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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF β-LACTAM ANTIBIOTICS, USING FLUORESCENCE DETECTION FOLLOWING POST-COLUMN DERIVATIZATION

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## SUMMARY

This paper describes a rapid, sensitive and specific technique for the determination in microbial fermentation broths of several naturally produced β-lactams. The method consists of reversed-phase, high-performance liquid chromatographic separation of the antibiotics on an octylsilane chemically bonded stationary phase. This is followed by reaction with *o*-phthaldialdehyde and quantitative spectrofluorimetric detection. Post-column reaction conditions including temperature, reagent pH, flow-rates and reactor coil length are investigated. Accuracy, reproducibility and detection limits of the method are also examined. The technique is applied to the analysis of fermentation media following sample preparation by centrifugation and filtration.

## INTRODUCTION

Quantitative determination of antibiotics has proved one of the more difficult areas of pharmaceutical analysis, conventionally being performed either by microbiological assay and/or a variety of physico-chemical methods. The bioassay, although a relatively sensitive technique, is time consuming, difficult to quantitate accurately and lacks specificity. This results in complications when more than one antibiotic is present. For the analysis of β-lactams several chemically-based techniques are available[1]. Colorimetric methods such as the nicotinamide assay[2], the hydroxylamine assay[3] and the molybdoarsenic acid–mercuric chloride assay[4] rely upon estimation of β-lactam reaction products rather than the antibiotic itself. In several methods including colorimetric titration[5] and the imidazole assay[6] the β-lactam is hydrolysed prior to estimation.

Several problems are encountered in the analysis of trace levels of β-lactams in complex fermentation media. First, the selectivity of most chemical assays is insufficient to determine individual β-lactam levels. Secondly, chemical reactions involved in the assay procedure are not limited to β-lactams and hence other broth constituents may contribute to the result. Finally, a method may lack intrinsic sensitivity or be subject to high background readings.

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In recent years chromatographic techniques have been developed which allow simple, rapid and specific determinations to be performed. Gas chromatography has been applied to the analysis of penicillins[7] although it is limited to those which are thermally stable after derivatization. High-performance liquid chromatography (HPLC) provides simultaneous separation and quantitation without the need for volatilization or extraction of antibiotics from biological media. This technique has developed rapidly over the last decade from low efficiency ion-exchange chromatography[8] to the use of ultra high-performance, microparticulate, reversed-phase columns[9]. An extensive range of stationary and mobile phases has been reported for the analysis of β-lactams[10–12]. More recently ion-pairing reagents have been employed to increase the capacity factor of highly polar antibiotics and facilitate separations hitherto impossible[13].

Many HPLC detection systems are documented[14] but the most widely used is UV absorption which allows sensitive and quantitative determination. However the detection wavelength of compounds without chromophores is limited to end absorption below 220 nm. Most organics absorb strongly in this spectral region resulting in high sensitivity but absence of specificity.

Our interest lies in the microbial biosynthesis of β-lactams and their metabolic precursors[15–17], many of which lack chromophores. A derivatization procedure was therefore considered necessary for their detection. Several chromophoric and fluorogenic labelling reagents are now available[18]. As fluorescence detection is inherently more sensitive than that provided by UV, and the majority of naturally produced β-lactams possess a primary amino function, the use of the readily available reagent, *o*-phthaldialdehyde (OPA)[19,20], was investigated. In recent work fluorescamine has been used for the derivatization of certain cephalosporins, allowing their spectrofluorimetric determination in urine and sera[21]. There are advantages and disadvantages to both pre-column and post-column derivatization techniques[22]. Coupling of OPA to primary amino groups in aqueous media usually requires a pH of 9–10[23]. Under these conditions β-lactams are rapidly hydrolysed[24] and therefore post-column derivatization was necessitated.

This paper describes the development of an HPLC procedure for the determination of β-lactams in fermentation media with sensitive fluorescence detection after post-column reaction with OPA in a tubular reactor. Conditions of the chromatography and reaction system are discussed.

## EXPERIMENTAL

### *Materials and reagents*

Acetonitrile, used in the mobile phase, and methanol used in the derivatization reagent were both HPLC grade and obtained from Rathburn Chemicals (Great Britain). Sodium dihydrogen orthophosphate, acetic acid, potassium hydroxide and boric acid were of "analar" grade and supplied by BDH (Great Britain). 2-Mercaptoethanol and *o*-phthaldialdehyde were obtained from Sigma London (Great Britain), whilst the ion-pairing agent, tetra-*n*-butylammonium hydroxide, was acquired from Fisons (Great Britain).

Standards of antibiotic preparations, cephamycin C (sodium salt), isopenicillin N (barium salt), cephalosporin C (sodium salt), penicillin N (barium salt) and 6-

aminopenicillanic acid (6-APA) (potassium salt), were kindly supplied by Beecham Pharmaceuticals (Great Britain), Ciba-Geigy (Switzerland) and Glaxo (Great Britain).

*Apparatus and operating conditions*

*Fluorescence detection.* A spectrofluorimeter (Model No. 650-10s, Perkin-Elmer, CT, U.S.A.) fitted with a 18-μl quartz flow cell was used for liquid chromatographic detection following post-column derivatization. The same instrument fitted with a cuvette holder was also used for fluorescence analysis after batch reaction with OPA.

*UV detection.* UV detection was carried out at 220 nm using a variable wavelength detector (Spectromonitor III; Laboratory Data Control, FL, U.S.A.) which was employed to monitor precision of the sample injection as post-column reaction conditions were varied.

*Chromatographic system.* The solvent delivery system used was a double reciprocating pump (Model No. 750/04; Applied Chromatography Systems, Great Britain). Injections were made using a Rheodyne valve (Model No. 7125; Anachem, Great Britain) fitted with a 20-μl loop. Chromatography was performed on a 20 × 4.6 mm I.D. analytical column filled with 5-μm Spherisorb C8 (Cat. No. S5C8; Phase Separations, Great Britain). This was slurry-packed[25] in acetonitrile and methanol using Stansted Air Amplified Pump (Model No. RR/OZ5; HPLC Technology, Great Britain). A 4 × 10 mm I.D. guard-column was fitted prior to and in series with the analytical column and was tap-packed[25] with the same stationary phase as the analytical column.

The eluent was prepared by mixing 40 ml of acetonitrile with 460 ml of 0.01 *M* $NaH_2PO_4$, containing 0.2% (v/v) glacial acetic acid. Tetra-*n*-butylammonium hydroxide was then added to a concentration of 0.01 *M*, and the solvent adjusted to pH 5.5 with 2.0 *M* NaOH. Degassing was carried out by sonication followed by continual passage of helium through the solution.

The OPA reagent was prepared from two stock solutions. Solution A was prepared by dissolving OPA in methanol at a concentration of 10 mg/ml. This may be stored in darkness at 4°C for up to 1 week. Solution B consisted of 0.4 *M* boric acid, containing 2% (v/v) 2-mercaptoethanol. The pH was adjusted with KOH to between 10 and 14, according to that required. 500 ml of solution B are then mixed with 25 ml of solution A, at room temperature. This reagent was prepared freshly each day and was vacuum degassed prior to use.

*Post-column reaction system.* Connection between the analytical column and UV detector was by a short piece of 0.15 mm I.D. PTFE tubing. A similar piece of tubing joined the detector outlet to a Swagelok union tee, from which the eluent and reagent passed to the fluorimeter via the reactor coil. The coil consisted of various lengths of 0.3 mm I.D. PTFE tubing, contained within a water-bath (Model No. DB 237; Chemlab Instruments, Great Britain) with thermostat control to ±0.1°C.

Reagent delivery was by a single piston Milton Roy pump (Milton Roy, FL, U.S.A.), equipped with a pulse damping system consisting of a large volume Bourdon gauge. Care was taken to thoroughly flush this system after use with distilled water followed by methanol, due to the corrosive nature of the reagent. A short piece of 0.15 mm I.D. PTFE tubing connected to the fluorimeter outlet was found very use-

ful in preventing bubble formation in the flow cell by creating a slight back-pressure.

Integration of peak areas and data handling was performed by a Hewlett-Packard reporting integrator (Model No. 3390A; Hewlett-Packard, PA, U.S.A.).

*Analytical procedures*

Preparation of fermentation broths for analysis was by centrifugation and filtration to remove particulates. Deproteinization was unnecessary as the analytical column was protected by a guard column. 1 ml of fermentation media was removed from a shake flask with a glass syringe and spun at 7900 *g*, in a tapered centrifuge tube, for 10 min at 4°C. The supernatant was then passed under pressure through a 0.22–$\mu$m filter (Cat. No. GSWP 01300; Millipore, MA, U.S.A.), and injected onto the column.

Standard solutions of the antibiotics, in the range 1–1000 $\mu$g/ml, were prepared daily by dilution with distilled water from a stock of 1 mg/ml. Stock solutions were maintained at −20°C until required for use. Fermentation broths were spiked with penicillin N as an internal standard at a concentration of 50 $\mu$g/ml, using an analytical microsyringe. Cephamycin C was then added to give a range of final concentrations from 1 to 100 $\mu$g/ml. Broth injections of 20 $\mu$l were used in each case.

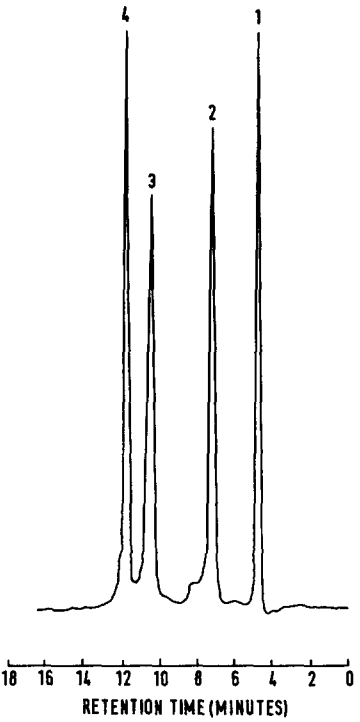


Fig. 1. HPLC of cephamycin C (35 $\mu$g/ml) (I), penicillin N (15 $\mu$g/ml) (2), cephalosporin C (40 $\mu$g/ml) (3) and 6-APA (30 $\mu$g/ml) (4), using fluorescence detection following post-column derivatization with OPA, (injection volume 20 $\mu$l). Chromatographic conditions were as described in the text. Flow-rate 1.5 ml/min. Post-column reaction conditions: reagent pH 12; reactor coil length 12 m; reaction temperature 90°C; reagent flow-rate 0.8 ml/min. Detector sensitivity 1.0; $\lambda_{ex}$ 350 nm, $\lambda_{em}$ 450 nm; slit widths of 5 nm.

## RESULTS AND DISCUSSION

### *Chromatographic conditions*

Optimum chromatographic conditions for a range of β-lactams were investigated by varying the stationary phase and nature, concentration and pH of the mobile phase constituents. The chromatographic procedure finally adopted for all analytical studies in this paper was as specified under *Apparatus and operating conditions*. Fig. 1 shows a typical chromatogram obtained using this system.

### *Detection wavelengths*

Excitation and emission maxima of the derivatized antibiotics were obtained using a "stop-flow" technique and are listed in Table I. Emission maxima are centred at 350 nm, whilst excitation maxima occur over a 5-nm range around 450 nm. Use of 5 nm slit widths with excitation and emission wavelengths of 350 nm and 450 nm respectively gave the maximum sensitivity and selectivity for the range of β-lactams studied.

### *Effect of temperature and reagent pH*

The effect of temperature and reagent pH on the fluorescence intensities of four β-lactams is illustrated in Fig. 2. A final pH of 11.5 resulted from mixing of the column eluent and derivatization reagent (pH 12.0), in the procedure described. Both cephamycin C and cephalosporin C show similar exponential responses as the temperature of the reactor coil is raised, this effect becoming more pronounced as the pH increases. Under the conditions described in Fig. 2, the highest sensitivity was therefore achieved at a reaction temperature of 100°C and a pH of 14.6-APA shows a less marked increase in fluorescence intensity as the reaction temperature is raised and this relationship tends to be more linear than that for cephamycin C and cephalosporin C. At high temperatures, and a pH of 12 or 14, the gradients decrease and approach a plateau, this effect being more marked at higher pH values. Thus maximum sensitivities are achieved at a pH of 12, above a temperature of 70°C, under the conditions described in Fig. 2. The overall effect of temperature and pH on the fluorescence intensity of penicillin N is less significant than that for the other β-lactams studied. Decreasing intensity occurs at pH 14 although this is slightly less pronounced at pH 12 above 65°C. No difference was observed between the response to pH and temperature of isopenicillin N or penicillin N.

TABLE I

FLUORESCENCE DETECTION OF DERIVATIZED ANTIBIOTICS

Maximum excitation and emission wavelengths of several β-lactams obtained by a "stop-flow" technique, following derivatization with OPA. Conditions for the reaction are described in the text.

| *Antibiotic* | *Excitation maxima (nm)* | *Emission maxima (nm)* |
|---|---|---|
| Cephamycin C | 347 | 450 |
| Cephalosporin C | 352 | 452 |
| Penicillin N | 347 | 452 |
| 6-Aminopenicillanic acid | 357 | 449 |

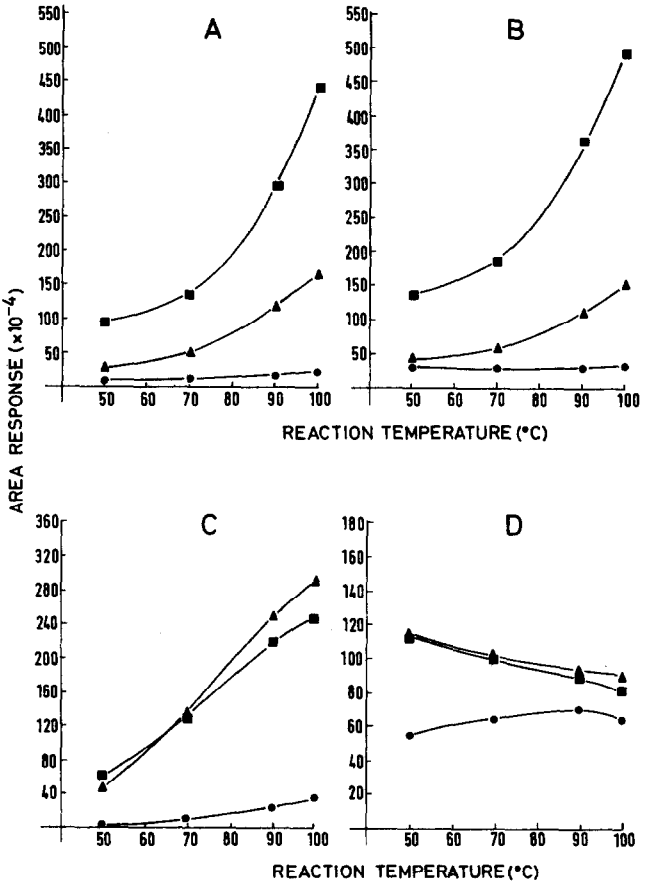


Fig. 2. The effect of reaction temperature and reagent pH on the fluorescence intensity of cephamycin C (140 μg/ml) (A), cephalosporin C (150 μg/ml) (B), 6-APA (165 μg/ml) (C) and penicillin N (45 μg/ml) (D) (injection volume 20 μl). Chromatographic conditions were as described in the text. Flow-rate 1.5 ml/min. Post-column reaction conditions: reagent pH of 10 (●), 12 (▲) or 14 (■); reactor coil length 5 m; reagent flow-rate 0.8 ml/min; reaction temperature 90°C. Detector sensitivity 1.0; $\lambda_{ex}$ 350 nm, $\lambda_{em}$ 450 nm; slit widths of 5 nm.

The influence of temperature and pH on the fluorescence intensity of the β-lactams may be ascribed to differences in the rates of one or more of the following processes:

alkaline hydrolysis
reaction with OPA
decay of the fluorescent derivative

The results suggest that alkaline hydrolysis of the β-lactams is of considerable importance in their derivatization with OPA. However our data is not in complete accord with hydrolysis rates already published[26] probably due to the influence of other reactions involved prior to, during and after the derivatization.

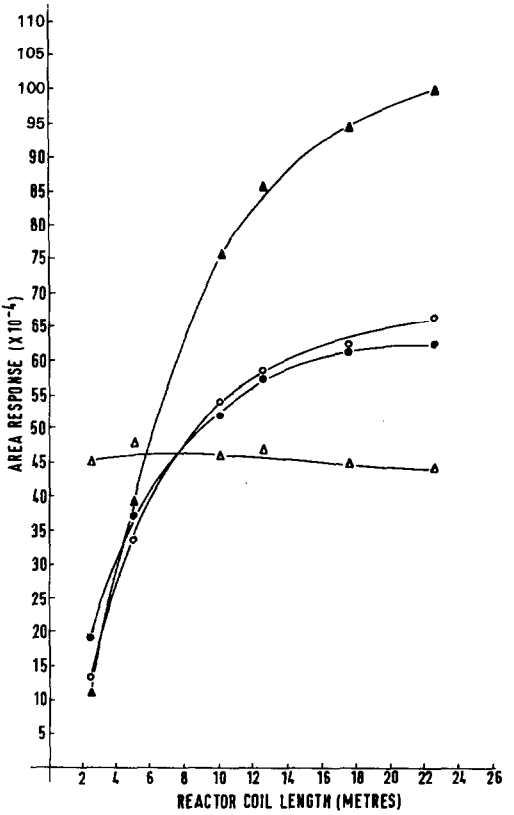


Fig. 3. Effect of reactor coil length on the fluorescence intensity of cephamycin C (40 μg/ml) (○), cephalosphorin C (50 μg/ml) (●), penicillin N (20 μg/ml) (△) and 6-APA (25 μg/ml) (▲) (injection volume 20 μl). Chromatographic conditions were as described in the text. Flow-rate 1.5 ml/min. Post-column reaction conditions: reagent pH 12; reaction temperature 90°C; reagent flow-rate 0.8 ml/min. Detector sensitivity 1.0; $\lambda_{ex}$ 350 nm, $\lambda_{em}$ 450 nm; slit widths of 5 nm.

Batch studies on the formation and decay of fluorescence are consistent with the HPLC data relating the effect of pH and temperature, although decay rates appear independent of these factors. It therefore seems likely that the observed effects of temperature and pH are due to both the rates of alkaline hydrolysis and the reaction with OPA. The decline in fluorescence intensity shown by the penicillins above pH 12 may be due to decomposition of the fluorescent derivative. The respective reaction rates depend on the exact stereochemistry and subsequent electronic effects of a particular β-lactam.

*Effect of reaction coil length and flow-rate*

A helically coiled capillary tube reactor was chosen for this investigation due to the rapid reaction of OPA with primary amines previously reported[27], and the low degree of peak broadening resulting from a rapid flow through the coil.

Fig. 3 shows the effect of reactor coil length on the fluorescence intensities of four β-lactams. Conditions of temperature and reagent pH were kept constant throughout

these studies. These conditions were chosen to provide adequate sensitivity whilst being less detrimental to the reagent pump and fittings than the more severe conditions required to obtain higher cephamycin C and cephalosporin C sensitivities. A significant increase in intensity was observed for cephamycin C, cephalosporin C and especially 6-APA, up to a reactor coil length of 12 m. For these β-lactams, extending the coil length to 22.5 m produced a more gradual rise in intensity although this was less pronounced for 6-APA. Increasing the reactor coil lengths produces a slight overall decline in the fluorescence intensity of penicillin N. Calculations, based on peak areas[28], indicated only a slight loss of resolution as the reactor coil was lengthened, probably due to its small internal volume.

The effect of reagent flow on fluorescence intensity was studied whilst maintaining the other conditions constant. Flow-rates of less than 0.46 ml/min produced excessive short-term noise probably due to ineffective pulse-damping of the single piston minipump. At flow-rates above 0.46 ml/min the intensities decreased only slightly, although above 1 ml/min a sharp decline was observed.

Lengthening the reactor coil or decreasing reagent flow-rate effectively increases the time for derivatization prior to detection. The rise in fluorescence intensity shown by cephamycin C, cephalosporin C and 6-APA implies that post-column reactions only approach completion, under the conditions of temperature and pH employed, with coil lengths above 12 m and reagent flow-rates lower than 1 ml/min. The gradual decrease in sensitivity for penicillin N implies complete derivatization followed by decay of the fluorescent product as described in the previous section.

*Accuracy and reproducibility*

During these studies, for reasons already stated, post-column reaction conditions were maintained at a reagent pH of 12, a temperature of 90°C, a reactor coil length of 12 m and a reagent flow-rate of 0.8 ml/min.

Linearity of the fluorescence response with respect to concentration of penicillin N and cephamycin C was checked. Serial dilutions of each β-lactam, between 0 and 100 μg/ml, were prepared in triplicate, and 20 μl of each solution injected onto the column. Peak areas were used to construct calibration lines, both of which gave correlation coefficients greater than 0.999. Gradients of 10,456 area units per μg per ml for penicillin N and 4100 area units per μg per ml for cephamycin C were calculated by regression analysis. Good reproducibility was obtained and assessed by repeating the calibration procedure on two different days each plot lying within a 95% confidence interval of the first. Reproducibility of a 20-μl, repeated injection ($n = 20$) of cephamycin C (100 μg/ml) gave a ±2% error from the mean.

Accuracy was investigated by spiking a blank broth with solutions containing known concentrations of cephamycin C. Using penicillin N as an internal standard at a concentration of 50 μg/ml, the ratio of peak areas was used to calculate cephamycin C concentrations. The results are listed in Table II.

*Sensitivity and detection limits*

Using 20-μl injections and optimum post-column reaction conditions, detection limits of less than 0.5 μg/ml and 1.0 μg/ml were achieved for penicillin N and cephalosporin C respectively. In samples of fermentation media (*Streptomyces* fermentation medium) similar sensitivities were obtained although in these studies ex-

TABLE II

DETERMINATION OF CEPHAMYCIN C IN FERMENTATION MEDIA

Cephamycin C was added to blank broths at a range of concentrations, together with penicillin N at a constant 50 μg/ml. Peak area ratios were used to calculate an experimental cephamycin C concentration. Chromatographic and post-column reaction conditions are described in the text.

| *Sample No.* | *Cephamycin C concentration (μg/ml)* | | *Recovery (%)* |
|---|---|---|---|
| | *Theoretical* | *Experimental* | |
| 1 | 125.0 | 124.1 | 99.2 |
| 2 | 80.0 | 77.9 | 97.3 |
| 3 | 45.0 | 45.7 | 101.5 |
| 4 | 40.0 | 38.9 | 97.2 |
| 5 | 15.0 | 14.7 | 98.0 |

cessive baseline noise, due to inefficient pulse-damping of the reagent pump, prevented maximum electronic amplification of the signals. A typical chromatogram showing natural levels of β-lactams in broths resulting from the fermentation of *Streptomyces clavuligerus* (ATCC 27064) is illustrated in Fig. 4.

Improved detection limits can also result from the use of larger injection volumes, although 20 μl was used throughout the present investigation to prevent the possibility of overloading the column when complex broths were injected.

The formulation of fermentation media, nature of the microorganism and the extent of growth can all influence detection limits primarily due to differences in the levels of interfering components, especially those eluting close to the solvent front.

## CONCLUSIONS

The post-column derivatization procedure described allows rapid and selective analysis of microbial fermentation broths for trace levels of several naturally produced β-lactams.

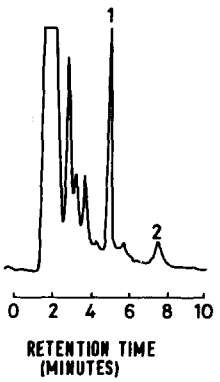


Fig. 4. HPLC of a fermentation broth after 72-h growth of *Streptomyces clavuligerus* (ATCC 27064) indicating the presence of cephamycin C (1) and penicillin N (2). The 20-μl sample was injected following centrifugation and filtration. Chromatographic and post-column reaction conditions were as described under Fig. 1.

Results obtained by varying post-column reaction conditions strongly suggest that hydrolysis of β-lactams is necessary prior to their derivatization with OPA. Once the reaction is complete decay rates of the fluorescent product become an important consideration in achieving maximum sensitivity.

The authors have also shown the method to be applicable to deacetylcephalosphorin C, deacetoxycephalosporin C determinations, and are investigating its application to other β-lactam biosynthetic precursors. Post-column reaction conditions may be varied to achieve maximum sensitivity for a particular analysis. If a medium contains both penicillins and cephalosporins a compromise in post-column derivatization conditions must be made and we have found the conditions described in Fig. 3 to be most suitable for such analyses. However it should be realised that differences in composition and flow-rate of the column eluent will also alter fluorescence intensity if post-column conditions are held constant.

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