# Densitometric determination of zinc bacitracin and nystatin in animal feed

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## Abstract

**BACKGROUND: The European Union has forbidden the use of antibiotics as additives in animal feed. Zn-bacitracin (Zn-BC) and nystatin (NYS) were frequently used for their growth-promoting effects and for feed conversion in poultry, pigs and cattle. An HPTLC method has been developed for separating Zn-BC and NYS in the mixture, for routine quality control.**

**RESULTS: The separation was obtained using RP-18 $F_{254S}$ coated HPTLC plates with acetonitrile/methanol (equal volumes):toluene:$KH_2PO_4$/KOH (buffer, pH 6.8) = 57:3:40 (v/v/v), adjusted with HCl to pH 8.2, as a mobile phase. The densitograms were monitored at 192, 215 and 305 nm and both antibiotics were assayed at 215 nm. The method was shown to be specific, accurate (recoveries were 98.7 ± 0.5% and 104.8 ± 0.7% for Zn-BC and NYS, respectively), linear over the tested range (correlation coefficients, 0.9982 and 0.9884), and precise (intermediate precision RSD below 2.2% for both analytes) with efficient separation ($R_s$ = 3.5).**

**CONCLUSION: The method was applied for determining Zn-BC and NYS as additives in spiked matrices of commercial animal feedstuffs. According to LOD values for each antibiotic, the minimum detectable amount in feed is 4.5 and 5.5 ppm of Zn-BC and NYS, respectively.**
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**Keywords:** zinc-bacitracin; nystatin; high-performance thin-layer chromatography; animal feed

## INTRODUCTION

Bacitracin (BC) is a mixture of many structurally related compounds including bacitracins A, B, C, D, E, F, G, H and I.[1] It is a polypeptide antibiotic produced by strains of *Bacillus licheniformis* and *Bacillus subtilis*. It has been shown that bacitracin A (Fig. 1) is the main component with the most potent activity. It is commonly used against Gram-positive organisms and especially as animal feed additive.[2] Since 1999, the European Union has forbidden its use as an additive in animal feed. It has frequently been used in association with zinc (Zn-BC), because this combination is more stable than BC alone[3] as an antibiotic with growth-promoting effects and feed conversion in poultry, pigs and cattle.

Nystatin (NYS) is an antifungal antibiotic used to treat yeast and yeast-like fungal infections. It is prescribed for treating superficial oral and intestinal infections caused by *Candida* species, and is used in a variety of pharmaceutical preparations. Liquid chromatographic determination has been used for its analysis.[4] Commercially available nystatin can be a mixture of two or more biologically active components of which nystatin $A_1$ (Fig. 1) is the most common. Nystatin is also used as an animal feed additive.[5]

Both antibiotics (BC and NYS) are unstable at high temperatures and in light.[6] In order to characterise the bacitracin components, thin-layer chromatography (TLC) was reported, which allowed eight to ten components to be separated.[7] Counter-current chromatography was also used for its separation.[8]

Thin-layer chromatography and high-performance liquid chromatography,[9] liquid chromatography[4] and derivative spectrometry[10] have been used for identification and determination of nystatin in pharmaceuticals. A rapid spectrophotometric method for the assay of NYS in feeds was developed, and the limit of quantification is 5 ppm.[5]

Micellar electrokinetic capillary chromatography (MEKC) is an important liquid separation technique that is complementary to LC. In MEKC, the successful separation of Zn-BC and NYS is based

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(*Received 20 December 2006; revised version received 4 August 2007; accepted 6 February 2008*)
Published online 12 May 2008; DOI: 10.1002/jsfa.3253

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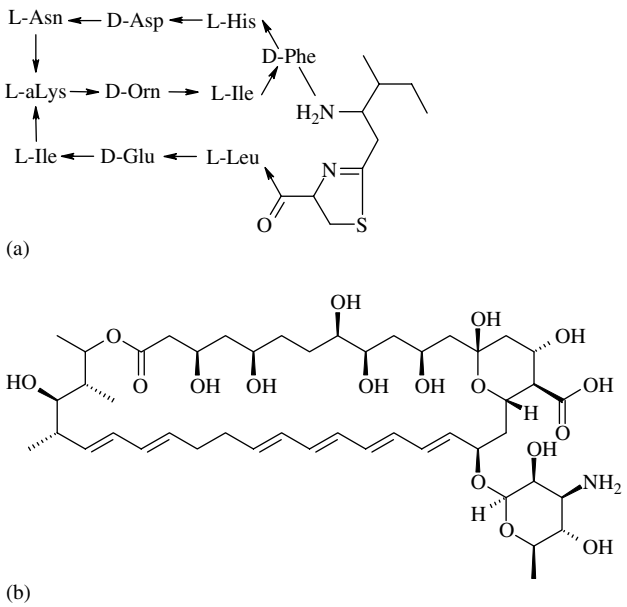


**Figure 1.** The chemical structures of (a) bacitracin A and (b) nystatin $A_1$.

mainly on appropriate selection of the surfactant, running voltage and buffer.[11–13]

The sensitivity of traditional TLC analytical procedures was markedly lower than that of the corresponding HPLC and GC methods. However, with the advent of modern HPTLC methods, sensitivity has been considerably enhanced and it is similar or identical to that of other chromatographic methods. The use of silica HPTLC plates coupled with scanning densitimetry resulted in good sensitivity for different kinds of sample.

The objective of this work was to establish the optimal conditions for HPTLC separation for densitometric determination of Zn-BC and NYS in mixtures, as well as to validate the method for assaying Zn-BC and NYS as additives in the extracts from spiked matrices of commercial animal feedstuffs. The HPTLC technique, coupled to densitometric determination, was selected due to its common use for separating the constituents in different matrices, such for herbal drugs,[14] and in addition the HPTLC technique is easy to optimise and avoids the time-consuming sample preparation step.

## EXPERIMENTAL

### Apparatus

A Camage densitometer TLC Scanner 3 'SCANNER 3' S/N 070 419 (1.14.16), a Linomat Camag IV sample applicator, and a TLC chamber, 10 cm × 10 cm, for plates were all from Camag (Muttenz, Switzerland). The HPTLC-RP-18 $F_{254S}$ plates, 10 cm × 10 cm, were from Merck (Darmstadt, Germany). A Consort C-831 pH meter (Turnhout, Belgium) was used for pH measurements.

### Reagents and feedstuffs

All solvents and reagents were of analytical grade unless indicated otherwise. Solutions were prepared with deionised water (Milli-Q quality).

Analytical reagent grade solvents, acetonitrile, methanol, toluene and pro analysis (p. a.) chemicals $KH_2PO_4$; KOH (Zorka, Sabac, Serbia) were used.

Zn-bacitracin and nystatin were obtained from SB Trade (Belgrade, Serbia), and the quality was in agreement with British Pharmacopoeia (BP) requirements (>98%).

Animal feed TS-2, for pigs over 60 kg (protein, min 14%; crude fat, max 10%; moisture, max 13.5%; crude fibre, max 6%; calcium, min–max 0.6–1.2%; phosphorus, min 0.5%; sodium, min 0.15%; methionine + cystine, 0.45%; lysine, 0.70%; and vitamins A, $D_3$, E, C, B complex, zinc, manganese, selenium, iodine, cobalt, copper and iron), and chicken feed TKN-2 (protein, min 15%; moisture, max 13.5%; crude fibre, max 8%; calcium, min–max 3–4%; phosphorus, min–max 0.5–0.8%; sodium, min–max 0.15–0.20%; methionine + cystine, 0.55%; and vitamins A, $D_3$, E, $K_3$, C, B complex, zinc, manganese, selenium, iodine, cobalt, copper and iron), were manufactured by Fooder (Zupanja, Croatia). Cattle feed KM-19 (protein, min 19%; ash, max 10%; crude fibre, max 15%; calcium, max 0.8%; phosphorus, min 0.50%; sodium, min 0.20%; and NaCl, max 1%) was manufactured by the Institute for Hygiene and Pathology of Animal Nutrition (Ljubljana, Slovenia).

### Chromatographic conditions

HPTLC was performed on 10 cm × 10 cm HPTLC-RP-18 $F_{254S}$ plates. Ascending chromatography was performed in a Camag TLC chamber (10 cm × 10 cm) with acetonitrile/methanol (equal volumes):toluene: $KH_2PO_4$/KOH (pH 6.8) = 57 : 3 : 40 (v/v/v) with the pH adjusted with HCl to approximately 8.2, as mobile phase. The optimal chamber saturation time for the mobile phase was determined as 30 min at room temperature (25 ± 2 °C) and at the relative humidity of 60 ± 5%.

Sample solutions, from 1 to 60 µL each, were applied in the form of bands (1 cm) on the chromatographic plate. Chromatograms were developed twice (for 30 and 20 min, respectively). After the first separation, the plate was air dried and the second development was carried out in the same mobile phase. After development (up to a height of 7 cm), the plates were air dried and visualised under UV light at 254 nm or 366 nm.

The chromatographic zones, corresponding to spots of Zn-BC and NYS, were scanned at 192, 215 and 305 nm with a Camag TLC scanner, in reflectance–absorbance mode, and densitograms obtained at 215 nm were used for the assay of both antibiotics.

### Preparation of standard stock solutions

Stock solutions of Zn-BC and NYS were prepared by accurate weighing of 100 mg of each drug and dissolving in 50 mL methanol:deionised water (1:1, v/v). Serial dilutions of standards, as well as the samples following extraction from spiked feed (10–80 mg $L^{-1}$ for Zn-BC and 10–100 mg $L^{-1}$ for NYS) were carried out using methanol:deionised water (1:1, v/v) to give the required concentrations ranges. Solutions were kept at 4 °C and used within 48 h.

### Procedure for extracting Zn-BC and NYS from feedstuffs

Spiked feed mixtures were prepared by grinding 1 kg of feedstuff and adding 50 mg of Zn-BC and of NYS. To prepare extracts, 100 g of blank feed mixture or feed mixture with added Zn-BC and NYS was extracted with methanol:deionised water (1:1, v/v; 2 × 20 mL), first by shaking, then in an ultrasonic bath for 15 min. The resulting extracts, obtained from the spiked feedstuff samples, as well as those extracts from blank feedstuff samples, were separately combined, filtered (filter paper), and filled up to 50 mL with 1:1 (v/v) methanol:deionised water. The aliquots (1, 5, 10, 20, 30, 40, 50 and 60 μL) were spotted on the HPTLC plate to give concentrations of 100, 500, 1000, 2000, 3000, 4000, 5000 and 6000 ng per spot of Zn-BC and of NYS.

## RESULTS AND DISCUSSION

### Method development

For separating Zn-BC and NYS in synthetic mixtures of standard solutions, several different plates (all from Merck) were used: (1) DC-RP-18 $F_{254S}$ (10 × 10 cm; 0.25 mm); (2) DC silica gel 60 (10 × 10 cm; 0.25 mm); (3) HPTLC-RP-18 $F_{254S}$ (10 × 10 cm); and (4) TLC silica gel 60 $F_{254S}$ (10 × 10 cm). The best result was obtained with HPTLC-RP-18 $F_{254S}$ (significantly higher $R_f$ values with good separation between migration spots) with different mobile phases.

A TLC method has been published[7,9] for determining impurities in Zn-BC and NYS and for their separation and determination in pharmaceutical preparations, but no reports were found for simultaneous separation and determination of Zn-BC or NYS by planar chromatography in real samples such as feedstuffs. In preliminary experiments, several previously reported mobile phases[8,9,15–19] were tested for separation and analysis of the investigated compounds.

Mobile phases of similar composition – chloroform:methanol:water (20:22:10, v/v/v)[9] for NYS by TLC, and chloroform:ethanol:methanol:water (5:3:3:4, v/v/v/v),[8] used as the mobile phase for separating Zn-BC components by counter-current chromatography – were not suitable for separating mixtures of Zn-BC and NYS along with the experiments during these studies by HPTLC. Proposed modifications of glacial acetic acid[9] for pH modifier, and of 25% $NH_3$ for TLC separation of Zn-BC[15] in a mixture with neomycin sulfate and polymyxin B sulfate (methanol:*n*-butanol:25% ammonia:chloroform, 14:4:9:12, v/v/v/v) were also not efficient for analysing Zn-BC and NYS mixtures in this study.

The separation using a mobile phase with different percentages of acetone, toluene, methanol and 25% ammonia gave some good results in the ratio (1:5:10:1, v/v/v/v), although with a low $R_f$ value for Zn-BC, and significant tailing of both spots. These results were the best in the preliminary studies and indicated that toluene could be the essential component in the mobile phase.

Mobile phases for Zn-BC analysis by HPLC were used acetonitrile/methanol (equal volumes):toluene:$KH_2PO_4$/KOH (pH 6.8) in similar volume ratios to those reported,[16–19] and gave some good results for HPTLC separation of Zn-BC and NYS. Toluene was added, at the concentrations from 1 to 10%, to acetonitrile/methanol equal volumes:$KH_2PO_4$/KOH (pH 6.8) = 60 : 40 (v/v). The presence of 3% toluene in the mobile phase resulted in the most sufficient increase of $R_f$ values and very efficient separation. The calculated value for resolution ($R_s = 3.5$) confirmed the efficient separation, since acceptable values must be higher or equal to 1 (baseline separation = 1.5).[20]

The finally selected mobile phase for separating mixtures of Zn-BC and NYS by HPTLC was: acetonitrile/methanol (equal volumes):toluene:$KH_2PO_4$/KOH (pH 6.8) at 57:3:40 (v/v/v) with the pH adjusted with HCl to approximately 8.2. For efficient separation the optimal pH is 8.2, as established previously,[13] but if toluene is added to the mixture of acetonitrile/methanol and buffer at that pH, an emulsion is formed. It is essential therefore that the starting buffer solution has a pH of 6.8 as subsequent addition of toluene increases the apparent pH to over 8.2, but without emulsion formation. The pH can then be adjusted to 8.2 with HCl. The densitogram recorded at 215 nm is shown in Fig. 2. The maximum absorbance is at 192 nm for Zn-BC and at 305 nm for NYS, but Zn-BC cannot be detected at 305 nm, and the best results are obtained at 215 nm for both antibiotics. The $R_f$ values for Zn-BC and NYS at 215 nm (Table 1), obtained for extracts of spiked feed samples and of standards, were used. The $R_f$ value for Zn-BC in extracts from spiked feedstuff was 0.231 which is 95.46% of the $R_f$ value for Zn-BC standard (0.242), and the corresponding $R_f$ value for NYS was 0.716 which is 101.85% of the $R_f$ value for NYS standard (0.703). The calculated values for the symmetry factor of Zn-BC and NYS peaks were 1.14 and 0.89, respectively.

### Method validation

Validation of the developed HPTLC method was carried out as per ICH guidelines Q2 (R1)[21] for selectivity, precision, accuracy, robustness, measurement range limits (limit of detection (LOD), recovery and specificity). Some other literature data were also used.[22–25]



*J Sci Food Agric* **88**:1576–1581 (2008)
DOI: 10.1002/jsfa

**Table 1.** Selected parameters for HPTLC method validation and separation Zn-BC and NYS in feedstuffs

| Parameter | Zn-BC | NYS |
|---|---|---|
| λ (nm) | 215 | 215 |
| $R_f$ (standards) | 0.242 | 0.703 |
| $R_f$ (spiked feed) | 0.231 | 0.716 |
| Detection limits (μg) | 0.18 | 0.22 |
| Linearity range (μg) | 0.8-3.2 | 1.1–5.4 |
| $r^2$ | 0.9964 | 0.9769 |
| Regression equations, $y = ax + b$ | $a = 879.69 \pm 2.32$; $b = 0.71 \pm 0.24$ | $a = 742.42 \pm 1.97$; $b = 0.23 \pm 0.19$ |

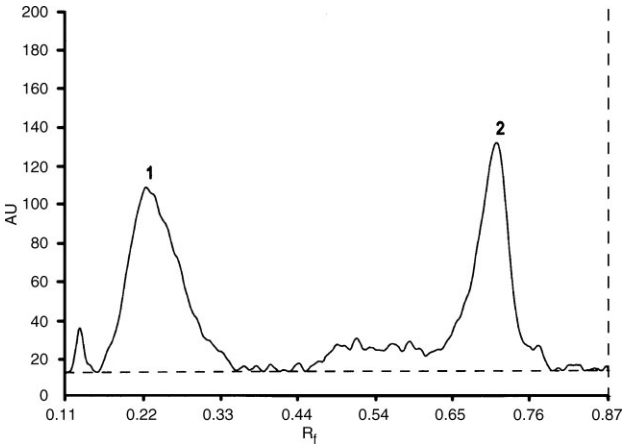


**Figure 2.** Densitogram of extracts from spiked animal feedstuff for pigs over 60 kg TS-2 with Zn-bacitracin (1) and nystatin (2), for concentrations 2 μg per 20 μL, at 215 nm. The mobile phase was acetonitrile/methanol (equal volumes):toluene:$KH_2PO_4$/KOH (pH 6.8) = 57 : 3 : 40 (v/v/v); HPTLC-RP-$18_{F254S}$ (10 × 10 cm); 25 °C.

The selectivity of the method was investigated by observing interfering peaks from matrix present in the feedstuffs. Three different feedstuff mixtures – for pigs, cattle and chicken – were tested for interference, using 20 μL of extracts obtained from blank feedstuff samples. The feed ingredients showed no interference with the HPTLC results in any of the tested feedstuff samples, indicating the selectivity of the method.

The quantification limit and detection limit were considered together, since densitometric analysis ensures that well developed peaks are obtained at low noise level. The sample signal to which a specified amount of analyte was added was at least three times greater than that of the blind signal (Table 1). LOD were 0.18 μg and 0.22 μg for Zn-BC and NYS, respectively.

Linearity was checked for six solutions of different concentrations ranging from 50 to 150% of the expected concentration of 2 μg per 20 μL. The results were analysed using the linear regression method for concentrations and peak areas (Table 1). Correlation coefficients were 0.9982 and 0.9884, in linear ranges from 0.8 to 3.2 μg and from 1.1 to 5.4 μg for Zn-BC and NYS, respectively.

The accuracy of the method was expressed as the values of recovery of both analytes. For this purpose

**Table 2.** Accuracy of the method of synthetic standard's mixtures for the assay of animal feed additives

| Amount added (μg) | Amount found (μg) | | Recovery (%) | |
|---|---|---|---|---|
| | Zn-BC | NYS | Zn-BC | NYS |
| 1.6 | 1.574 | 1.663 | 98.37 | 103.94 |
| 1.8 | 1.767 | 1.895 | 98.16 | 105.28 |
| 2.0 | 1.984 | 2.093 | 99.20 | 104.66 |
| 2.2 | 2.169 | 2.294 | 98.59 | 104.27 |
| 2.4 | 2.378 | 2.536 | 99.08 | 105.66 |
| Mean ± SD | | | 98.68 ± 0.45 | 104.76 ± 0.70 |

Zn-BC and NYS were added in amounts from 80 to 120% of 2 μg (20 μL of extracts obtained from spiked feedstuff samples) and analysed at five concentrations levels ($n = 6$). The results of quantitative analysis (in the range 1.6–2.4 μg for both antibiotics), are presented in Table 2.

Repeatability and intermediate precision were determined by calculating the relative standard deviation (RSD) for $R_f$ and for peak areas, using 20 μL of extracts obtained from spiked feedstuff samples. The RSD for repeatability ($n = 10$) was ≤1.7% for both values, which is acceptable. Intermediate precision was carried out independently ($n = 6$) by two analytical chemists (RSD ≤ 2.2%).

The parameters of the optimum HPTLC conditions were modified slightly in order to evaluate the robustness. The effects of different concentrations of organic modifier (28.5 ± 0.5% acetonitrile, 28.5 ± 0.5% methanol and 3 ± 0.5% toluene), as well as the effects of buffer pH (6.8 ± 0.06), temperature (25 ± 5 °C) and detection wavelength (±3 nm), were assessed. Mobile phase volume (10 ± 1 mL), duration of saturation (30 ± 10 min), development distance (7 ± 1 cm), spot size (1 ± 0.2 cm), volume of sample (20 ± 1 μL) and development times (30 ± 1 min and 20 ± 1 min) were also determined. The fractional factorial design was applied.[26] No significant variations in accuracy, specificity and precision were found over the tested ranges (RSD ≤ 1.2% for peak area and RSD ≤ 0.2% for $R_f$ value for both antibiotics in all cases), which indicated good reproducibility of the method.

## Stability

The stability of Zn-BC and NYS was checked in acidic (pH < 6) and basic (pH > 10) solutions in test extracts at room temperature (23 ± 3 °C) for 24 and 48 h, and the recoveries were lower (96.7 and 97.8% for 24 h and 95.1–96.3% for 48 h), indicating a low degree of degradation.

The possibility of photo-degradation of Zn-BC and NYS in 1:1 (v/v) methanol:deionised water solution was also studied. Solutions were exposed to direct sunlight, UV light or kept in the dark for 7 days. The samples kept in the dark at room temperature showed no significant degradation (≥98.5% recovery). However, Zn-BC and NYS solutions exposed to UV

**Table 3.** Application results for spiked feedstuff samples

| | | Zn-BC | | NYS | |
|---|---|---|---|---|---|
| Sample tested | Amount expected (mg) | Amount found (mg) | Recovery (%) | Amount found (mg) | Recovery (%) |
| TS-2 | 5 | 4.89 ± 0.37 | 97.8 | 5.09 ± 0.53 | 101.8 |
| TKN-2 | 5 | 4.98 ± 0.28 | 99.6 | 5.18 ± 0.34 | 103.6 |
| KM-19 | 5 | 4.94 ± 0.31 | 98.8 | 5.13 ± 0.19 | 102.6 |

light and direct sunlight showed photo-degradation of around 9% for Zn-BC and 7.5% for NYS, respectively.

### Application

The method was tested for assaying Zn-BC and NYS in animal feed. When analysing the spiked commercial products, the amounts and recoveries obtained were determined by comparing the results with a standard solution containing the same concentration as that in the spiked commercial products after extraction. The results presented in Table 3 show significant agreement between expected and found values after spiking ($n = 6$).

In comparison to the MEKC method for separation and determination of Zn-BC and NYS,[13] HPTLC is sufficiently accurate and can be applied for rapid and economical routine quality control of feedstuffs at the market with acceptable precision and sensitivity.

According to LOD values for each antibiotic, the minimum detectable amounts with the proposed method are 4.5 and 5.5 mg kg$^{-1}$ (ppm) of Zn-BC and NYS, respectively, in feed. Zn-BC and NYS were used as additives in animal feed at efficiency levels of 5–250 mg kg$^{-1}$ and 50–200 mg kg$^{-1}$, respectively.[5,27,28] The sensitivity of the proposed method is acceptable in relation to the efficiency levels of Zn-BC and NYS as additives and can be utilised for routine control of animal feedstuffs, but not for monitoring residues of non-authorised medicinal substances in food from animal origin (completely different samples and concentration levels of banned veterinary drugs and even metabolites) so, in that case validation should be performed according to European Directive 657/2002/EC.[29]

## CONCLUSION

The results show that HPTLC is a valuable technique for separating Zn-BC and NYS with good reproducibility. A new mobile phase has been proposed in which the toluene content was substantial for separation, at the low, but crucial concentration of 3%. The pH is very important for stability of both molecules and the best separation and stability was at pH 8.2. Chromatograms were developed twice, and the second development was carried out in the same mobile phase, resulting in peaks without tailing (values for symmetry factor of Zn-BC and NYS peaks were 1.14 and 0.89, respectively) and with higher $R_f$ and efficient separation at $R_s = 3.5$. This system was also applied successfully for qualitative and quantitative analysis of the investigated components in animal feedstuffs spiked with Zn-BC and NYS.

## ACKNOWLEDGEMENTS

This work was financed in part by the Ministry of Science and Environment protection, Belgrade, Serbia, as part of Project no. 142072.

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