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Analysis of neomycins A, B and C by high-performance liquid chromatography with post-column reaction detection

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Abstract: The analysis of the antibiotics neomycins A, B and C was investigated. The separation of the components was studied using reversed-phase and reversed-phase ion-pair chromatography. The optimum separation was obtained utilizing a Lichrosorb RP-2 column with a mobile phase consisting of 75 mg/l sodium dodecyl sulphate, 0.5M Na_2SO_4 and 0.015M sodium acetate buffer at pH 7.0. Using this mobile phase, baseline separation was obtained for all three compounds in approximately 20 min. Detection was via post-column derivatization of the analytes with *ortho*-phthalaldehyde in the presence of mercaptoethanol to form fluorescent iso-indole products. This system is applied to the analysis of a number of formulated products containing neomycin.

Keywords: *Neomycins; reversed-phase ion-pair high-performance liquid chromatography; post-column reaction detection; fluorimetric detection.*

Introduction

The antibiotic neomycin is a mixture of aminoglycosides, consisting primarily of two isomeric bacteriocidal components, neomycins B and C, and one of their degradation products, neamine or neomycin A (Fig. 1). Since the therapeutic activity of neomycin depends on the B:C ratio, it is useful to be able to determine the concentration of each component in the mixture independently. The analysis of neomycins has been reviewed extensively by Heynes [1]. Various techniques have been applied to this analysis including microbiological methods, countercurrent distribution, paper, thin-layer, ion exchange, liquid and gas–liquid chromatography.

For quantitative analysis, a column chromatographic method is preferable. Classical ion exchange separations have been performed [2], but this approach is very time-consuming. A high-performance liquid chromatographic (HPLC) method based on ion exclusion [3] using a Bio-Rad AG-X2 column has been reported which is complete in approximately 25 min. The column, however, proved to be not very stable, requiring replacement after 25–30 analyses. A separation has been reported using normal-phase

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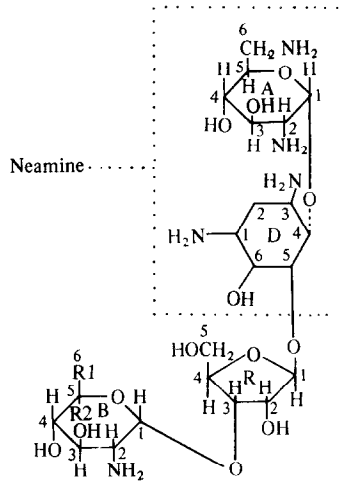

Figure 1
Structure of neomycins. Neomycin B: $R_1 = H$, $R_2 = CH_2NH_2$. Neomycin C: $R_1 = CH_2NH_2$, $R_2 = H$.

HPLC following pre-column derivatization with 1 fluoro 2,4-dinitrobenzene; however, the pre-column derivatization was time-consuming [4, 5]. Although ion-pair chromatography has been applied to the analysis of antibiotics [6], and shows promise for the separation of the components of neomycin, no such application has been reported.

Since the neomycins do not exhibit significant absorption in the 200–340 nm region and possess no native fluorescence, sensitive detection of these compounds presents a problem. Although refractive index [3] and polarimetric [7] detection have been applied, they have limited sensitivity. Post-column reaction detection has been applied using ninhydrin as a reagent [8], but this method suffers from a requirement of a long reaction time. Post-column derivatization with orthophthalaldehyde (OPA) has been applied previously to a number of aminoglycosides [9], although not specifically to the analysis of neomycins.

The goal of the present work was to develop a rapid and efficient method for the separation and detection of neomycins A, B and C, using HPLC and post-column reaction detection with OPA as a reagent.

Experimental

Apparatus

A schematic of the HPLC system used in the major part of this work is shown in Fig. 2. A multi-head Orlita model 1515 pump (Giessen, GFR) with a home-made pulse dampening system was used for both mobile phase and OPA reagent delivery. A Rheodyne (Cotati, CA, USA) model 7010 injection valve with a 20 μl sample loop was used for sample introduction. A Varian Associates (Walnut Creek, CA, USA) Fluorochrom filter fluorometer was used for detection, equipped with a tungsten lamp, Corning 7 — 54 and 7 — 60 excitation filters and 4 — 76 and 3 — 73 emission filters. The detector response was recorded using a Kipp and Zonen (Delft, The Netherlands) model BD-8 recorder.

The post-column reaction system consisted of a home-made mixing tee constructed from three 0.25 mm i.d. × 1/16″ stainless steel capillaries joined in a stainless steel body at 30° angles, and a reaction coil of 3.5 m × 0.25 mm i.d. spiral stainless steel capillary.

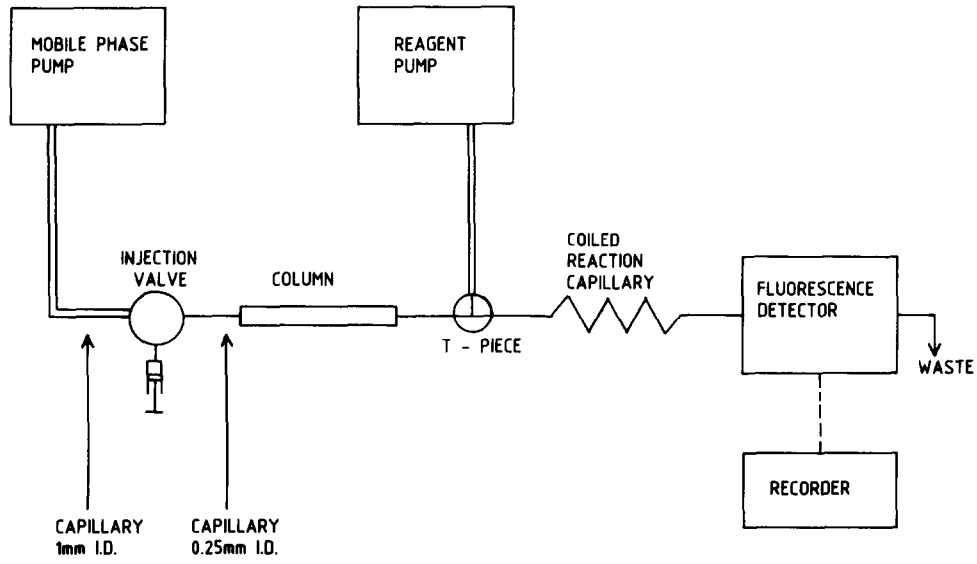

Figure 2
Schematic of HPLC system.

The use of the following columns was investigated:
(1) 25 cm Supelcosil 5 μm LC-1 (Supelco, Bellefonte, PA, USA);
(2) 10 cm Brownlee MPLC 10 μm CN (Santa Clara, CA, USA);
(3) 10 cm Brownlee MPLC 10 μm DIOL;
(4) 15 cm Spherisorb 5 μm C-1 (Phase Separations, Queensferry, UK);
(5) 15 cm LiChrosorb 5 μm RP-2 (Merck, Darmstadt, GFR);
(6) 15 cm LiChrosorb 5 μm RP-8 (Merck, Darmstadt, GFR);
(7) 15 cm LiChrosorb 5 μm RP-18 (Merck, Darmstadt, GFR).

All columns were 4.6 mm i.d. Columns 1, 2 and 3 were purchased pre-packed, while columns 4, 5, 6 and 7 were packed in-house using a standard slurry packing technique. A 20 × 4.6 mm i.d. guard column packed with 10 μm LiChrosorb RP-18 was inserted in the system between the mobile phase pump and the injection valve to increase analytical column lifetime.

The final experiments to test the routine and automation potential of this method were carried out with a Varian Model 5000 liquid chromatograph equipped with a Varian post column reactor PCR-1 and the above-mentioned Varian fluorimeter.

Chemicals and reagents

Methanol, chloroform, sodium acetate, sodium tetraborate, sodium sulphate and potassium chloride were obtained from J. T. Baker (Deventer, The Netherlands). Sodium dodecyl sulphate and heptane sulfonic acid were obtained from Kodak (Rochester, NY, USA). *Ortho*-phthalaldehyde and mercaptoethanol were obtained from Merck (Darmstadt, GFR). Water was purified in-house using a Milli Q system (Waters Millipore, Millford, MA, USA). Neomycin samples and standards were obtained as gifts from Glaxo Operations (UK) Limited and Gist-Brocades NV (Delft, The Netherlands).

Methods

The OPA solutions were prepared daily. 160 mg/l OPA and 0.2% (v/v) 2-mercaptoethanol were dissolved in a 0.2M sodium tetraborate buffer at pH 9.3. The mobile phase flow rates were (unless otherwise noted) 1.8 ml/min and the reagent was added at 0.7 ml/min.

All neomycin samples were injected in a 0.015M sodium acetate solution at pH 7.0. Samples in a tablet form were simply weighed, powdered and dissolved in buffer (1 tablet/500 ml buffer). Lotions, ointments and creams were first dispersed in chloroform (5g/50ml) and then extracted into 40 ml buffer. Although the sample extracted into the buffer was used for the analysis, the organic phase was saved and re-extracted with buffer to check the extraction efficiency.

Results and Discussion

Optimization of separation

Reversed-phase HPLC. Table 1 summarizes the resolution data obtained using reversed phase HPLC. Three separate columns were evaluated: a 25 cm Supelcosil LC-1, a 10 cm Brownlee MPLC CN and a 10 cm Brownlee DIOL. In all experiments, a 10 mM phosphate buffer at pH 8.1 was used with varying amounts of methanol and potassium chloride in the mobile phase. For both the LC-1 and the CN column, no components were eluted in the absence of potassium chloride. The salt concentration exhibited a large effect on retention and, by adjusting it, some separation of the neomycins could be obtained. Using the LC-1 column sufficient selectivity could be obtained for all three neomycin components; however, in all cases, peak tailing was severe and resolution poor (see Fig. 3). With the CN column, neomycin pairs A, B and A, C could be separated, but not B, C. Some separation could be obtained using the DIOL column, retention was

Table 1

Column	% Methanol	(KCL) (M)	Result
SUPELCOSIL	40	0	No elution
LC-1	0	0	No elution
Brownlee	0	0	No elution
MPLC CN	40	0	No elution
	60	0	No elution
	0	1.0	No retention
	0	0.1	Separation: A, B and A, C. No separation: B, C.
Brownlee MPLC DIOL	40	0	Separation: A, B and A, C.
	0	0	Separation: A, B and A, C.
	0	1.0	No retention.
	0	0.1	Almost no retention.
	0	0.027	Separation: A, B and A, C.
	0	0.01	Separation: A, B and A, C. Beginning of B, C separation.

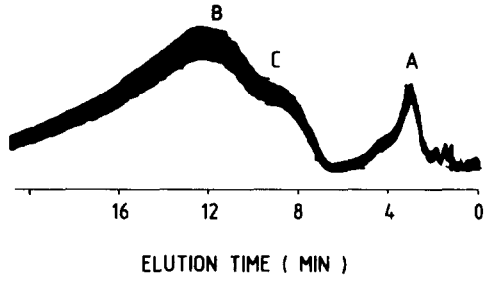

Figure 3
Typical separation of standard mixture using reversed phase HPLC. Column: 10 cm Brownless MPLC Diol. Mobile phase: 0.01 M phosphate buffer, pH 8.1. Flow rate: 1.8 ml/min.

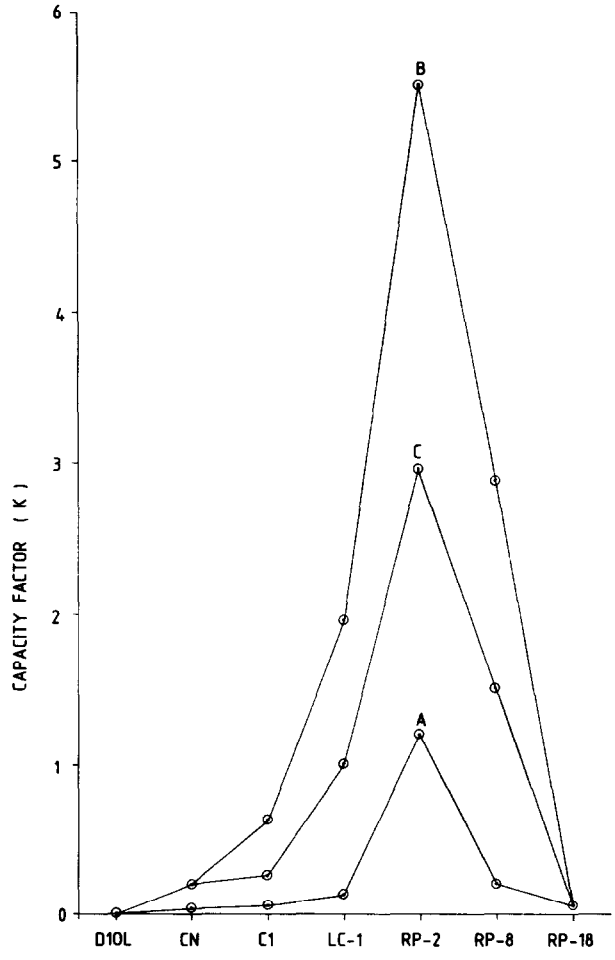

Figure 4
Capacity factor vs column. Mobile phase: 65 mg/l SDS, 0.5 M Na_2SO_4, 0.015 M acetate buffer, pH 7.5. Flow rate: 1.8 ml/min.

significantly lower with low potassium chloride concentrations. Even in the absence of salt neomycin pairs A, B and A, C could be resolved, but B, C could not. Due to the severe peak tailing and consequent poor resolution, the use of reversed-phase HPLC was abandoned in favour of reversed-phase ion pair HPLC.

Reversed-phase ion pair chromatography. By adding the ion pairing agent sodium dodecyl sulphate (SDS) the separation of neomycins could be vastly improved. A range of reverse phase conditions were examined. Figure 4 shows the effect of the column type on the capacity factor for a given mobile phase (65 mg/l SDS, 0.5M Na_2SO_4, 0.015M acetate buffer at pH 7.5). The retention of all three components was maximized using a LiChrosorb RP-2 column, but acceptable retention was also present for the LiChrosorb RP-8 and Supelcosil LC-1.

The SDS concentration exhibited a large effect on the retention. Figure 5 shows the capacity factor as a function of the SDS concentration using the LiChrosorb RP-2 column. Below approx. 50 mg/l SDS, no retention was observed.

The effect of the pH of the mobile phase is shown in Fig. 6. Retention was maximized at pH > 7. Although these data were obtained for the Supelcosil LC-1 column, comparable results were obtained for all the columns examined, as might reasonably be expected.

The linearity of response of the reaction system was investigated and found to be satisfactory over the analytical region of interest (Fig. 7).

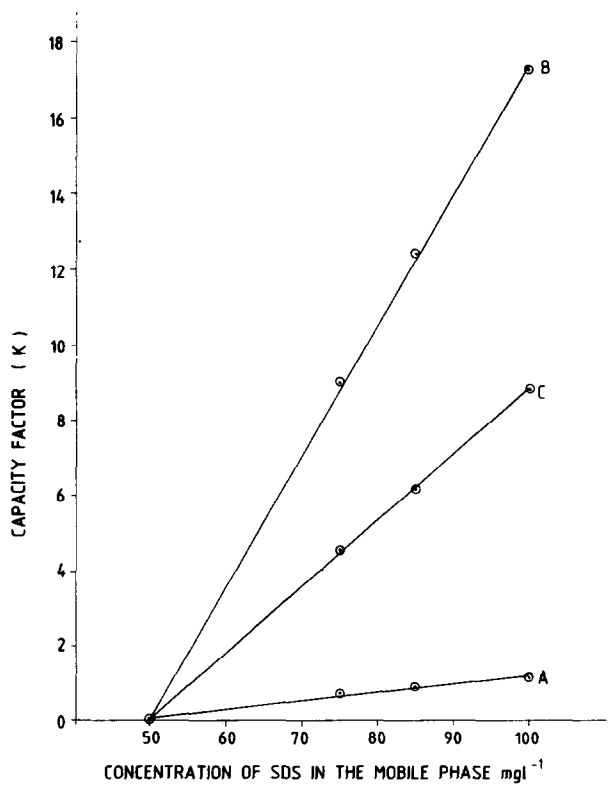

Figure 5
Capacity factor vs SDS concentration. Column: 15 cm LiChrosorb RP-2. Mobile phase: as above (except SDS).

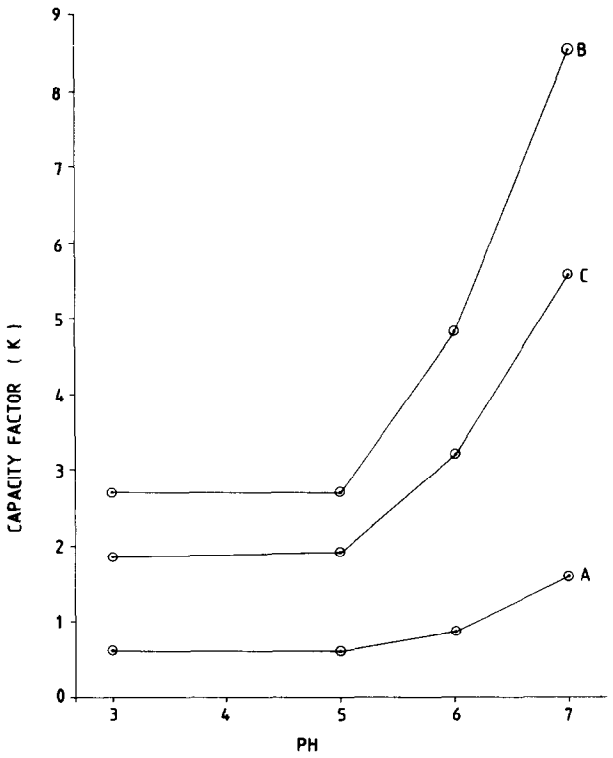

Figure 6
Capacity factor vs pH. Column: Supelcosil 25 cm LC-1. Mobile phase: 112 mg/l SDS, 0.5 Na_2SO_4 0.015 M acetate buffer.

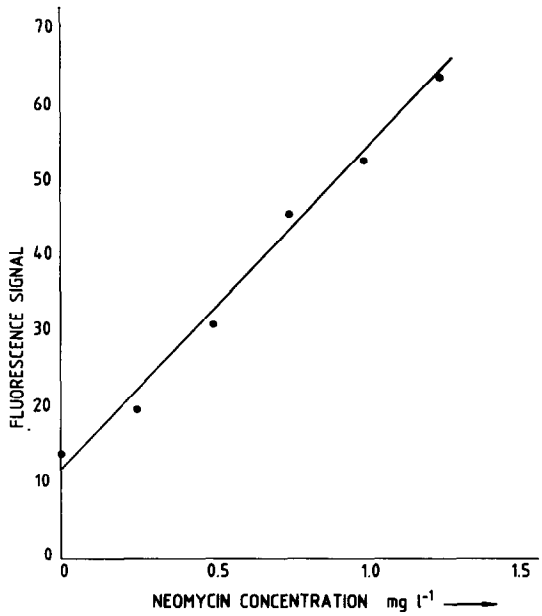

Figure 7
Linearity of detection system response. Chromatography conditions as per Fig. 6.

For all columns and mobile phases investigated, two major problems persisted. The first concerned the slow equilibration time and degradation of the analytical column. When changing mobile phases, all columns required at least 10–12 h equilibration time before reproducible results could be obtained.

After several days use, however, back pressure and retention times increased drastically. Examination of the packing material at the column entrance showed as much as 1 cm depth of coarse discoloured material. This degradation could be prevented, however, by placing a guard column packed with 10 μm LiChrosorb RP-18 material between the mobile phase pump and the injector. The guard column required periodic repacking.

The second problem concerns the band broadening of the neomycins, and has not been totally alleviated using any of the columns or mobile phase evaluated in this study. By injecting test substances (such as amino acids), it can be demonstrated that the band broadening is not due to poor flow dynamics in either the analytical column or the post-column reaction system. It must therefore be due to the slow mass transfer process of the neomycins during separation. This may be due to the interaction with residual silanol groups on the packing material or the size and multifunctional character of the

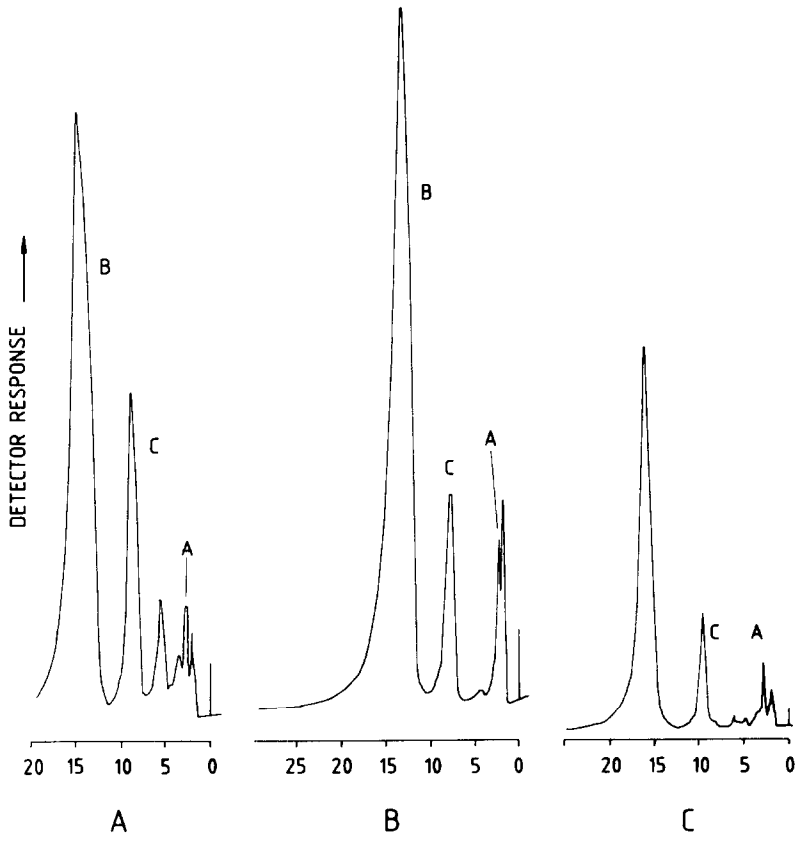

Figure 8
Analysis of pharmaceutical preparations. Column: 15 cm LiChrosorb RP-2. Mobile phase: 75 mg/l SDS, 0.5 M Na_2SO_4, 0.015 M acetate buffer. (a) Corticosteroid drops containing neomycin 0.62 mg/ml sample. (b) Neomycin eyedrops 0.68 mg/ml sample. (c) Corticosteroid cream containing neomycin 0.42 mg/ml sample.

neomycins. The exact mechanism of this anomalous band broadening has not been determined.

Application. Using a 15 cm LiChrosorb RP-2 column with a mobile phase consisting of 75 mg/l SDS, 0.5M Na_2SO_4 and 0.015 15M acetate buffer at pH 7 and a flow rate of 1.4 ml/min, the chromatograms of several pharmaceutical neomycin preparations shown in Fig. 8 were obtained. Under these conditions, the reproducibility of the peak height given as the relative standard deviation (n = 5) was A:2.9%, B:1.8% and C:1.5%. The detection limit for A was 0.4 ng and for B and C it was approximately 5 ng. Since the pharmaceutical preparations are typically in the concentration range of 0.5–1.0 mg/ml, these detection limits are more than adequate.

The extraction procedures for lotions, ointments and creams were examined by re-extracting the organic phase used originally to disperse the lipophilic samples and measuring the neomycin concentrations. The extraction efficiency was found to be 100% in all instances.

Conclusions

A method has been developed for the separation and detection of neomycins A, B and C in pharmaceutical preparations. The method allows complete quantitation of these analytes within the required detection limits within a period of approximately 20 min per sample. Under the best separation conditions we found, there was sufficient separation to allow baseline resolution of all three components in spite of excessive band broadening presumed to be due to slow mass transfer of the analytes during separation.

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References

[1] W. F. Heynes, *Analytical Profiles of Drug Substances*, Vol. 8, pp. 399–488. Academic Press, New York (1979).
[2] H. Maehr and C. P. Schaffer, *Anal. Chem.* **36**, 104 (1964).
[3] W. Decoster, P. Claes and H. Vanderheghe, *J. Chromatogr.* **211**, 223 (1981).
[4] K. Tsuji, J. F. Goetz, W. Van Meter and K. A. Gusciora, *J. Chromatogr.* **175**, 241 (1979).
[5] P. Melboe and S. Kryger, *J. Chromatogr.* **235**, 215 (1982).
[6] E. R. White and J. E. Zarenko, *J. Antibiot.* **34**, 836 (1981).
[7] P. De Rossi, *Analyst (Lond.)* **100**, 25 (1975).
[8] S. Inouye and H. Ogawa, *J. Chromatogr.* **13**, 536 (1964).
[9] J. P. Anhalt, F. D. Sancilio and T. McCorkle, *J. Chromatogr.* **153**, 489 (1978).

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