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High-performance liquid chromatography determination of Zn-bacitracin in animal feed by post-column derivatization and fluorescence detection

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Abstract

A sensitive and selective method is presented for the determination of Zn-Bacitracin in adulterated animal feed by reversed-phase ion-pair high-performance liquid chromatography and post-column derivatization with *o*-phthalaldehyde prior to fluorescence detection. The calibration function was estimated to be between 8.0 and 65.0 mg l^{-1} of Zn-BC. The detection and quantification limits of the chromatographic method were 2.5 and 7.5 mg l^{-1}, respectively. Using the extraction procedure of Zn-Bacitracin from the feedstuff that we recently proposed and applying this new chromatographic method, it was possible to detect this antibiotic at levels below 5 mg kg^{-1} in different kinds of feedstuffs with a standard deviation less than 6.0%. © 2002 Published by Elsevier Science B.V.

Keywords: Food analysis; Derivatization, LC; Zinc; Bacitracin

1. Introduction

Bacitracin (BC) is one of the most common antibiotics used in the world as an animal feed additive due to its growth-promoting effects [1,2]. It is a basic and cyclic polypeptide antibiotic produced by the strains of *Bacillus licheniformis* and *Bacillus subtilis* [3]. Commercial BC is a mixture of similar polypeptides, which may differ by only one amino acid. Frequently it has been used in association with zinc (Zn-BC), because this combination is more stable than BC alone as an antibiotic for improving growth rates and feed conversion in poultry, pigs and cattle. Since 01/01/99, the European Union has forbidden its use as an additive in animal feed [4]. In the absence of chemical methods, microbiological methods [5–8] have traditionally been used for the qualitative and quantitative determination of bacitracin, although counter-current distribution [9,10] and column chromatography have also been applied [11,12] for its extraction. Nevertheless, owing to the great importance of investigating and determining the presence of banned substances in feedstuffs, several chemical methods have been reported recently to determine BC in animal feeds. One of them is an HPLC method to determine bacitracin methylene disalycilate, another stable form of BC also used as an additive in animal diets, in feedstuffs at levels

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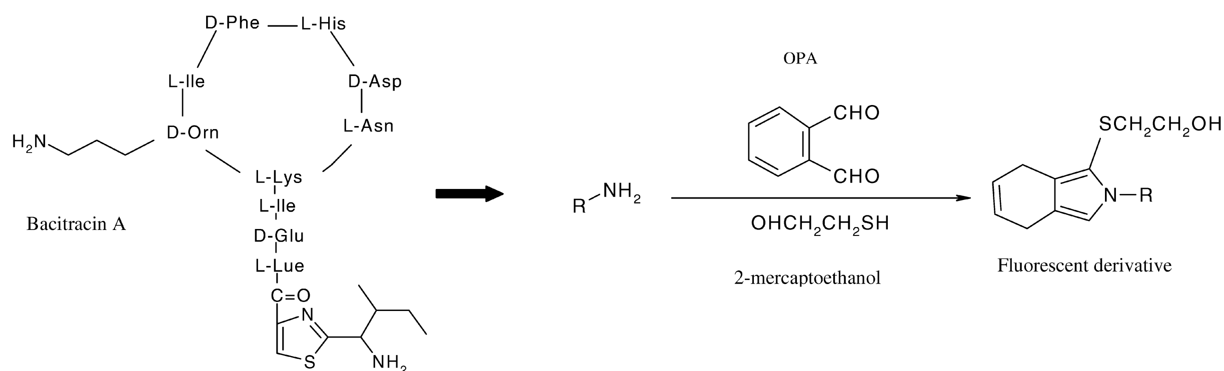

Fig. 1. Zn-Bacitracine fluorescence reaction with *o*-phthalaldehyde and 2-mercaptoethanol.

between 50.0 and 250.0 mg kg^{-1} [13]. Recently a rapid assay for the determination of Zn-BC in chicken and swine feed by liquid chromatography–mass spectrometry at levels between 5.0 and 50.0 mg kg^{-1} was presented [14]. In a previous work, we have proposed a method to determine Zn-BC in feedstuffs by HPLC with ultraviolet detection at levels from 5.0 to 200.0 mg kg^{-1} [15]. Here, using the same extraction procedure of Zn-BC from the feedstuffs developed by us in the cited work, an HPLC method is proposed with post-column derivatization and fluorescence detection with the aim to improve the results of the previous method. The derivatization of primary amine groups present in the lateral chain of the amino acids that make up the BC structure (Fig. 1) with *ortho*-phthalaldehyde (OPA) in presence of 2-mercaptoethanol [16] produce intensely fluorescent isoindole products and enhanced the detection of the Zn-BC. The method developed has been applied successfully to the determination Zn-BC in different kinds of feedstuffs at levels below 5.0 mg kg^{-1}.

2. Experimental

2.1. Apparatus and software

A Hewlett-Packard HPLC model 1090 equipped with fluorescence detector (Waldbronn, Germany) and connected to a Pentium 200 PC fitted with ChemStation $HPLC^{3D}$ (Win95) software were used. A Waters Reagent Manager (Milford, MA, USA) was used. A Perkin-Elmer (Norwalk, CT, USA) LS-50 luminescence spectrometer interfaced with an IBM (Armonk, NY, USA) PC 300-100DX4 microcomputer supplied with FL Data Manager Software (Perkin-Elmer) for spectral acquisition and the subsequent manipulation of spectra was used to study the derivatization of Zn-BC.

A multiplace-heating block from Selecta (Barcelona, Spain) and a centrifuge Hettich, universal model 16 (Tuttlingen, Germany) were used. The solid-phase extraction clean-up steps were done with 500 mg C_{18} cartridges (Waters Corporation, Milford, MA, USA). A mill equipped with a 1-mm screen was used.

Software programs used for the statistical treatment of the data were the Statgraphics Plus 6.0 software package (Statistical Graphics System Corporation, USA, 1992) and the Excel ver 8.0 software package in Microsoft Office 97, 1997. The lack-of-fit test was used to check the linearity of the calibration graphs in accordance with the guidelines of the Analytical Methods Committee [17].

2.2. Reagents

All reagents were of analytical reagent grade unless stated otherwise. Reverse-osmosis type quality water (Milli-RO plus Milli-Q station from Millipore) and HPLC quality were used throughout. A stock solution of Zn-BC (from Sigma, Barcelona, Spain) containing 1000 mg l^{-1} was prepared in phosphate buffer solution and was stored in dark bottles at 4°C, remaining spectrophotometrically and chromatographically stable, without change, for at least 1 week. Working solutions were prepared daily

by appropriate dilutions with 0.3 *M* phosphate buffer solution, pH 3.

Ethyl acetate, methanol, ethanol, acetonitrile, phosphoric acid, sodium tetraborate, and sodium hydroxide were supplied from Panreac (Barcelona, Spain); electrophoresis grade sodium dodecyl sulphate was supplied from Bio-Rad Laboratories (Alcobendas, Madrid, Spain); *o*-phthalaldehyde (OPA) was supplied by Fluka (Barcelona, Spain) and 2-mercaptoethanol was supplied by Sigma (Barcelona, Spain).

OPA reagent solution was prepared by dissolving 540 mg in 10 ml of ethanol, adding 0.4 μl of 2-mercaptoethanol and adjusting the volume to 100 ml with 0.1 *M* pH 9.5 sodium tetraborate buffer solution. The reagent solution was kept at 4°C for 24 h prior to use. The reagent strength was maintained by addition of 0.4 μl of 2-mercaptoethanol every 3–4 days. When necessary, a dilution of the reagent solution with 0.1 *M* borate buffer solution (pH 9.5) was made.

2.3. Sample treatment procedure

2.3.1. Extraction procedure

For analysis of Zn-bacitracin at levels between 5.0 and 1.0 mg kg^{-1}, ground feed, typically between 1.0 and 5.0 g, previously crushed with a mill equipped with a 1-mm screen, and mixed, was accurately weighed and placed into a suitable conical flask. Then it was extracted by mechanical shaking for 20 min with 8.0 ml of the extracting solution. This extracting solution was composed of 3:1 (v/v) phosphate buffer solution (0.5 *M*, pH 2) and acetonitrile 5% (v/v) in water; 20 mg of sodium sulphide was added to each sample. The extracts were then transferred to a glass centrifuge tube and centrifuged at 3000 RFC (Relative Force Centrifuge) for 10 min to remove any undissolved feed matrix. The undissolved feed matrix was again treated twice with 5 ml of the extraction solution, centrifuged and it was combined with all the extracts.

2.3.2. Clean-up procedure

The pH of the combined centrifuged extract was adjusted at pH 8 with sodium hydroxide. The extract was again centrifuged at 3000 RFC for 5 min to remove the precipitate produced at this pH value. The extract was then transferred to a glass tube and the precipitate was treated three times with 0.2 ml of the same phosphate buffer solution used above to extract the Zn-BC from the feed, and was centrifuged each time for 2 min at 3000 RFC. All the solutions were combined and added to the extract obtained at pH 8. At this point the extract was cleaned up by liquid–liquid extraction with 3 ml of ethyl acetate three times.

A clean-up by solid-phase extraction was performed with a C_{18} cartridge fitted with a reservoir. The cartridge was preconditioned by adding dichloromethane (3 ml), methanol (5 ml) and pH 8 phosphate buffer solution (5 ml). The clean extract was transferred to the reservoir and allowed to run through the C_{18} cartridge at a flow-rate of 1 ml min^{-1}. The bacitracin was eluted with 3 ml of methanol at a flow-rate of 1 ml min^{-1} and collected in a glass tube. The eluate was evaporated to dryness on a hot-block at a temperature not exceeding 30°C under a gentle stream of nitrogen. The residue was dissolved in 0.2 ml of a mixture of 0.3 *M* phosphate buffer solution at pH 2, and acetonitrile 70:30 (v/v); 10 μl of this solution was used for HPLC analysis.

2.4. Chromatographic procedure

Appropriate dilutions of the stock solution of Zn-BC with 0.3 *M* phosphate buffer solution (pH 2) were prepared daily to obtain standard solutions containing between 8.0 and 65.0 mg l^{-1}. In order to obtain the calibration function, 10 μl of the standard solutions were injected into the chromatograph provided with a precolumn ODS 5 μm 10×2.1 mm and a Hypersyl column ODS 5 μm 200×2.1 mm, working at a temperature of 45°C. The mobile phase used was composed of 50:50 (%v/v) solution A and B; solution A being 0.3 *M* phosphate buffer solution at pH 3 containing sodium dodecyl sulphate (20 m*M*) and solution B, a mixture of acetonitrile/methanol 19:1 (v/v%). The flow-rate was maintained at 0.5 ml min^{-1}. The reaction coil was made of steel tubing for HPLC (7.0 m×0.12 mm I.D.) and it was maintained at a temperature of 4°C using an ice bath. The OPA reagent (OPA 4.02×10^{-3} *M*) was pumped at a flow-rate of 0.9 ml min^{-1}. The chromatograms were obtained at an emission wavelength of 455 nm with an excitation wavelength of 331 nm.

3. Results and discussion

The derivatization of proteins using OPA as fluorescent reagent has been previously described [18–21] and it has been established that the fluorescence of the products that come from the derivatization of amino acids depends strongly upon pH value, derivatization time, temperature and OPA concentration.

3.1. Study of the fluorescent products

Before the study of the conditions for post-column derivatization of bacitracin, the formation of fluorescent derivatives was investigated using a spectrofluorimeter.

In the consulted literature, different compositions for the OPA reagent solution were found [16,22,23], but the composition used to react with amino acids and related substances was selected from Ref. [23].

Since the medium where subsequently Zn-BC has to react with the OPA reagent is the chromatographic mobile phase, all the experiments were made with Zn-BC dissolved in this mobile phase. Although the excitation and emission wavelengths are well known for the fluorescent products formed using the OPA reagent, they were determined for Zn-BC in mobile phase medium. The excitation and emission spectra of Zn-BZ derivative in mobile phase show peaks of excitation and emission located at 331 and 455 nm, respectively. So, the fluorescence intensity measured in the above conditions was selected as the analytical signal. These fluorescence conditions are very similar to those cited in bibliography, 340 nm for λ_{ex} and 455 nm for λ_{em} [16].

The effect of the pH on the derivatization of Zn-BC in mobile phase medium was studied using a 1:1 (v:v) volume ratio Zn-BC solution/OPA reagent obtained by mixing the OPA reagent solution (4.02×10^{-2} M, pH 9.5) with Zn-BC solutions of 10 mg l^{-1} in mobile phase at pH values between 1.0 and 5.2. The experimental results showed that fluorescence intensity decreases drastically under pH 2.5 remaining constant upon reaching this value. This was because the buffer capacity of the OPA reagent at pH 9.5 was lost at pH lower than 2.5 (pH 8) whereas it was maintained at pH values higher than 2.5. pH values higher than 5.2 were not investigated because the optimal pH for the chromatographic Zn-BC separation was 3. In the same way it was confirmed that the fluorescence signal was maintained when the SDS content was varied from 0.10 to 30 mM. Thus no change in the pH value and SDS content in mobile phase was necessary to proceed with the post-column fluorescence reaction.

A study of the time of derivatization was carried out using a 1:1 (v/v) volume ratio Zn-BC solution/OPA reagent, with a Zn-BC concentration of 10 mg l^{-1} in mobile phase and 4.02×10^{-2} M, pH 9.5, OPA. Results obtained in this experiment were in agreement with results cited in Refs. [20,21]. The OPA derivatives were rapidly generated, within 30 s; after this time, the intensity of fluorescence decreased drastically (40% in 5 min) because the OPA derivatives were labile again. For this reason, the post-column derivatization technique was selected and also because of the main disadvantage of pre-column derivatization, i.e. the formation of side products which influences the chromatographic analysis or reproducibility of the derivatization reaction [24].

3.2. Study of the conditions for post-column derivatization

As stated above, in a previous work we established the conditions for the chromatographic separation of Zn-BC as an ionic pair with sodium dodecyl sulphate (SDS) in a C_{18} column [15]. In accordance with this type of study, to obtain the entire analytical signal of Zn-BC separated from the matrix interferences in a single chromatographic peak, the selected mobile phase was composed of 50:50 (v/v%) phase A and B, phase A being a pH 3, 0.3 M phosphate buffer solution containing SDS (20 mM) and phase B being a mixture of acetonitrile/methanol 19:1 (v/v%) operating at a flow-rate of 0.5 ml l^{-1}. With these conditions, it was not possible to close the whole Zn-BC analytical signal in a single chromatographic peak, so it was decided to have the largest peak in the chromatogram register at 254 nm to obtain the analytical parameters of the chromatographic method. In the present work, these chromatographic separations conditions have also been selected and the analytical characteristic of the chromatographic method has been estimated using the largest chromatographic peak of Zn-BC obtained in the

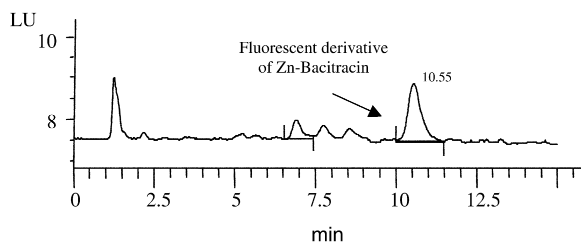

Fig. 2. Chromatogram of a standard solution of 25.0 mg l^{-1} of Zn-Bacitracin.

chromatogram registered at λ_{em} of 455.0 nm and λ_{ex} of 331.0 nm (Fig. 2). The concentration of OPA reagent, the OPA reagent flow, the reaction time as length of the reactor coil and the reaction temperature were then optimised using these chromatographic conditions.

The influences of the flow reagent and OPA reagent concentration on the fluorescent reaction were studied simultaneously. Using a Zn-BC standard solution of 300.0 mg l^{-1}, the dependence of flow reagent was studied between 0.1 and 1.3 ml min^{-1} at three different OPA reagent concentrations, 4.02×10^{-2}, 4.02×10^{-3} and 4.02×10^{-4} *M* (Fig. 3). The higher analyte responses for all the flow reagents tested were obtained for a concentration of OPA of 4.02×10^{-3} *M*, and in accordance with these results, a flow of 0.9 ml min^{-1} was then selected.

Furthermore, different lengths of the coil reaction were tested and the optimum was 7.0 m; longer coil lengths did not improve the analytical signal, confirming again that the OPA derivatives were rapidly generated. Following this experiment, the temperature was studied from 4 to 45°C introducing the

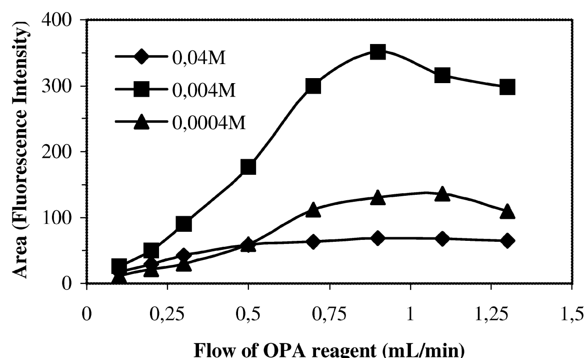

Fig. 3. Influence of the flow reagent and OPA concentration.

reaction coil into an ice bath (at a temperature of 4°C) or into an oven (at the other temperatures tested). The best results were obtained when the reaction coil was placed in an ice bath as is usual in fluorescence behaviour.

3.3. Analytical parameters

Finally, the analytical parameters of the chromatographic method were obtained using the selected conditions described above. The linear relationship between Zn-BC concentration and the analytical signal (peak area) was estimated to be from 8.0 to 2000.0 mg l^{-1}. As the Zn-BC levels that will be analysed by the application of the chromatographic method will not be greater than 50.0 mg l^{-1}, a calibration function for standard samples prepared as described in the Chromatographic procedure was obtained between 8.0 and 60.0 mg l^{-1} of Zn-BC.

In order to check the linearity of the calibration function, the *lack-of-fit* test was applied to four replicates of each standard sample. The results for the intercepts (a), slopes (b), correlation coefficients (R^2), analytical sensitivity and probability levels of the *lack-of-fit* test (P_{lof} (%)) are summarised in Table 1. The data yield shows a good linearity within the ranges studied. The detection limit (DL) and the quantification limit (QL), calculated from the standard calibration function [25], were 2.0 and 7.0 mg l^{-1}, respectively. These limits, compared with the

Table 1
Analytical parameters

Intercept (*a*) and RSD (*a*)	−2.27 (AU), 31.9%
Slope (*b*) and RSD (*b*)	2.06, 1.20%
Correlation coefficient (R^2)	0.998
Analytical sensitivity (mg l^{-1})	1.1
RSD (%)	
c_1=60.0 mg l^{-1}	1.34
c_2=30.0 mg l^{-1}	2.18
c_3=15.0 mg l^{-1}	4.21
c_4=8.0 mg l^{-1}	8.87
Lack-of-fit test (*P*-value)	0.73
Linear dynamic range studied (LDR) (mg l^{-1})[a]	8.0–65.0
Linearity (100−RSD (*b*)%)	98.80
Detection limit (mg l^{-1})[a]	2.4
Quantification limit (mg l^{-1})[a]	7.3

AU, area units; RSD, relative standard deviation.
[a] Indicated as concentration, for an injection volume of 10 μl.

results obtained in our previous work (72.0 and 142.0 mg l^{-1}, respectively, for DL and QL), have been improved considerably.

3.4. Zn-BC in feedstuffs

Once the chromatographic method was established, the validation was carried out by applying it to spiked feedstuffs. A sheep feed, a poultry feed and a cattle feed taken from the available Spanish commercial feedstuffs, all of which were free of Zn-BC, were selected to apply the method. These kind of feedstuffs were chosen because Zn-BC was used, when it was not a banned substance, as an antibiotic for improving growth rates and feed conversion in cattle, sheep and poultry.

The method was then validated by using it to analyse animal feed samples with a Zn-BC concentration between 5.0 and 1.0 mg kg^{-1}. To analyse levels of 5.0 mg kg^{-1} of Zn-BC, 1.0 g of spiked animal feed was extracted as has been described in the Extraction procedure. To analyse levels of 1.0 mg kg^{-1}, 5.0 g of spiked animal feed was extracted in the same way. The critical point in the extraction procedure was the first step in which Zn-BC was extracted from the feed. In our previous work, to analyse levels of 5.0 mg kg^{-1}, 10.0 g of spiked feedstuff were necessary. In the present work, to obtain a cleaner extract, a lower quantity of feedstuff was extracted. Then 1.0 and 5.0 g of feedstuff were extracted to analyse levels of 5.0 and 1.0 mg kg^{-1}, respectively. Different mixtures of the phosphate buffer solution/organic solvent were tested for solvent extraction. Finally, the best results were obtained using a mixture of phosphate buffer solution (0.5 *M* and pH 2)/5% of acetonitrile in water, 6:2 (v/v). When the Zn-BC was added to the extract from the feed, levels above 80% recovery were found.

In spite of the heterogeneity of the different kinds of feedstuffs, the extraction method allowed for a clean extract from all of them. This was tested before carrying out the analysis of the spiked Zn-BC feedstuff, and representative chromatograms of the blank feedstuff extracts were obtained. The chromatograms shown in Fig. 4 for the poultry feedstuff extracts (blank and spiked) demonstrated that the method's selectivity is satisfactory, allowing for the resolution of the analyte peak from matrix interferences. Moreover, the reproducibility of this procedure in terms of Zn-BC retention time was also satisfactory.

The reproducibility and recovery for the two concentrations tested were estimated by analysing

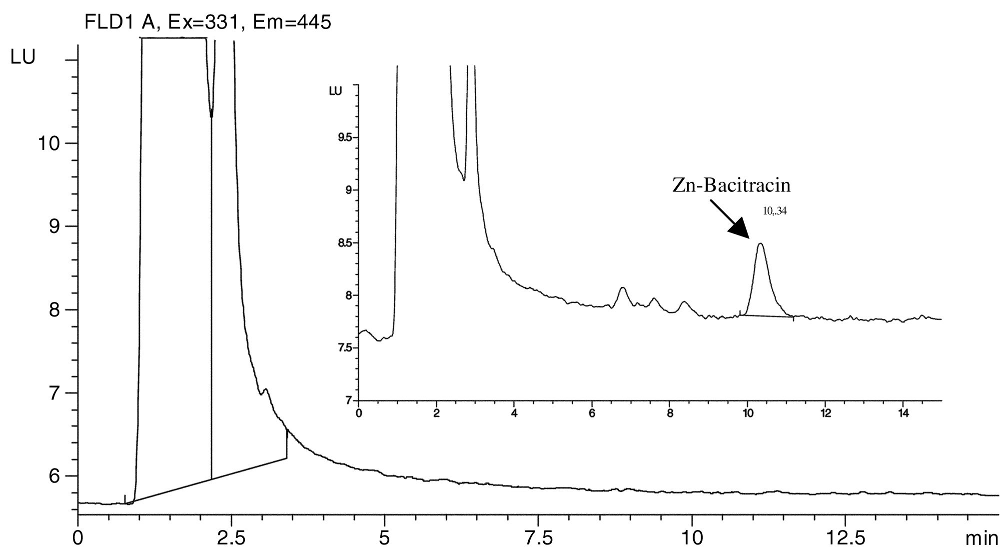

Fig. 4. Chromatogram of poultry feedstuff free from Zn-Bacitracin and chromatogram of poultry feedstuff spiked with 5.0 mg kg^{-1} of Zn-Bacitracin.

Table 2
Validation data for feedstuff fortified with Zn-BC

[Zn-BC] added to feedstuff (mg kg^{-1})	Mean recovery (%)	RSD (%)
5.0 (n=9)	55.5	3.2
1.0 (n=9)	43.5	4.3
Recovery and 5-day precision		
5.0 (n=6)	53.5	4.1
1.0 (n=6)	41.4	5.4

n, number of determinations; RSD, relative standard deviation.

nine samples corresponding to three replicates of each kind of feedstuff and processed individually through the sample treatment procedure. Table 2 shows that the recovery of Zn-BC from the spiked samples was independent of the type of feedstuffs. In spite of the recovery values, about 40.0% for 1.0 mg kg^{-1} and 50.0% for 5 mg kg^{-1}, the high sensibility of the proposed method allowed for a satisfactory detection of Zn-BC. As has been cited above, the critical point was always the first extraction from the feed, because when the antibiotic was added to the feedstuff extract, the recovery rose to 80%.

Reproducibility values, expressed as a percentage of relative standard deviation (RSD), below 6.0% are satisfactory taking into account the complexity of the matrix from which Zn-BC was extracted and the low levels at which it was analysed. The inter-day precision (5 days) was also investigated. Three replicates, one for every kind of feed, were processed independently again. Table 2 shows that the inter-day precision values, expressed as RSD (%), were comparable with the reproducibility previously obtained and in all cases, the values were satisfactory, with RSD below 6%.

4. Conclusion

The procedure described provides a selective and sensitive method for the determination of the antibiotic zinc-bacitracin at low levels in animal feed. The main problem of determining banned substances at low levels in this kind of complex matrix, the incomplete extraction from the feedstuff, was overcome by using a fluorescence reaction before the detection step. Thus, with the proposed method, low levels — the appropriate levels with regard to the recent European ban — of zinc-bacitracin in feedstuff could be analysed in order to detect fraudulent animal feeds to which this antibiotic could have been added.

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