suitability for performing multi-analyte determinations of macrolide antibiotics, preferred for regulatory purposes, has been demonstrated through previous publications [11,12]. The use of a new generation of electrochemical detectors, so called coulometric array detectors, capable of working under gradient elution conditions, resulted in a dramatic improvement of method selectivity allowing the development and/or validation of chromatographic methods for the separation of a high number of macrolides in biological matrices [13,14]. Contrary to traditional electrochemical detectors, coulometric array detectors provide the possibility to obtain "on-line" hydrodynamic voltammograms (HDV) which can be considered as the electrochemical equivalent to the absorbance or mass spectrums and therefore constitute a powerful tool for confirmation purposes [15].

When coming to the determination of macrolide antibiotics in feedingstuffs using electrochemical detection, until now and to our knowledge the methods available in the literature are not suitable for the intended purpose. In this frame the objective of this paper was, thus to develop a sample preparation strategy suitable for the simultaneous detection and determination of banned, authorised or non-authorised macrolides in target and non-target feedingstuffs applying liquid chromatography coupled to a coulometric electrochemical detector in order to obtain a whole post-screening analytical procedure suitable for these matrices. Due to the lack of European legislation establishing specific performance requirements for the analysis of antibiotics in feedingstuffs, the performance criteria established in this work have been based on several harmonized guidelines internationally accepted [16–19].

## 2. Experimental

### 2.1. Reagents

The commercial macrolide standards, rosamicin (ROS), oleandomycin phosphate salt (OLE), tylosin tartrate (TYL) and roxithromycin (ROX) were obtained from Sigma–Aldrich (St. Louis, MO, USA) whereas kitasamycin (KIT) and josamycin (JOS) were provided by ICN Biomedicals (Asse-Relegem, BE). Erythromycin (ERY) was purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA) and Spiramycin 1 (SPI) pure standard was obtained from the European Growth Project G6RD-CT-2000-00431 [20] (hereinafter SIMBAG-FEED project).

Potassium phosphate monobasic (reagent P 8709) and potassium phosphate dibasic (reagent P 8584) 1.0 M solutions, 0.2 μm filtered, were provided by Sigma–Aldrich (St. Louis, MO, USA).

Acetonitrile (ACN) HPLC grade and methanol (MeOH) HPLC grade were purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA) and *tert*-buthyl-methyl-ether (MTBE) HPLC grade from Aldrich (Sigma–Aldrich, St. Louis, MO, USA).

Acidic alumina was obtained from Merck.

HLB and MCX Oasis cartridges were obtained from Waters (Milford, MA, USA).

Pure water (18.2 MΩ cm quality) used for the preparation of all the aqueous solutions was obtained from a MilliQ Plus 185 System (Millipore, Molsheim, France).

Feedingstuffs blanks (hereinafter blank samples) and feedingstuffs samples fortified with different antibiotics and growth promoters including spiramycin and tylosin at different concentration levels, so called materials 1–3 (poultry) materials 4–5 (pig) and materials 6–7 (cattle), were obtained from the SIMBAG-FEED project and analyzed as unknown samples (hereinafter real samples).

To carry out the recovery assays additional feed samples were also prepared "in-house" by adding to the blank feedingstuffs the appropriate volume of a mixture containing all the target macrolides (hereinafter spiked samples). These samples were stored overnight at +4 °C protected from the light before submitting them to the sample preparation procedure.

### 2.2. Preparation of standard solutions

Stock solutions of OLE (2.53 g $L^{-1}$), ERY (2.47 g $L^{-1}$), ROX (2.50 g $L^{-1}$), SPI 1 (2.50 g $L^{-1}$), TYL (2.47 g $L^{-1}$), ROS (1.00 g $L^{-1}$), JOS (2.50 g $L^{-1}$) and KIT (2.53 g $L^{-1}$)

were prepared by dissolving the commercial compounds in acetonitrile. These stock solutions were stored away from the light in polypropylene tubes at −28 °C until used.

Working individual and mixture solutions of macrolides were prepared daily from the respective stock solutions by dilution in a mixture of (phosphate buffer 1 M pH 6.3:$H_2O$:ACN (12.5 + 587.5 + 150), hereinafter solvent A, at the appropriate concentrations. All working solutions were kept at +4 °C and protected from the light.

### 2.3. Sample preparation

The grinding and homogeneity of both blank and real samples have been studied in the course of the SIMBAG-FEED project. One outcome of the homogeneity study in particular was the proven homogeneity of all materials for 5 g sample size.

A 5.0 ± 0.1 g sized feedingstuff sample was therefore selected as appropriate sample size and placed in a 50 mL falcon tube, the powder was then thoroughly mixed with 10 mL of water in order to wet all the feed, and subsequently another 10 mL of MeOH was added, manually mixed and submitted to head over heels agitation for 30 min. After that, the sample was centrifuged at 4000 × *g* for 10 min. A 5 mL aliquot of the supernatant was collected and reduced up to ∼2 mL under a gentle stream of nitrogen at 37 °C. Once the volume was reduced, the mixture was centrifuged at 4000 × *g* for another 5 min. The obtained supernatant was loaded on a home-made acidic alumina column (1.0 ± 0.1 g) and collected by gravity on a HLB Oasis cartridge previously conditioned with 3 mL of MeOH followed by 3 mL of water. Once all the liquid was passed by gravity through the HLB Oasis cartridge a washing step, aimed to remove matrix interferents, was performed by passing through it another 4 mL of a mixture $NH_4OH$: $H_2O$: MeOH 5:35:60 v:v:v. After the washing step the macrolides were eluted from the HLB Oasis cartridge with 2 mL of *tert*-methylbutyl ether (MTBE) and collected on a glass tube. The washing and the elution steps have been performed by gravity. The collected eluate was then evaporated up to dryness under a gentle stream of nitrogen at 37 °C. The dried residue was finally re-dissolved in 1 mL of solvent A containing the internal standard (ROX) at a concentration of 2 μg $mL^{-1}$.

### 2.4. Matrix-matched calibration standards

In order to prepare the matrix-matched calibration standards, a blank feedingstuff was submitted to the procedure detailed in Section 2.3. The dried residue obtained after the whole procedure was dissolved back in 1 mL of MTBE, aliquoted, re-evaporated and finally re-dissolved in the same volume of solvent A containing the internal standard (ROX) at a concentration of 2 μg $mL^{-1}$ and the target macrolides at the appropriate concentration level.

### 2.5. Chromatographic separation and electrochemical detection

All chromatographic measurements were performed with a HPLC system SERIE VP (Shimadzu Benelux, Hertogenbosh, NL) followed by a multichannel COULARRAY coulometric detector (ESA Belford, MA, USA). Separation was performed with a Waters Corp. (Milford, MA, USA) SymmetryShield RP8 (150 mm × 3.9 mm; 5 μm particle size) analytical column. A binary mobile phase was constituted by a mixture of solvent A (phosphate buffer 1 M pH 6.3:$H_2O$:ACN (12.5 + 587.5 + 150)), and solvent B (phosphate buffer 1 M pH 6.3:$H_2O$:ACN (12.5 + 237.5 + 600)). The content of the solvent B was increased from 20 to 80% over 30 min. The initial proportion of solvent B (20%) was then pumped for 10 min before the next injection was started. The mobile phase flow-rate was set at 0.8 mL $min^{-1}$ and the separation was performed at 50 °C.

The selection of the quantification channel required a trade-off between the larger signal given by the target macrolides and the lowest signal given by the matrix components. The cumulative signal of the channels set at 800 mV and at 850 mV was found optimum to perform the quantification of the macrolides in feedingstuffs.

## 3. Results and discussion

### 3.1. Optimisation of the sample preparation procedure

Even when there are a relatively large number of publications related to the analysis the macrolides most of them are developed for the analysis of a single macrolide. Preliminary tests, performed with sample preparation procedures that were successfully applied for the determination of a similar group of macrolides in animal tissues, or biological fluids lead to dirty extracts not suitable for the quantification of the selected antibiotics at the target level in poultry feed when using electrochemical detection [13,21,28]. In the present method, our challenge was therefore to develop a sample preparation procedure suitable for the electrochemical detection and quantification of several banned or non-authorised macrolide antibiotics in feedingstuffs using liquid chromatography. Bearing in mind the specific characteristics of our determination, mainly in terms of detection requirements and chemical nature of the target antibiotics, several studies were performed, initially with standard mixtures and afterwards with spiked feedingstuffs, in order to optimize the different steps involved in the sample preparation procedure. The main conclusions obtained from each of the performed assays can be summarized as follows:

- Based on the solubility of the target macrolides and the composition of the feedingstuffs a mixture of MeOH and $H_2O$ was considered as the most appropriate to carry out the solid-liquid extraction.
- The obtained extract was partially evaporated under a $N_2$ stream at 37 °C before the clean-up procedure for reducing the percentage of MeOH below 5% [21] in order to prevent the breakthrough of the macrolides from the SPE cartridges within the loading step.
- The SPE method was selected for performing the clean-up of the extracts. Based on literature [21–28] polymeric and cationic stationary phases were tested.

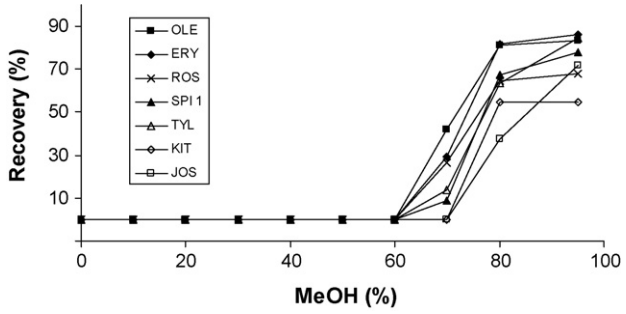


Fig. 1. Elution profiles of standard mixtures of oleandomycin (OLE), erythromycin (ERY), rosamicin (ROS), spiramycin 1 (SPI 1), tylosin (TYL), kitasamycin (KIT) and josamycin (JOS) prepared in MeOH:$H_2O$ 50:50 using Oasis HLB cartridges. Full experimental conditions provided in the text.

- Elution profiles were established with different MeOH: $NH_4$: $H_2O$ mixtures in both kind of SPEs. Better behaviour was observed when using a polymeric phase, therefore HLB Oasis cartridges were selected (Fig. 1). A washing step with 4 mL of a mixture MeOH:$NH_4$:$H_2O$ (60:5:35) was found optimum to minimize the interferents from the feedingstuff matrix without having a significant breakthrough of the target compounds.
- Different elution solvents were tested with spiked feedingstuffs. An elution step with 2 mL of MTBE shows the best trade-off between recovery and selectivity.
- The loading, washing and eluting flow rate have been proven to have a critical effect on the obtained recoveries as well as in their reproducibility. The best results were obtained when all the steps were carried out by gravity.
- Two major endogenous signals appearing around the retention time of spiramycin and oleandomycin (Fig. 2a) have been notably reduced by passing the partially evaporated extract through a $1.0 \pm 0.1$ g home-made acidic alumina column before loading it on the HLB Oasis cartridge (Fig. 2b).

The whole sample preparation procedure was detailed in Section 2.3.

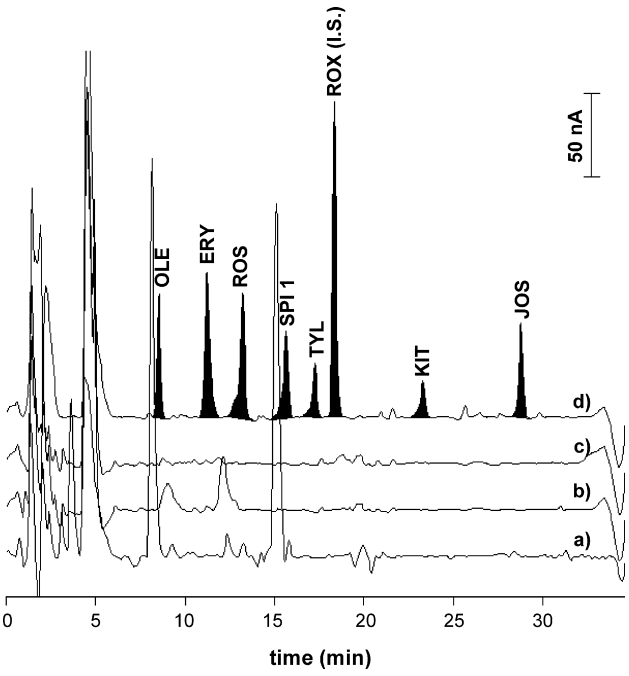


Fig. 2. Chromatogram profiles obtained with blank poultry feedingstuffs extracts submitted to different clean-up procedures. (a) After Oasis HLB clean-up, (b) after acidic $Al_2O_3$ plus Oasis HLB clean-up, (c) after acidic $Al_2O_3$ plus Oasis HLB clean-up plus an additional back extraction in MeOH and (d) standard mixture of the seven target macrolides at a concentration of 1.0 mg $L^{-1}$. See text for other experimental conditions.

### 3.2. Quantification approach

The sample preparation procedure developed in this work lead to reasonable clean extracts. This observation raised the possibility of using a standard calibration curve to attempt the quantification of the target macrolides in feedingstuffs. This approach is always advisable, whenever possible, because it overcomes the difficulty of finding representative blanks, which is a handicap for the analysis of samples such as feedingstuffs characterised by their very complex composition. Unfortunately,

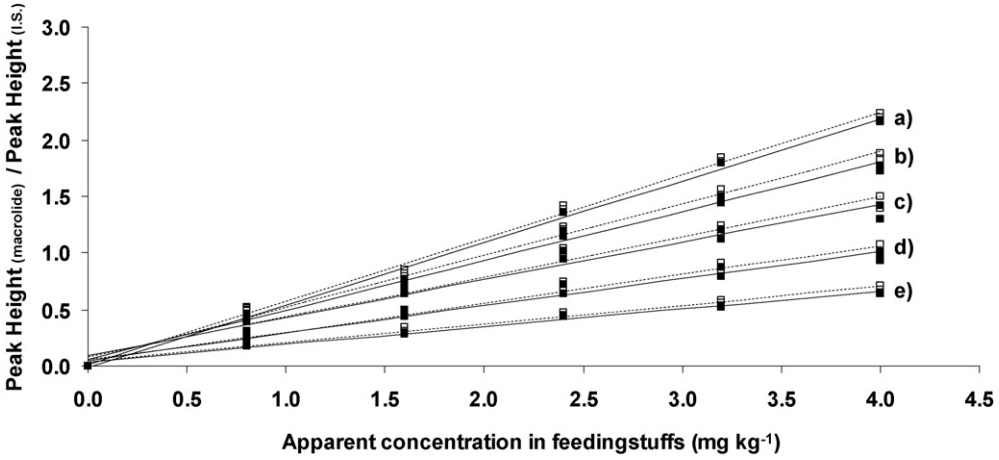


Fig. 3. Typical calibration curves obtained by using standard calibrants and matrix-matched calibrants. (a) ERY, (b) OLE, (c) JOS, (d) TYL and (e) KIT. Dashed lines and hollowed squares represent matrix-matched calibrants and solid lines and filled symbols represent standard calibrants. See text for other experimental conditions.

Table 1a
Recoveries obtained in poultry feed blank spiked at different levels of concentration

| | Poultry feed spiked at 0.5 mg kg$^{-1}$ ($N$ = 6) | | Poultry feed spiked at 1.0 mg kg$^{-1}$ ($N$ = 6) | | Poultry feed spiked at 2.0 mg kg$^{-1}$ ($N$ = 6) | | Averaged ($N$ = 18) | |
|---|---|---|---|---|---|---|---|---|
| | Recovery (%) | S.D.[a] | Recovery (%) | S.D.[a] | Recovery (%) | S.D.[a] | Recovery (%) | S.D.[a] |
| OLE | 136.3 | 5.6 | 70.6 | 6.1 | 84.7 | 14.4 | 97.2 | 30.4 |
| ERY | 84.4 | 3.4 | 78.1 | 4.6 | 82.8 | 3.2 | 81.8 | 4.5 |
| ROS | 77.5 | 11.1 | 64.9 | 4.9 | 80.1 | 6.4 | 74.2 | 10.1 |
| SPI 1 | 82.1 | 2.8 | 94.3 | 10.8 | 97.6 | 2.8 | 91.3 | 9.3 |
| TYL | 98.4 | 8.8 | 82.6 | 9.0 | 97.7 | 5.1 | 92.9 | 10.5 |
| KIT | 110.9 | 12.2 | 95.8 | 8.0 | 108.0 | 4.6 | 104.9 | 10.7 |
| JOS | 108.4 | 9.6 | 101.7 | 9.5 | 111.6 | 4.6 | 107.2 | 8.8 |

[a] Standard deviation expressed as percentage.

performing the quantification with matrix matching or with standard calibration curves usually lead to significant differences in the obtained signals, due to the effect of the matrix on the detector, and therefore in the final results. In this case in the frame of routine control analyses, the standard addition approach has to be applied since the matrix of the unknown samples is in general not available to the laboratories.

In order to investigate the occurrence of matrix effects, the target analytes in the poultry feed matrix were quantified against calibration solutions containing the pure standard compounds and calibration solutions prepared on cleaned extracts from a blank matrix. The calibration curves obtained with the two sets of solutions show the equivalence of both approaches (Fig. 3). Furthermore the values of the slopes and the origin ordinates calculated with one set of calibrants (matrix-matched calibration approach) are within the confidence limits ($P$ = 0.05) of the ones obtained using the other set of calibrants (standard calibration approach). Therefore it can be concluded that both calibration curves are equivalent at this probability and can be used indistinctly. The limits of detection (LODs), calculated as three times the signal-to-noise ratio of different poultry blanks ($n$ = 8), obtained within the retention time plus minus the width of the peak for each macrolide ranged from 0.02 to 0.61 mg kg$^{-1}$ (expressed as apparent concentration of the macrolides in poultry feed).

### 3.3. Recovery assay in spiked poultry samples

In order to estimate the recovery of the analytical procedure, different poultry feeds were spiked in duplicate at three concentration levels (three repetitions of two independent samples for each concentration level, i.e. $N$ = 6). The spiked samples were left overnight and then submitted to the sample procedure and analyzed as detailed above. The quantification was carried out with standard calibration curves (Table 1a). No pattern has been found with the concentration, which suggests a non-dependence of the obtained recoveries with the level of concentration. The unexpected high recovery obtained for OLE of about 140% at the lowest concentration level tested (0.5 mg kg$^{-1}$), seems to suggest an overestimation of this macrolide at low concentrations, probably due to an endogenous interferent appearing nearby the retention time of OLE (Fig. 2b). Nevertheless the abovementioned sample preparation is fully suitable for the determination of the six remaining macrolides (ERY, ROS, SPI 1, TYL, KIT and JOS) showing mean recoveries between 74 and 107% with relative standard deviation values below 13.6%.

#### 3.3.1. Analysis of real poultry samples

The sample preparation described above was applied for the identification and quantification of several real samples of poultry feed, so called materials 1, 2 and 3. The three different materials were analyzed in duplicate (three repetitions of two independent samples for each material, $N$ = 6). The analysis of the materials results on the successful identification of one or two target macrolides. The experimental mean recoveries of the materials are stated in Table 2. These results are within the range of recoveries (mean ± 2S.D.) calculated with spiked poultry feedingstuffs which demonstrate a good fitting between real and spiked samples.

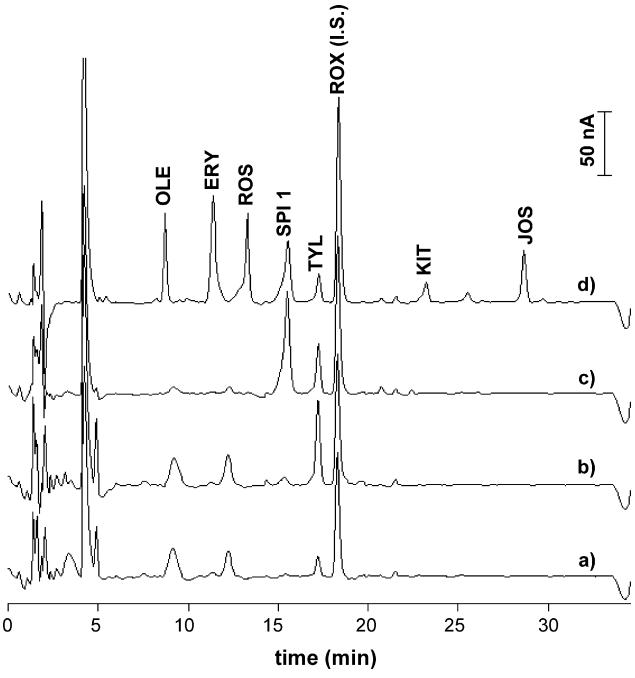


Fig. 4. Typical chromatograms obtained with real poultry feedingstuffs samples without the back extraction in MeOH. (a) Material 2, (b) material 1, (c) material 3 diluted 1:3 and (d) standard mixture of the seven target macrolides at a concentration of 1.0 mg L$^{-1}$.

Table 1b
Recoveries obtained in poultry feed blank spiked at different levels of concentration with a back extraction in MeOH

| | Poultry feed spiked at 0.5 mg kg$^{-1}$ ($N$ = 6) | | Poultry feed spiked at 1.0 mg kg$^{-1}$ ($N$ = 6) | | Poultry feed spiked at 2.0 mg kg$^{-1}$ ($N$ = 6) | | Averaged ($N$ = 18) | |
|---|---|---|---|---|---|---|---|---|
| | Recovery (%) | S.D.[a] | Recovery (%) | S.D.[a] | Recovery (%) | S.D.[a] | Recovery (%) | S.D.[a] |
| OLE | 75.9 | 4.6 | 74.7 | 2.0 | 65.8 | 6.4 | 72.2 | 6.4 |
| ERY | 86.4 | 1.3 | 81.6 | 3.5 | 78.1 | 3.0 | 82.0 | 4.3 |
| ROS | 54.3 | 5.1 | 63.2 | 2.4 | 66.0 | 3.0 | 61.2 | 6.2 |
| SPI 1 | 67.6 | 1.6 | 80.0 | 3.0 | 76.7 | 3.6 | 74.8 | 6.0 |
| TYL | 88.6 | 4.1 | 76.6 | 4.9 | 77.2 | 3.2 | 80.8 | 6.9 |
| KIT | 92.7 | 0.9 | 84.3 | 3.7 | 90.0 | 3.1 | 89.0 | 4.5 |
| JOS | 101.0 | 2.2 | 94.2 | 1.8 | 96.8 | 2.6 | 97.3 | 3.5 |

[a] Standard deviation expressed as percentage.

Table 2
Precision and recoveries obtained with poultry SIMBAG materials

| | Nominal concentration (mg kg$^{-1}$) | Experimental concentration (mg kg$^{-1}$) | | Recovery (%) | S.D.[a] |
|---|---|---|---|---|---|
| | | Range | Mean | | |
| Material 1 | | | | | |
| TYL ($n$ = 6) | 4.0 | 3.24–4.02 | 3.59 | 89.9 | 8.4 |
| Material 2 | | | | | |
| TYL ($n$ = 6) | 1.0 | 0.77–0.94 | 0.85 | 86.0 | 6.4 |
| Material 3 | | | | | |
| SPI 1 ($n$ = 6) | 7.2 | 5.37–6.27 | 5.58 | 77.5 | 4.8 |
| TYL ($n$ = 6) | 6.9 | 6.07–6.57 | 6.32 | 91.4 | 2.8 |

[a] Standard deviation expressed as percentage.

Table 3a
Precision and recovery for different feedingstuffs spiked at the target level with a back extraction in MeOH

| | Cattle feed spiked at 1.0 mg kg$^{-1}$ ($N$ = 10) | | Pig feed spiked at 1.0 mg kg$^{-1}$ ($N$ = 10) | | Poultry feed spiked at 1.0 mg kg$^{-1}$ ($N$ = 6) | | Averaged ($N$ = 26) | |
|---|---|---|---|---|---|---|---|---|
| | Recovery (%) | SD[a] | Recovery (%) | SD[a] | Recovery (%) | SD[a] | Recovery (%) | SD[a] |
| OLE | 80.6 | 2.0 | 84.5 | 1.4 | 74.7 | 2.0 | 80.7 | 4.1 |
| ERY | 115.0 | 8.8 | 92.2 | 7.7 | 81.6 | 3.5 | 98.5 | 15.0 |
| ROS | 59.0 | 4.8 | 54.5 | 3.5 | 63.2 | 2.4 | 58.2 | 5.1 |
| SPI 1 | 72.1 | 6.1 | 68.7 | 7.6 | 80.0 | 3.0 | 72.6 | 7.5 |
| TYL | 73.4 | 4.5 | 69.7 | 2.8 | 76.6 | 4.9 | 72.7 | 4.7 |
| KIT | 88.8 | 6.5 | 80.6 | 3.0 | 84.3 | 3.7 | 84.6 | 5.9 |
| JOS | 100.3 | 5.8 | 93.2 | 4.1 | 94.2 | 1.8 | 96.1 | 5.5 |

[a] Standard deviation expressed as percentage.

### 3.4. Inclusion of the determination of oleandomycin

The analysis of spiked poultry feedingstuffs indicated some problems in the quantification of OLE at the lowest concentration tested (0.5 mg kg$^{-1}$) revealed by extremely high recoveries obtained for this macrolide. This can be explained for the remaining endogenous interferent in some poultry blanks nearby the retention time of OLE. Even when the recovery and precision results obtained including those measurements are within the limits commonly accepted when multilevel recovery is calculated for this family of antibiotics [22,29] the presence of this endogenous signal could lead to false positives when real samples are analyzed (Fig. 4). It should therefore not be advisable to use the method to carry out the detection and/or quantification of OLE. In order to extend the applicability of the method for OLE,

Table 3b
Overall precision and recovery values with a back extraction in MeOH

| | $N$ = 38 | |
|---|---|---|
| | Recovery (%) | S.D.[a] |
| OLE | 77.6 | 7.06 |
| ERY | 93.4 | 14.8 |
| ROS | 58.8 | 5.8 |
| SPI 1 | 72.5 | 6.8 |
| TYL | 75.9 | 7.2 |
| KIT | 86.7 | 6.0 |
| JOS | 97.0 | 5.0 |

[a] Standard deviation expressed as percentage.

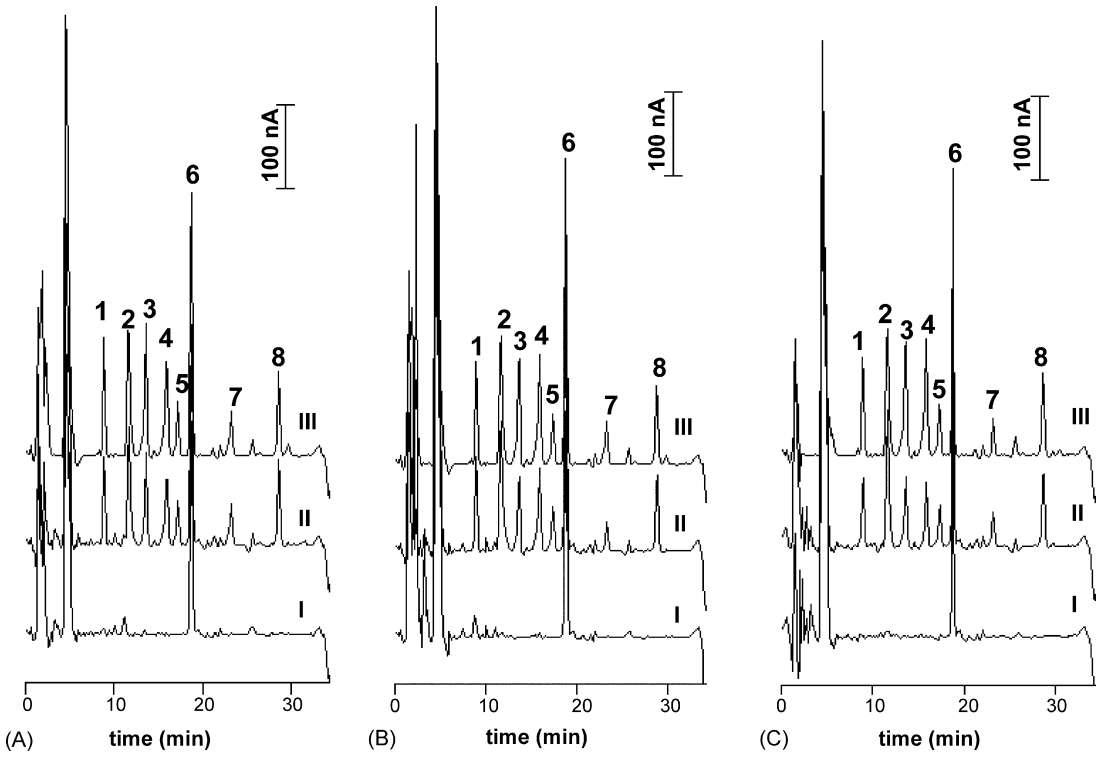


Fig. 5. Chromatogram profiles obtained with different types: (A) cattle, (B) pig and (C) poultry feedingstuffs extracts submitted to the sample preparation including the back extraction in MeOH. (I) Blank feedingstuffs, (II) feedingstuffs spiked with the target macrolides at a concentration of 1.0 mg $kg^{-1}$ and (III) standard mixture of the seven target macrolides at a concentration of 1.0 mg $L^{-1}$. Peak identification—(1) OLE, (2) ERY, (3) ROS, (4) SPI 1, (5) TYL, (6) ROX (IS), (7) KIT and (8) JOS.

Table 4
Precision and recovery for different SIMBAG materials with a back extraction in MeOH

| | Nominal concentration (mg $kg^{-1}$) | Experimental concentration (mg $kg^{-1}$) | | Recovery (%) | S.D.[a] |
|---|---|---|---|---|---|
| | | Range | Mean | | |
| Poultry feed | | | | | |
| Material 1 | | | | | |
| TYL | 4.0 | 3.05–3.26 | 3.14 | 79.1 | 2.7 |
| Material 2 | | | | | |
| TYL | 1.0 | 0.68–0.69 | 0.68 | 68.9 | 0.8 |
| Material 3 | | | | | |
| SPI 1 | 7.2 | 4.87–6.10 | 5.41 | 75.2 | 8.7 |
| TYL | 6.9 | 5.06–5.67 | 5.26 | 75.7 | 4.9 |
| Pig feed | | | | | |
| Material 4 | | | | | |
| SPI 1 | 0.7 | 0.48–0.54 | 0.51 | 73.1 | 3.9 |
| Material 5 | | | | | |
| SPI 1 | 3.6 | 2.21–2.57 | 2.37 | 65.8 | 4.1 |
| Cattle feed | | | | | |
| Material 6 | | | | | |
| SPI 1 | 1.4 | 1.02–1.07 | 1.04 | 74.6 | 1.7 |
| TYL | 2.0 | 1.53–1.64 | 1.58 | 79.6 | 2.4 |
| Material 7 | | | | | |
| SPI 1 | 2.9 | 1.64–1.90 | 1.77 | 61.1 | 3.4 |
| TYL | 5.0 | 3.05–3.51 | 3.25 | 65.5 | 3.7 |

[a] Standard deviation expressed as percentage.

an additional back extraction in MeOH was introduced. Therefore the MTBE eluate residue obtained from the SPE procedure was subsequently re-dissolved in 2 mL of MeOH. After closure of the tube, it was submitted to sonication for 5 min in order to facilitate the re-dissolution of the dry residue. The resulting solution was then aliquoted, re-evaporated and finally re-dissolved in the appropriate volume of solvent A containing the internal standard at a concentration of 2.0 $\mu g\,mL^{-1}$. This additional step leads to a further clean-up of the samples making the whole procedure more selective. As expected this additional step lead to a cleaner chromatogram pattern without any endogenous signal which allowed the reliable quantification of OLE (Fig. 2c) making thus the latter method suitable for the determination of seven macrolides in poultry feed in a single run.

### 3.4.1. Recovery assay in poultry spiked samples

The influence of this additional step in the recovery of the macrolides was evaluated by repeating the recovery assay described in Section 3.3. Therefore independent poultry feeds were again spiked in duplicate at three concentration levels (three repetitions of two independent samples for each concentration level i.e. $N = 6$) and submitted to the sample preparation described in Section 2.3. but now including the additional back extraction in MeOH. The new procedure lead to a general decrease on the mean recoveries as well as an increment of the precision for all the target macrolides at the tested concentration levels (Table 1b). Moreover likewise without the additonal back extraction step, no pattern has been found with the concentration which proved the validity of the previous conclusions regarding the concentration level. Furthermore the obtained recoveries for all tested macrolides ranged from 61 to 97% and are within the values reported for this kind of analysis [4,28]. Experimental detection limits, calculated as three times the signal-to-noise ratio of different poultry blanks, obtained within the retention time plus minus the width of the peak for each macrolide ranged from 0.04 to 0.22 $mg\,kg^{-1}$. Considering the target level of 1.0 $mg\,kg^{-1}$ for each compound the obtained LODs are considered acceptable.

Overall the new sample procedure lead to lower but still satisfactory and more precise recoveries (relative standard deviation values below 10.1%). In addition and conversely to the previous method no interference was now observed for any of the tested macrolides which makes this new procedure more selective, reducing the possibility of false positives and suitable for

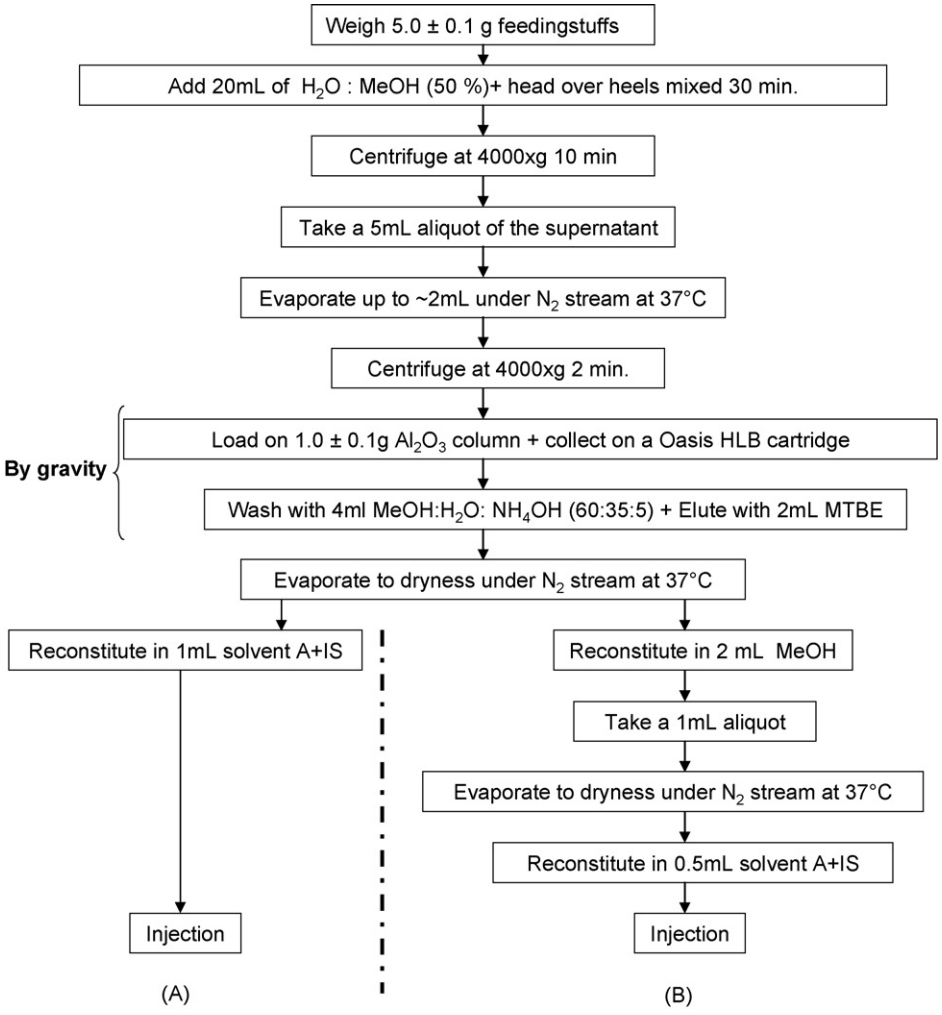


Fig. 6. Scheme of the sample preparation procedures: (A) without the back extraction in MeOH and (B) with the back extraction in MeOH.

the reliable determination of the seven macrolides at a lower concentration level.

*3.4.2. Extension of the recovery assay to other animal feed*

Once checked that there was no dependence of the recoveries obtained with the concentration level (with and without the additional back extraction in MeOH) another assay was performed in order to investigate a possible dependence of the recovery rate with the type of feedingstuffs. The aim of this study was to prove the feasibility to extend the applicability of the proposed method to other animal feedingstuffs, enlarging therefore the scope of the method.

Different feedingstuffs (cattle and pig) were thus spiked in duplicate at the target level ($1.0\,mg\,kg^{-1}$) and after leaving them overnight the samples were submitted to the latest sample preparation procedure involving the additional methanol back extraction. Table 3a depicts the obtained recoveries for each kind of feedingstuffs (poultry, cattle and pig). As can be seen in Fig. 5 similar chromatogram profiles were obtained for each macrolide in the three kinds of feedingstuffs tested, which suggest the validity of the conclusions previously stated with the poultry feedingstuffs. Furthermore, no relevant effect was observed on the obtained recoveries as regards the kind of feedingstuffs which indicates the absence of a matrix mismatch.

Once proved that the recoveries obtained are independent from the concentration level (Section 3.4.1) and from the nature of feedingstuffs analyzed (Section 3.4.2) experimental recovery limits can be established for each target macrolide by pooling the results obtained in the sections mentioned above and defined as previously i.e. mean ± 2S.D. (Table 3b).

*3.4.3. Analysis of real cattle, pig and poultry samples*

The assumptions described above were corroborated by analysing real samples of different materials so called materials 1, 2 and 3 (poultry feedingstuffs) materials 4 and 5 (pig feedingstuffs) and materials 6 and 7 (cattle feedingstuffs). Description of the nominal content of each macrolide in the tested materials is stated in Section 2.1. The obtained results are depicted in Table 4.

The analyses of the different materials lead to the identification of one or two target macrolides in the materials. The experimental concentration obtained with the test samples were within the recovery ranges established with spiked samples. Therefore the fit for purpose of the method proposed for those macrolides, as well as a good fitting between real and spiked samples was demonstrated.

A scheme of both sample preparation procedures is shown in Fig. 6.

## 4. Conclusions

A full sample preparation procedure, suitable first for the analysis of up to six macrolides (ERY, ROS, SPI 1, TYL, KIT and JOS) in poultry feedingstuffs by liquid chromatography coupled to an electrochemical detector has been developed in this paper. Due to the complexity of the matrices and the instrumental requirements, the development was focussed in the clean-up of the obtained extracts. A recovery assay performed in poultry feed indicated a non-dependence of the recoveries with the concentration level.

It has been proven that the optimised sample preparation, mainly focussed on the clean-up procedure, allows the quantification of the target macrolides in poultry feedingstuffs by using standard calibration curves. The proposed method has been successfully applied in test poultry feed samples demonstrating a good fitting between spiked and real samples.

The inclusion of a back extraction in methanol as an additional step within the sample preparation procedure makes possible to extend the determination up to seven macrolides (OLE, ERY, ROS, SPI 1, TYL, KIT and JOS). This additional step lead to lower recoveries in spiked poultry feed and moreover also provided cleaner chromatograms profiles and more precise recoveries without a dependence on the concentration level. The suitability of the modified sample preparation procedure in different animal feedingstuffs (poultry, cattle and pig) has been successfully proven at the target level of concentration. All matrices lead to similar recovery values which indicates a non-dependence of the recoveries with the kind of feedingstuffs and consequently the applicability of the method to different animal feedingstuffs. In addition, the analysis of test samples poultry cattle and pig lead to similar results than the ones obtained with spiked samples.

Both methods gave recovery and precision values within the range of the methods previously published for this kind of analysis and therefore demonstrate that the electrochemical detection is a sound alternative to carry out the analysis of the target macrolides in feedingstuffs within the frame of a control strategy (post-screening method) and/or monitoring surveillances.

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