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Journal of Chromatography A, 1066 (2005) 1–7
JOURNAL OF CHROMATOGRAPHY A
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Review

# Dynamic ultrasound-assisted extraction of colistin from feeds with on-line pre-column derivatization and liquid chromatography-fluorimetric detection

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Received 16 September 2004; received in revised form 15 December 2004; accepted 5 January 2005
Available online 20 January 2005

**Abstract**

A dynamic ultrasound-assisted extraction (UAE) method with on-line pre-column derivatization/high performance liquid chromatography (HPLC) and fluorimetric detection is proposed for the analysis of colistin in feed. A flow injection manifold is used for the development of the extraction and derivatization steps and for interfacing them with the separation/detection step, thus providing an on-line approach with the advantage of minimum sample handling. The derivatization was performed with *ortho*-phthaldialdehyde and 2-mercaptoethanol. The optimum conditions for colistin extraction and formation of the fluorescent derivative have been obtained by experimental design methodology. The use of a high-intensity probe sonication makes UAE an expeditious (7 min versus >1 h) and efficient (93.1–98.2% versus 87.5–94% of recovery) alternative as compared with extraction using an ultrasonic bath. The within-laboratory reproducibility and repeatability, expressed as percentage of relative standard deviation, were 5.2 and 5.8, respectively.


*Keywords:* Ultrasound-assisted extraction; HPLC; Colistin; Feed

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0021-9673/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.chroma.2005.01.010

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## 1. Introduction

Colistin (polymyxin E), an antibiotic produced by *Bacillus polymyxa* subsp. *colistinus* [1], consists of a cyclic heptapeptide and a side-chain of three amino acids acylated at the N-terminus by a fatty acid (Fig. 1). It is a complex mixture of at least 13 different components [2], the two main ones being colistin A (polymyxin E1) and colistin B (polymyxin E2), which differ only in the fatty acid side-chain. The sulfate salt of this antibiotic is widely used in veterinary practice as drug or feed additive against certain infectious diseases caused by Gram-negative bacterias. In particular, colistin commonly used in the treatment of intestinal infections [3], is often not administered in a conventional unit dosage, but by addition of the drug (liquid and/or solid medicated premix) to animal feed.

To prevent errors with veterinary doses, European authorities pressurize to check homogeneity, stability and storability of the antimicrobial formulations, both in the medicated premixes and the medicated feed produced with them [4]. So, this antibiotic has been measured in pharmaceutical formulations [5,6] and feed [7,8], and also in plasma [9], tissues [10], etc. Conventional methods described in the literature for colistin analysis are based on microbiological or immunological assays [11,12]. These methods, subjected to problems, such as low sensitivity, and low stability, are time consuming. Recently, this antibiotic has been determined by electrophoresis [13], and by high performance liquid chromatography (HPLC) with UV [8], fluorimetric [7–9], or mass spectrometry detection [5,6]. Although direct HPLC determination of colistin employing UV detection is possible, the sensitivity is not high enough. Thus, HPLC with derivatization and fluorimetric detection is preferable. An easy, well established way for derivatization of compounds, containing free amino groups is the reaction of the fluorogenic reagent *ortho*-phthaldialdehyde (OPA) with these groups at alkaline pH in the presence of a nucleophilic substance, such as 2-mercaptoethanol (ME) or mercaptopropionic acid (MPA), which forms fluorescent isoindole derivatives [14–16]. Fluorescamine and naphthalene 2,3-dicarboxaldehyde [17] and dansyl chloride [9] has also been used for the derivatization of this antibiotic. HPLC-fluorimetric approaches using both manual pre-column [7] and automatic post-column [8] derivatization with OPA-ME have been used for the analysis of colistin in feed. Pre-column derivatization is preferred over post-column reaction methods in HPLC because, with the latter, the fluorescence background resulting from the reaction between impurities in the mobile phase and the derivatization reagent interferes with the determination of the analyte.

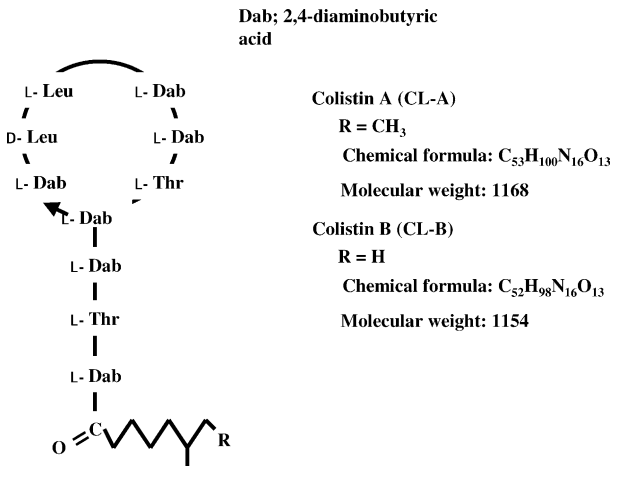


Fig. 1. Structure of colistin.

The analysis of colistin in feed requires sample preparation prior to the determination of the analyte. This preparation usually consists of an extraction step, which involves analyte removal from the solid sample to the appropriate liquid phase, in order to obtain a solution where the analyte can, subsequently, be determined by a chromatographic-detection technique. The extraction of colistin from solid samples, such as feed has traditionally been performed by sonication in an ultrasonic bath with subsequent mechanical shaking [8,10]. This sample pre-treatment is time consuming and involves the use of high volume of extractant. Moreover, although ultrasonic baths have been more widely used for the ultrasound-assisted extraction (UAE) of a number of analytes from different types of samples [18–20], including colistin from feed, ultrasonic probes (UP) are more efficient as they focus the ultrasonic energy on the sample zone [21], which results in more experimental reproducibility and repeatability. Furthermore, UP are more versatile as they can be incorporated in continuous extraction systems, with miniaturization of the extraction step, thus expediting the extraction process considerably and facilitating the coupling of extraction with other steps of the analytical process.

The aim of the present research was to develop a simple, sensitive and efficient method for the analysis of colistin in feed. The proposed approach consists of the cou-

pling of dynamic UAE, continuous pre-column derivatization with OPA-ME, and HPLC-fluorimetric detection of the target analyte. The dynamic UAE approach, assisted by an UP, consists of a closed system in which a preset volume of extractant is circulated to and fro through the solid sample until the partition equilibrium of the target analyte between the solid sample and a given volume of extractant is established.

## 2. Experimental

### 2.1. Instruments and apparatus

Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (Branson, Danbury, CT, USA) equipped with a cylindrical titanium alloy probe, which was immersed in a water bath in which the extraction cell was placed. An extraction chamber consisting of a stainless steel cylinder (12 cm in length and 10 mm i.d.) closed with screws at either end was used, allowing circulation of the leaching solvent through it. The screw caps were covered with a cellulose filter to ensure that the sample remained in the extraction chamber.

A Gilson Minipuls-3 low-pressure programmable peristaltic pump (Gilson, Worthington, OH, USA), programmed for changing the rotation direction at preset intervals, a low-pressure injection valve (Rheodyne, Cotati, CA, USA) and Teflon tubing of 0.8 mm i.d. were used to build the flow manifold.

For conventional extraction, a mechanical shaker and a centrifuge (both from Selecta, Barcelona, Spain) were used.

The determination was carried out with an HP1100 liquid chromatograph (Hewlett Packard, Avondale, PA, USA) consisting of a G1311A high-pressure quaternary pump, a G1322A vacuum degasser, a Rheodyne 7725 high-pressure manual injector valve (20 μl injection loop) and a fluorimetric detector (Hitachi, Tokyo, Japan). An Ultrabase $C_{18}$ (250 mm × 4.6 mm; 5 μm particle size from Scharlau, Barcelona, Spain) was used as analytical column.

### 2.2. Standards and reagents

Colistin sulphate (COL) (Sigma-Aldrich, Steinheim, Germany) was used for preparing the stock standard solution (1000 μg/ml) in distilled water. Distilled water was also used as extractant. Hydrochloric acid (Panreac, Barcelona, Spain) at different concentrations in aqueous solution was tested as extractant. Phthaldialdehyde (OPA) of synthesis grade and 2-mercaptoethanol (ME) for analysis (Sigma-Aldrich) were used as derivatization reagent, which was prepared by mixing the OPA (80 mg) in methanol (15 ml) with borate buffer (15 ml) and ME (0.1 ml), as described Decolin et al. [10]. Methanol, boric acid and sodium hydroxide were from Panreac. HPLC-grade acetonitrile (Panreac) was used as mobile phase in the chromatographic step.

### 2.3. Samples

A hundred grams of feed spiked with COL to obtain a final concentration of 150 μg/g were used to carry out the study. This concentration corresponds with the usual dose administered to the animals mixed with feed [4]. Commercial medicated feed was also analyzed.

### 2.4. Procedures

#### 2.4.1. Conventional extraction

Five grams of feed were weighed and introduced in a 250 ml glass-stoppered Erlenmeyer. A hundred ml of 50% methanol aqueous solution were added. Then, the mixture was mechanically shaken at room temperature during 60 min and centrifuged for 10 min at 2000 rpm. Four hundred and 50 μl of the resulting extract was manually injected into the continuous derivatization/separation/detection system.

#### 2.4.2. Ultrasound-assisted extraction

An amount of 0.1 g of feed was placed in the extraction chamber, which was assembled with the flow inyection (FI) manifold shown in Fig. 2. This chamber was immersed in a water bath at room temperature, and then, the ultrasonic probe was located at 1 mm from the top surface of the chamber. The closed system (with a total volume of 3 ml) was filled with the leacher-carrier (water) impelled by the programmable peristaltic pump PPP by maintaining selected the leacher-carrier (L-C) through valve SV1 by valve SV2 in the closed-circuit position. The water was then circulated to and fro through the solid sample at 2.5 ml/min (with the help of PPP) during 7 min under ultrasonic irradiation (duty cycle 1 s, output amplitude 100% of the converter nominal amplitude). The forward-and-backward movement of the extractant hinders to compactness of the sample in the chamber that could cause overpressure of the system. After extraction, the closed system was unloaded by switching valve SV2 and the extract was driven to the on-line derivatization/detection system.

#### 2.4.3. Derivatization

When the extraction was finished, the extract was driven to the 450-μl loop of the injection valve IV. At the same time, the peristaltic pump PP was activated and both the carrier (C) and the derivatization reagent (DR) were circulated through the FI manifold at 0.3 and 0.7 ml/min, respectively, thus establishing the baseline in the detector. Once the sample loop was filled, the injection valve IV was switched and the content of the loop was injected into the FI manifold. The derivatization product was formed in the reaction coil RC (2-m length) before reaching the HPLC system.

For calibration, the derivatization procedure was the same as for the samples, but in this case the loop of valve IV was manually filled with the standard solutions with the help of a syringe.

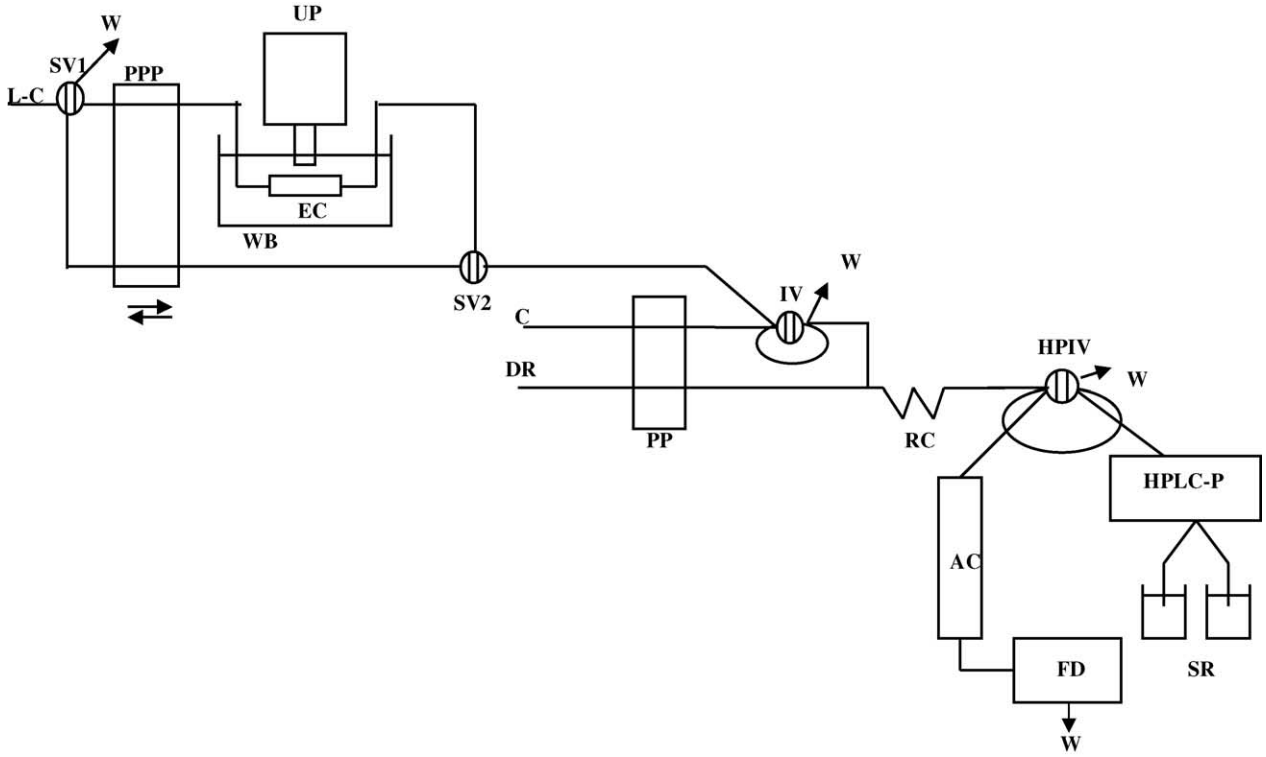


Fig. 2. Experimental set-up. L-C, leacher-carrier; SV, selection valve; PPP, programmable peristaltic pump; W, waste; UP, ultrasonic probe; EC, extraction chamber; WB, water bath; DR, derivatization reagent; C, carrier; PP, peristaltic pump; IV, injection valve; RC, reaction coil; HPIV, high-pressure injection valve; SR, solvent reservoirs; HPLC-P, high-pressure liquid chromatography pump; AC, analytical column; FD, fluorimetric detector.

#### *2.4.4. Chromatographic separation-fluorimetric determination*

The derivatization product formed was driven to the high-pressure injection valve of the chromatograph (HPIV), which was in its filling position. After the loop of HPIV was filled, the valve was switched to the inject position and 20 μl of the derivatized product was injected into the chromatographic column. The HPLC separation of the analytes was performed at a flow rate of 1.5 ml/min using an isocratic elution program in which 75:25% acetonitrile–water was used as mobile phase. Fluorimetric detection was performed at 340 and 440 nm for the excitation and emission wavelengths, respectively.

The elution profiles of the isoindole derivatives exhibited two main peaks corresponding to each native polymyxin ($E_1$ and $E_2$). The isoindole derivative correponding to polymyxing $E_2$ elutes first ($t_R$ = 14 min), since its native polymyxin has a less hydrophobic fatty acid moiety than polymyxin $E_1$ ($t_R$ = 18 min).

## 3. Results and discussion

### *3.1. Optimization of the method*

The experimental design methodology was used to carry out the optimization study of the proposed method. The order used for the optimization of the steps involved in the overall method was as follow: first, the chromatographic determination of the analyte was optimized; then, the variables affecting the derivatization step and, finally, the extraction step. The sum of the chromatographic peak areas of the derivatized polymyxins $E_1$ and $E_2$ was used as indicator of the fluorescence response obtained in the optimization process.

#### *3.1.1. Optimization of the chromatographic separation-fluorimetric determination step*

For the optimization of this step, colistin aqueous solutions of 5 μg/ml were selected and derivatized as described by Cancho-Grande et al. [7]. The derivatization products were manually injected into the chromatograph.

The experimental variables optimized were the composition of the mobile phase, the flow rate and injection volume. Different mixtures of acetonitrile/water were tested with an Ultrabase $C_{18}$ column. The optimum elution performance, for resolution of the two derivatized polymyxin peaks, was obtained under isocratic conditions with acetonitrile–water 75:25%. The influence of the flow rate of the mobile phase was studied within the range 1.0–2.0 ml/min, and the best separation was obtained for a flow rate of 1.5 ml/min. An injection volume of 20 μl was selected to obtain a quantifiable fluorimetric signal.

#### *3.1.2. Optimization of the derivatization step*

Colistin aqueous solutions of 5 μg/ml were selected for optimization of this step.

Table 1
Optimization of the method

| Variable | Tested range | Analysis result | Effect on the fluorescence signal | Optimum value |
|---|---|---|---|---|
| (a) Optimization of the derivatization step | | | | |
| *Screening study* | | | | |
| OPA (mg) | 20–80 | Non-significant | Positive | 80 |
| Derivatization reagent flow rate (ml/min) | 0.3–0.7 | Significant | Negative | – |
| Carrier flow rate (ml/min) | 0.3–0.7 | Significant | Positive | – |
| Sample volume (μl) | 150–450 | Non-significant | Positive | 450 |
| *Full factorial design* | | | | |
| Derivatization reagent flow rate (ml/min) | 0.1–0.3 | Non-significant | Positive | 0.3 |
| Carrier flow rate (ml/min) | 0.7–1 | Non-significant | Negative | 0.7 |
| (b) Optimization of the ultrasound-assisted extraction | | | | |
| *Screening study* | | | | |
| Probe position (mm) | 1–20 | Non-significant | Negative | 1 |
| Radiation amplitude (%) | 10–50 | Significant | Positive | – |
| Duty cycle (%) | 20–60 | Significant | Positive | – |
| Extractant flow rate (ml/min) | 0.5–2.5 | Non-significant | Positive | 2.5 |
| Extraction time (min) | 3–7 | Non-significant | Positive | 7 |
| Extractant volume (ml) | 1–3 | Non-significant | Positive | 3 |
| Extractant composition (M HCl) | 0–1 | Non-significant | Positive | 0 |
| *Full factorial design* | | | | |
| Radiation amplitude (%) | 50–100 | Non-significant | Positive | 100 |
| Duty cycle (%) | 60–100 | Non-significant | Positive | 100 |

The variables optimized were the amount of OPA in the derivatization reagent (as it is the fluorogenic reagent), the flow rates of both the derivatization reagent and the carrier (water), and the sample volume. A full $2^4$ factorial design involving 16 randomized runs plus three centered points [22] was built for a screening study of these variables. Table 1a shows the range tested for each variable in this first screening design.

The conclusions were that the amount of OPA and the sample volume were not influential variables in the ranges under study; however, better signals were obtained with the highest values tested. Thus, 80 mg of OPA and 450 μl of sample were used for subsequent experiments. The flow rate of the carrier and the derivatization reagent were influential variables. Better signals were obtained with the highest carrier flow rate and the lower derivatization reagent flow rate tested; therefore, a full factorial design involving four randomized runs plus three centered points [22] was built for the study of these variables (Table 1a).

The results of this study showed that both variables were non-significant statistically, but the lowest value tested for the carrier flow rate (0.7 ml/min) and the highest value (0.3 ml/min) tested for the derivatization reagent were selected as these values provided higher signals.

The repeatability and reproducibility of the derivatization reaction was studied by repeating the derivatization reaction of different colistin solutions (5 μg/ml) and injecting the derivatives into the HPLC system in an automatic manner. Relative standard deviations (RSD) of 4.6% and 5.8%, respectively, were obtained for nine analyses.

### 3.1.3. Optimization of the extraction step

The variables affecting the ultrasound-assisted extraction were the probe position, the ultrasound radiation amplitude, the percentage of duty cycle of ultrasonic exposure, the extractant flow rate, the irradiation time, the extractant volume and the extractant composition. The probe position was established as the distance between the tip horn of the ultrasonic probe and the top surface of the extraction chamber. A Plackett-Burman design $2^7 \times 3/32$ type III resolution allowing four degrees of freedom and involving 12 randomized runs plus three centered points [22] was built for a screening study of the behavior of the main variables affecting the extraction process (Table 1b).

The conclusions of this screening study were that the radiation amplitude and the duty cycle were key variables of the extraction, with a positive influence. The probe position, the irradiation time and the extractant flow rate, volume and composition were not statistically influential variables in the ranges under study. However, the results showed better recoveries with the minimum value of the probe position (1 mm) and with the maximum values of the other variables. Thus, 7 min irradiation time, 2.5 ml/min extractant flow rate, 3 ml extractant volume and water as extractant were selected for subsequent experiments.

For the radiation amplitude and the duty cycle, higher values were tested using a full two level factorial design involving four randomized runs plus three centered points [22] (Table 1b).

Analyzing the results of this second experimental design, both variables had non-significant effect on the extraction; however, better recoveries were obtained with the highest

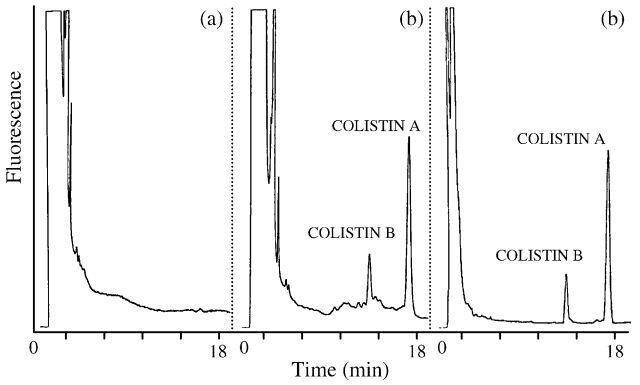


Fig. 3. Chromatograms of a blank feed (a), a colistin-spiked feed at 150 μg/g (b), and a standard of 5 μg/g (c). Colistin A, $t_R$ = 17.9 min; colistin B, $t_R$ = 13.7 min.

values tested. Thus, 100% of both amplitude and duty cycle were selected for subsequent experiments.

Although some matrix components (proteins, amino acids) and the analyte have a common moiety susceptible of reacting with the derivatization reagent, a clean-up step after UAE was not necessary because the proposed extraction has shown to be highly selective and no interference from the matrix was observed as shown in the chromatograms in Fig. 3.

### 3.2. Recovery and precision of the method

The linearity of the method was checked by running a calibration graph in the range 0.5–10 μg/ml (10 points). The equation was obtained by regressing the sum of the derivative peak areas versus the concentration of colistin standards. The calibration graph was linear within the range studied and used for calculation of the extraction recovery.

The recovery of colistin was studied by the analysis of feeds spiked with standards at two concentration levels, 50 and 150 μg/g, corresponding to the minimal and usual doses, respectively, administered with feed in order to meet or exceed the animal requirements [4]. When these samples were quantified with the calibration graph obtained above, mean recoveries of 98.2 ± 4.8% ($n$ = 3) and 95.1 ± 4% ($n$ = 3), respectively, were obtained.

The precision of the proposed approach was evaluated with two measurements of colistin per day during 7 days [23]. In all experiments, 0.1 g of feed spiked with 150 μg/g of the analyte was used under the optimum working conditions. The repeatability and within-laboratory reproducibility, expressed as RSD, were 5.8 and 5.2%, respectively.

The optimization of the detection and derivatization steps were performed using colistin solutions with the highest level of antibiotic that could be found in the extract, in order to ensure the applicability of the conditions found.

Table 2
Comparison between the proposed and the conventional methods

| | UAE | | Conventional extraction | |
|---|---|---|---|---|
| Sample | Recovery (%) | RSD[a] (%) | Recovery (%) | RSD[a] (%) |
| Spiked | 95.1 | 4.0 | 92.4 | 6.5 |
| Commercial | 93.1 | 5.2 | 91.5 | 8.5 |

[a] $n$ = 3.

### 3.3. Detection and quantification limits

The detection (LOD) and quantification (LOQ) limits were calculated experimentally by analyzing a blank feed (which did not give any response at the retention times of colistin derivatives). LOD and LOQ were expressed as the mass of analyte, which gives a signal that is 3σ and 10σ above the mean blank signal, respectively. Under the optimal experimental conditions, the LOD was 2.46 μg/g and the LOQ was 2.52 μg/g.

### 3.4. Comparison of the UAE with the conventional extraction

As certified reference material was not commercially available, the proposed approach had to be validated by comparing with a conventional extraction method for colistin in feed. Among the official methods described in the literature for the analysis of antibiotics in feed [24] there are none specific for colistin. However, in most of them the extraction procedure is similar, consisting on mechanical shaking of the feed with a high volume of a methanol–water solution. Thus, this procedure was used and compared with the proposed UAE method. With this aim, the extracts, thus obtained, were analyzed by the HPLC-fluorimetric method proposed.

The optimized and conventional methods were applied to the extraction of colistin in both a spiked feed at 150 μg/g and a commercial medicated feed with a concentration of

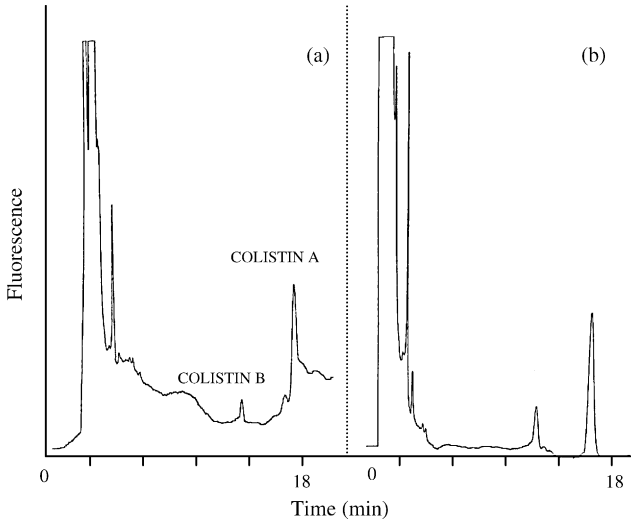


Fig. 4. Chromatograms of a commercial medicated feed obtained by the conventional (a) and the proposed (b) methods.

100.5 μg/g. The samples were analyzed in triplicate and the recoveries obtained are shown in Table 2. As can be seen, the recoveries were similar for both extraction methods, but the precision of the proposed extraction was better.

Fig. 4 shows the chromatograms obtained from a commercial medicated feed by both the proposed method and the conventional extraction. As can be seen, the UAE is more selective than the method consisting of mechanical shaking and subsequent centrifugation because the former is free from matrix interferences.

## 4. Conclusions

A method for the analysis of colistin antibiotic in feed has been developed. The proposed approach consists of dynamic ultrasound-assisted extraction (UAE) with on-line pre-column derivatization and high performance liquid chromatography (HPLC) separation with fluorimetric (FL) detection.

The joint use of a dynamic approach and an ultrasonic probe facilitates and accelerates the extraction step considerably as high extraction efficiency in short processing time is achieved with small solvent consumption. The proposed extraction takes 7 min instead of 1 h for extraction with ultrasonic baths [7,8], with recoveries of 93.1–98.2% versus 87.5–94%. Pure water can be used as extractant because of the highly effective extraction power of the UP. In applications where ultrasonic baths are used, the addition of an acid to water was necessary for efficient recovery of the antibiotic [7,8].

A derivatization reaction prior to HPLC-FL determination of colistin endows the method with a high sensitivity and avoids the use of complex conventional microbiological or immunological methods. OPA-ME chemistry was used as it is a well-established reaction for the derivatization of compounds with amino groups. Although this is a non-selective reagent, which can react with the protein and amino compounds of feed samples, the extraction technique used has shown to be selective and free from matrix interferences. The choice of the pre-column derivatization avoids the use of sophisticated equipment, baseline noise resulting from reaction between impurities in the mobile phase and the derivatization reagent, and greater reagent consumption.

The proposed approach was compared with a conventional method, obtaining similar results but in a shorter time and with better precision and selectivity. Thus, we can say that the proposed on-line approach provides an excellent via for determining the colistin concentration in medicated feed, with the advantage of minimum sample handling and high reproducibility.

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