Journal of Chromatography, 509 (1990) 347–356
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 345

Analysis of δ-L-α-aminoadipyl-L-cysteinyl-D-valine by ion chromatography and pulsed amperometric detection

M.J. DONALDSON* and M.W. ADLARD
Polytechnic of Central London, 115 New Cavendish Street, London W1 1ST (U.K.)
(First received September 22nd, 1989; revised manuscript received February 13th, 1990)

ABSTRACT

A novel high-performance liquid chromatography (HPLC) method is presented for the detection and trace level determination of the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV). The tripeptide, an intermediate in penicillin production, is derived from fungal fermentation. The technique relies on ion-exchange separation of the tripeptide on an anion-exchange column followed by detection by reduction on a gold electrode using pulsed amperometry. The sensitivity of direct determination of ACV is increased by employing pulsed amperometric detection (PAD) over direct ultraviolet detection. Choice of the working electrode and optimisation of electrode potentials was based on cyclic voltammograms recorded for the tripeptide in the mobile phase.

A linear regression equation was obtained over the range 0–100 μg ml^{-1}. The detection limit in fermentation broths was found to be 0.1 μg ml^{-1} whereas in buffer the detection limit was found to be 10 ng ml^{-1}. A good correlation coefficient was observed when ACV concentrations determined by ion chromatography–PAD were compared with measurements obtained by pre-column derivatisation with fluoromethylorthochloroformate followed by HPLC separation on a reversed-phase C_{18} silica column with UV detection.

The procedure has been applied to the measurement of natural levels of ACV in fermentation broths of selected strains of *Aspergillus nidulans* and *Penicillin chrysogenum*.

INTRODUCTION

Many of the classical β-lactam sulphur-containing antibiotics are derived from the same amino acid precursors, L-cysteine, D-valine and α-L-aminoadipic acid. In certain micro-organisms these amino acids form the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV), the condensation reaction being catalysed by ACV synthetase[1]. The biosynthetic pathway for the production of penicillin antibiotics in penicillium and cephalosporium species is now well established[2], and the production of ACV in fermentations represents the committed step in the production of these

0021-9673/90/$03.50

antibiotics. As such, its determination is important in the monitoring of antibiotic fermentations, the study of novel β-lactam-producing organisms and the assessment of blocked mutants. The available methods for the determination of ACV by high-performance liquid chromatography (HPLC) rely upon direct ultraviolet (UV) detection[3] or pre-column and post-column derivatisation[4,5] followed by UV or fluorescence detection.

Direct UV detection of ACV is limited in sensitivity because ACV is a poor chromophore with little absorbance above 210 nm. Derivatisation techniques are much more sensitive, although they are difficult to automate and can yield anomalous results when applied to ACV derived from fermentation broths containing large amounts of other derivatisable compounds. Pre-column derivatisation methods also have a major disadvantage in that they require several time-consuming pre-treatment steps which introduce inaccuracies and imprecision into the analysis.

ACV exists in two forms: within the fungal hyphae it is present as a monomer and, on release from the hyphae into the external medium, it is rapidly oxidised to form a dimer in which two tripeptide molecules are linked by a disulphide bond. Recently, sensitive HPLC methods have become available in which detection is based upon the electrochemical activity of the analytes[6,7]. In this report, the basis for the detection of ACV dimer is the reduction of the disulphide bond. Many organic materials have been studied using solid electrodes, however, sulphur-containing compounds rapidly poison noble-element electrodes due to strong adsorption[6]. With pulsed amperometric detection (PAD) the working electrode undergoes a cycle of reverse polarisation steps that serves to detect, clean and regenerate the surface of the electrode.

This report has set out to optimise the electrode materials, potentials and pulse durations for PAD of ACV and to use the optimised conditions for the determination of ACV in fermentation broths. The technique has been applied to the assay of ACV produced in fermentation broths by several fungal species which exhibited a wide variation in the levels of ACV production.

EXPERIMENTAL

Fungal strains

Penicillium chrysogenum strain P2 (kindly donated by Pan Labs.) is a fungal strain which has been used in the industrial production of penicillin antibiotics. The strains were grown in 25 ml of a defined medium described by Jarvis and Johnson[8] for three days in an orbital shaker at 150 rpm and 27°C. *Aspergillus nidulans* wild type is a fungus that produces low levels of penicillin[1]. *A. nidulans* was grown in Aspergillus Complete Medium (ACM)[9] for three days in an orbital shaker (150 rpm at 27°C) at which point the fermentation broths were assayed. *A. niger*, a fungal strain that does not produce penicillin, was used as a blank for recovery and spiking experiments. This species was grown in an orbital shaker (150 rpm at 27°C) in 25 ml of ACM for three days. All cultures were filtered through 0.22-μm Duraporc (Millipore Waters Chromatography) filters prior to analysis.

Reagents

Mobile phase solutions consisting of 100 m*M* sodium hydroxide (BDH, Poole,

U.K.) and 25 m*M* sodium acetate (Sigma) were prepared by dilution from a stock solution of 12.5 *M* sodium hydroxide and a stock solution of 1 *M* sodium acetate. HPLC-grade water was prepared from a Nanopure II water system (Barnstead). A standard sample of ACV dimer was a gift obtained from Glaxo U.K. Fluoromethylorthochloroformate (FMOC) was purchased from Sigma (Poole, U.K.). Acetonitrile and acetone, used in HPLC mobile phases and the preparation of the FMOC reagents, were HPLC grade and purchased from Rathburn (Walkerburn, U.K.). Boric acid, sodium dihydrogenphosphate and sodium hydroxide, used for the preparation of buffer solutions, were obtained from BDH and were AnalaR grade. Pentane was purchased from BDH and was chromatographic grade.

Chromatographic apparatus

Equipment supplied by Dionex U.K. used for ACV analysis was based on a Dionex BioLC quaternary gradient pump. The anion-exchange column employed for the separation of ACV was an AS6 column with an AG6 guard column containing the same stationary phase. The detector used was a Model PAD-2 pulsed amperometric detector operated with a gold working electrode. The working electrode potentials used were $E_1 = -0.8$ V, $E_2 = 0.05$ V, $E_3 = -0.95$ V, where E_1 was the sampling voltage, and E_2 and E_3 cleaning and regenerating voltages, respectively, and the durations of these potentials were 300, 60 and 60 ms, respectively. An Ag/AgCl reference electrode was employed.

The eluents were sparged and pressurised with helium using a Dionex Eluent degas unit. Sample injection was achieved using a Dionex autosampler module with a 50-μl injection volume. Chromatographic data were collected and analysed with a Trio 2 data station.

Bioassay of recovered ACV

Bioassays were carried out by a well-plate assay method with partially purified isopenicillin synthetase derived from *P. chrysogenum*[10,11]. The reaction mixture consisted of lyopholized HPLC eluent fractions resuspended in 50 μl of 3-(N-morpholino)propanesulphonic acid (MOPS) buffer (pH 8, 0.1 *M*), 10 μl of dithiothreotol (2 m*M*) and 10 μl of iron sulphate (0.1 m*M*), all purchased from Sigma. To this mixture 160 μl of the enzyme preparation were added and the reaction was allowed to proceed for 10 min at 30°C in 10 cm × 1.4 cm test tubes gently shaken in a reciprocating water bath. Finally, the reaction was quenched by the addition of 10 μl of disodium ethylenediaminotetraacetic acid (40 m*M*).

The production of isopenicillin N was then bioassayed with *Staphylococcus aureus* (strain 750), grown overnight at 36°C for 12 h, by means of the well-plate assay, using 6-mm diameter plug holes.

Cyclic voltammetry

Cyclic voltammetry was carried out with gold, glassy carbon and platinum electrodes. The equipment supplied by Ursar Scientific Equipment (Cannington, Oxford, U.K.) could be ramped between two set voltage values over a variable time period. In this way the rate of voltage change could be varied. The voltage change was measured against a standard calomel electrode.

RESULTS AND DISCUSSION

Calibration and limits of detection

Calibration over the range 0.35–100 μg ml^{-1} ACV in buffer at a sensitivity setting of 1000 nA on the pulsed amperometric detector gave the following linear regression equation: concentration (μg ml^{-1}) = $-1.2 + 2.2 \cdot 10^{-4}$ area counts (arbitrary units).

The limits of detection of ACV in fermentation broth, with a detector sensitivity of 1 nA, was 0.1 μg ml^{-1} (signal-to-noise ratio of 2:1). The calibration range for ACV spiked into fermentation media derived from *A. niger* (ACV-free) was linear between 0.1 and 100 μg ml^{-1}. The linear regression equation obtained for this concentration range took the form: concentration (μg ml^{-1}) = $-1.5 + 3.3 \cdot 1.0^{-4}$ area counts.

When solutions of ACV dissolved in phosphate buffer (pH 9, 10 m*M*) were analysed on a day-to-day basis the correlation coefficients for respective linear calibration equations were in the range 0.954–0.993.

The technique of anion-exchange chromatography has been applied to amino acid analysis[12]. However, amino acid analysers of this type require post-column derivatisation facilities. The detection of amino acids is usually based upon there reaction with orthophthaldialdehyde[13] or ninhydrin[12]. There are now alternative

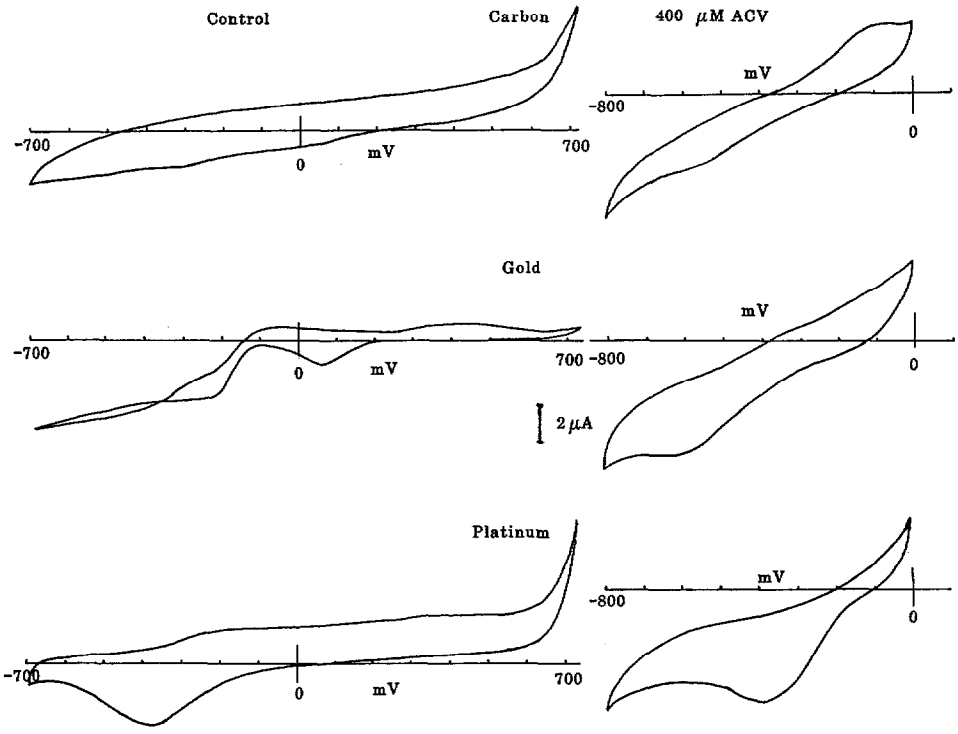

Fig. 1. Cyclic voltammograms for IC buffer and ACV (1.46 mg ml^{-1}) dissolved in IC buffer for different electrode materials. Conditions: scan rate, 20 mV s^{-1}; 25 m*M* sodium acetate, 100 m*M* sodium hydroxide.

pre-column derivatisation methods available for the analysis of amino acids based upon their reaction with FMOC[3], dansylaziridine[5] and phenylisothiocyanate[14]. These methods unfortunately have specific drawbacks in that they either require post-column pumps and reaction coils or require time-consuming sample clean-up, derivatisation and extraction steps.

PAD of biological materials has been demonstrated to be a versatile, sensitive technique[6,7]. This type of detection is based upon either oxidation or reduction at an electrode. The technique can be made selective for particular analytes by operating at electrode potentials at which these analytes are electroactive and interfering components are inactive.

Fig. 1 illustrates the cyclic voltammograms for different electrode materials in the ion chromatography (IC) mobile phase and the mobile phase containing dissolved ACV. Only the gold electrode shows a significantly different cyclic voltammogram for the IC buffer after the addition of ACV. A prominent trough is observed at −600 mV using the gold electrode, whereas glassy carbon only showed a small change in current at −550 mV with ACV present. No detectable change in the cyclic voltammogram for the reference solution and for the ACV solution was observed with the platinum electrode.

Fig. 2 shows the effect of the rate of change of applied potential on the observed current–voltage plot for ACV using a gold electrode. The diagram illustrates that the electrolytic reduction of ACV is a comparatively slow reaction. This necessitates a long E_1 value of 300 ms for the operation of the pulsed amperometric detector to improve the sensitivity of the detection system.

Fig. 3 shows the detector response at different electrode potentials for the reduction of ACV. The final electrode potential of −0.8 V was chosen to give a relatively simple chromatogram, with good separation between analytes and contaminating peaks, for the analysis of ACV in complex fermentation broths. When electrode potentials below −0.9 V were used, a high background noise was observed and other components in the broths, which eluted close to ACV, were detected.

IC patterns of a standard solution of ACV and samples of fungal broths containing ACV are shown in Figs. 4–6. The identity of the ACV peak was confirmed by collecting the column eluate and subjecting the fractions to enzymic reaction; the

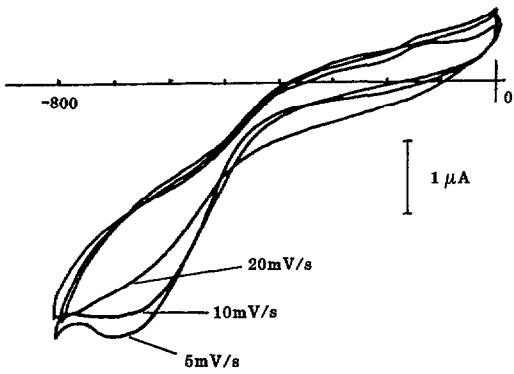

Fig. 2. Effect of voltage scan rate on the response of a gold electrode in the presence of 1.46 mg ml^{-1} ACV dissolved in IC buffer.

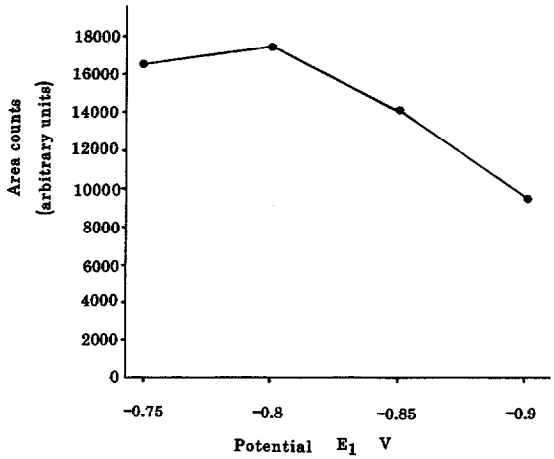

Fig. 3. Detector response for 83 μg ml^{-1} ACV at different E_1 potentials. Conditions: 100 mM sodium hydroxide, 25 mM sodium acetate with a flow-rate of 1 ml min^{-1}, E_1 potentials: -0.75, -0.8, -0.85 and -0.9 V. The potentials E_2 and E_3 were kept constant at 0.05 and -0.95 V, respectively.

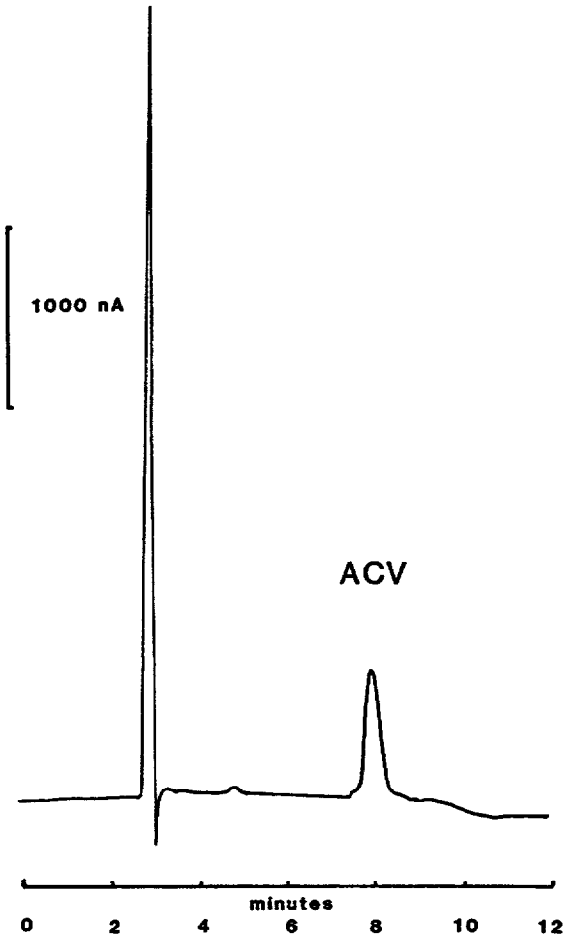

Fig. 4. IC analysis of a standard solution of ACV (300 μg ml^{-1}) determined by IC–PAD. Chromatographic conditions: 100 mM sodium hydroxide, 25 mM sodium acetate with a flow-rate of 1 ml min^{-1}. The chromatography was performed on a AS6 ion-exchange column with an AG6 guard column. The pulsed amperometric detector settings were $E_1 = -0.8$ V, $E_2 = 0.05$ V and $E_3 = -0.95$ V.

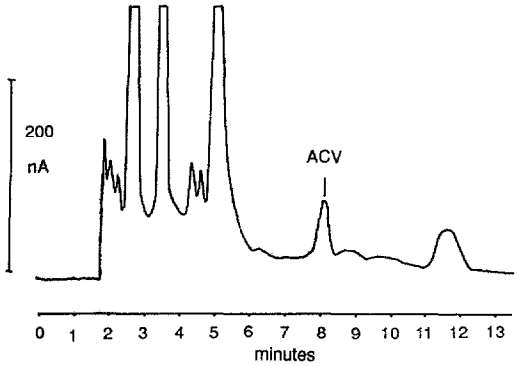

Fig. 5. IC analysis of *P. chrysogenum* grown in shake flasks and assayed for extracellular ACV concentration on day 3 of fermentation. The ACV peak illustrated represents a concentration of 12 $\mu g\ ml^{-1}$. Chromatographic conditions were as described in Fig. 4.

production of isopenicillin was then assayed by a standard well-plate assay technique. The relative simplicity of the chromatograms is an indication of the high selectivity with PAD. *A. nidulans* represents a species of fungus that produces β-lactam antibiotics and β-lactam biosynthetic pathway intermediates at very low extracellular concentrations. To detect and quantify ACV at these low concentrations in broths, derivatisation was previously necessary[4,5]. The IC method presented here allows direct detection of ACV in fermentation broths at concentrations down to 0.1 $\mu g\ ml^{-1}$. *P. chrysogenum* is an industrially important organism used for the production of β-lactam antibiotics. The analysis of ACV derived extracellularly from this species

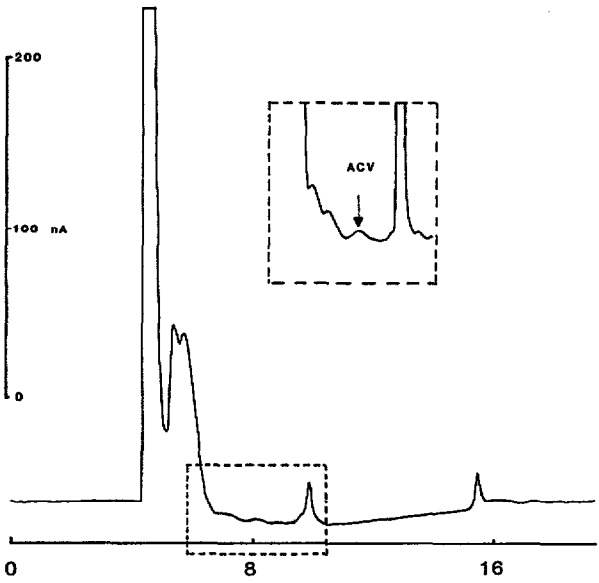

Fig. 6. IC analysis of a fermentation broth derived from *A. nidulans* for the production of extracellular ACV after 72 h of growth. Chromatographic and column conditions were as described in Fig. 4.

TABLE I

RELATIVE REPRODUCIBILITIES FOR THE IC–PAD ANALYSIS OF ACV PRODUCED NATURALLY IN FERMENTATION BROTHS DERIVED FROM PENICILLIN-PRODUCING ORGANISMS.

Chromatographic conditions are as described in Fig. 4.

Sample	*Concentration (mean ± S.D.) (μg ml^{-1})*	*Coefficient of variation (%)*
A. nidulans		
GH 108	2.6 ± 0.14	5.4
GH 1	3.8 ± 0.08	2.1
P. chrysogenum		
P2	56.8 ± 0.70	1.2

of fungus is an important parameter in the monitoring of β-lactam antibiotic production during fermentation processes. Table I shows results obtained for typical fermentation analysis of β-lactam-producing organisms.

IC separation and PAD of ACV has several advantages over previously reported techniques of ACV determination. The method is simple to operate, with a relatively short analysis time (20 min) and lends itself to automation for the analysis of large numbers of samples. Fig. 7 shows the linear regression line for two sets of data for the determination of ACV by the IC–PAD and by a previously reported

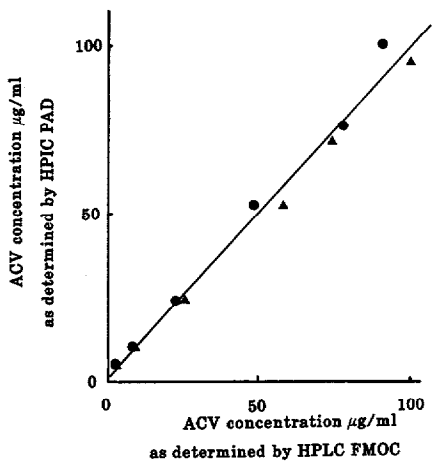

Fig. 7. Correlation between two different techniques for the detection of ACV in buffer. (●) ACV determined by FMOC derivatisation followed by HPLC with UV detection; (▲) ACV determined by IC–PAD. Chromatographic conditions are as described in Fig. 4 for IC–PAD and by Shah and Adlard[4] for FMOC HPLC determination of ACV. Standard solutions containing ACV at different concentrations were determined experimentally by both procedures. The experimentally determined concentrations were then used in regression analysis.

TABLE II

DETERMINATION OF ACV IN FERMENTATION BROTH

ACV was added to blank broth derived from *A. niger* in a range of concentrations after fermentation. Peak areas were used to determine experimentally the ACV concentration. Chromatographic conditions are as described in Fig. 4.

Sample No.	*ACV concentration (μg ml^{-1})*		*Recovery (%)*
	Theoretical	*Experimental*	
1	10	9.8	98.0
2	25	25.9	103.6
3	50	48.1	96.2
4	70	69.3	99.0
5	110	108.0	98.2

technique[4]. The correlation between this IC–PAD method of determining ACV concentrations and the alternative FMOC-HPLC method described by Shah and Adlard[4] gave a correlation factor of 0.947 for standard ACV solutions in buffer (see Fig. 7). The procedure of Shah and Adlard[4] was carried out as described with the exception that detection was achieved by UV absorbance at 275 nm in place of fluorimetric detection. The data presented in Table II indicate that this analytical procedure provides an acceptable level of precision and reproducibility.

CONCLUSION

The IC analysis of ACV in fungal fermentation broths has been demonstrated. The detection of ACV using PAD has been optimised on a gold electrode, and the method relies on the reduction of ACV at the electrode. IC determination of ACV gives good limits of detection for ACV in broths derived from fungal fermentations and compares well with conventional pre-column derivatisation methods for the analysis of ACV.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the assistance of Drs. Cass and Dachyar at Imperial College, (London, U.K.). This work was supported by an SERC Grant GR/D73850.

REFERENCES

1 H. Van Liempt, H. Von Dohren and H. Kleinkauf, *J. Biol. Chem.*, 264 (1989) 3680.
2 P. B. Loder and E. P. Abraham, *Biochem. J.*, 123 (1971) 471.
3 J. J. Usher, M. Lewis and E. P. Hughs, *Anal. Biochem.*, 149 (1985) 105.
4 A. Shah and M. W. Adlard, *J. Chromatogr.*, 424 (1988) 325.
5 C. D. Orford, D. Perry and M. W. Adlard, *J. Chromatogr.*, 481 (1989) 245–254.
6 R. Reid, M. R. Hardy, O. Hindsgaul and Y. C. Lee, *Anal. Biochem.*, 174 (1988) 459.
7 S. R. Rudge, D. Perret, P. L. Drury and A. J. Swannell, *J. Pharm. Biomed. Anal.*, 1 (1983) 205.

8 F. G. Jarvis and M. J. Johnson, *J. Bacteriol.*, 59 (1950) 51.
9 J. Pontecarvo, J. A. Roper, C. M. Hemons, K. D. MacDonald and A. W. Bufton, *Adv. Genet.*, 4 (1953) 141.
10 C. P. Pang, B. Chakravarti, R. M. Adlington, H. H. Ting, R. L. White, G. S. Jayaulake, J. E. Baldwin and E. P. Abraham, *Biochem. J.*, 222 (1984) 789.
11 D. Perry, E. P. Abraham and J. E. Baldwin, *Biochem. J.*, 255 (1988) 345.
12 R. E. Ferrel, S. K. Stroup, R. J. Tanis and R. E. Tashian, *Biochim. Biophys. Acta*, 533 (1978) 1.
13 M. Roth, *Anal. Chem.*, 43 (1971) 80.
14 S. A. Cohen, T. L. Tarvin and B. A. Bidlingmeyer, *Am. Lab.*, August (1984) 48–59.