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ON-LINE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF CEPHALOSPORIN C AND BY-PRODUCTS IN COMPLEX FERMENTATION BROTHS

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SUMMARY

A fully automated high-performance liquid chromatographic system was developed for optimization of cephalosporin C production. Using a simple, isocratic, reversed-phase method, cephalosporin C, deacetylcephalosporin C, deacetoxycephalosporin C, penicillin N, methionine and its decomposition product 2-hydroxy-4-methylmercaptobutyric acid were determined. The method worked reliably for 250 h and supplied information on the influence of many parameters of the process.

INTRODUCTION

Cephalosporin C (CPC) is a β-lactam antibiotic and its biosynthesis (Fig. 1) is similar to that of penicillin. CPC is produced by the mold *Cephalosporium acremonium* by a fed-batch fermentation technique[1]. Optimization of this process requires accurate and reliable measurements of substrates and products over a long period. Process analysis allows the detection of contamination at an early stage and the monitoring of the ratio of product to by-products to estimate the end-point of cultivation. Further, the influence of dissolved oxygen tension and the feed of substrate solutions [such as methionine (MET) and phosphate] on the production of CPC is particularly interesting. The determination of MET is important because it is a source of sulphur for antibiotic production.

An on-line method for cephalosporin determination using an automatic analyser system has been described[1]. The cephalosporins show an absorbance at 260 nm that disappears when they are treated with cephalosporinase, a β-lactamase. The reaction is specific for all cephalosporin derivatives, so that it only indicates the overall concentration of CPC, deacetylcephalosporin C (DAC) and deacetoxycephalosporin C (DAOC). This disadvantage prompted us to employ high-performance liquid chromatography (HPLC), which offers the possibility of determining several substances simultaneously. Möller *et al.*[2] used an on-line HPLC system for the determination of penicillin V. For several years, HPLC has been used for cephalosporin analysis[3–8] but not for process control. The aim of our investigations was the optimization and automation of a chromatographic method for on-line cephalosporin determination that would work reliably for 250 h. For on-line analysis, a continuous,

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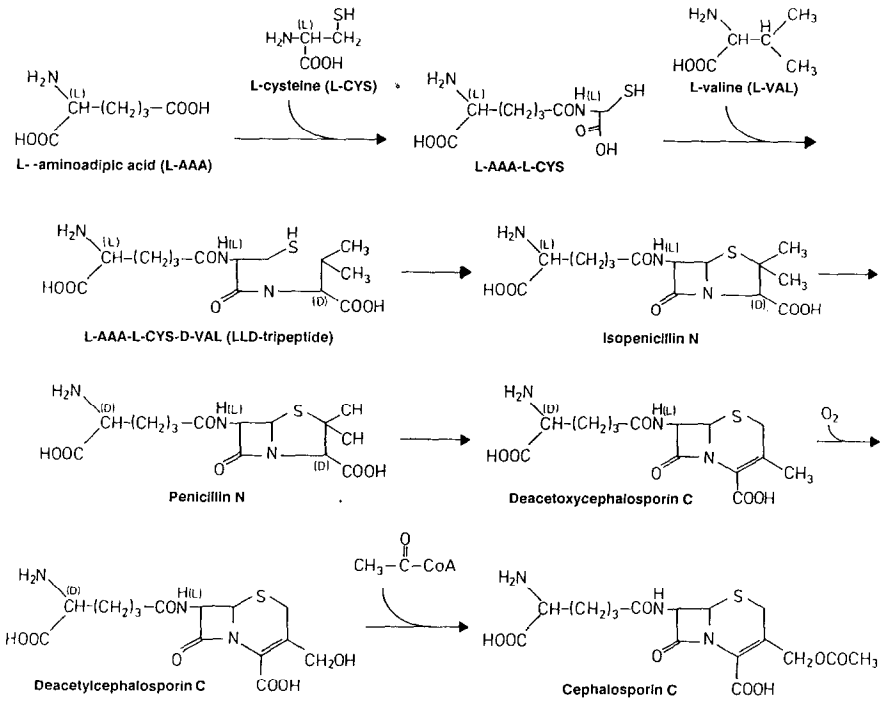

Fig. 1. Biosynthetic cephalosporin pathway.

sterile and cell-free sampling stream is required. The sampling module must work dependably over a period of days. The results are presented in this paper.

EXPERIMENTAL

Fermentation conditions

CPC production was carried out in a 20 l stirred-tank reactor by fed-batch operation. The complex fermentation medium contained 50–100 g/l of solid peanut flour and other substrates such as glucose, salts and antifoam. A glucose–methionine solution containing 500 g/l of glucose and 20 g/l of MET was used as the feed. In order to avoid catabolic repression, continuous feeding was started after the initial glucose in the medium had been used up. By varying the feeding rates (0.25–1.5 ml/min), a low glucose level could be maintained during the production phase.

Materials

CPC, DAC, DAOC, penicillin N (PEN N) and 2-hydroxy-4-methylcaptobutyric acid (MMBS) were supplied by Ciba-Geigy (Basle, Switzerland), tetrabutylammonium hydrogensulphate (TBAHS), phosphate salts and MET by Fluka (Neu-Ulm, F.R.G.) and HPLC-grade methanol by Merck (Darmstadt, F.R.G.). Doubly distilled water was obtained using a deionization system from Millipore (Eschborn, F.R.G.). Mobile phases and standard solutions were prepared with Milli-Q water (Millipore). The eluents were degassed with a stream of helium before and during the analysis.

Sampling system

For continuous sampling, different laboratory-built systems[2,7,8] are in use in our Institute. Two modules were employed for CPC fermentation. One is a flat, porous-plate filter[2,8] with polysulphone membranes (molecular weight cut-off 100 000; Sartorius, Göttingen, F.R.G.). The active filtration area is 47.5 cm^2 and the dead volume is 2.5 ml. For a low solid content in the medium (50 g/l of peanut flour) this sampling system showed very good results, but when using 100 g/l of peanut flour, the filtration rate decreased. This prompted us to employ another type of module (Fig. 2), which had been developed for *E.coli* fermentation. It turned out that it could also be used for CPC production. The module (ABC, Puchheim, F.R.G.) is made of stainless steel and contains a tubular membrane (polypropylene, 200 × 5.5 mm I.D.; pore size, 0.2 μm; Enka, Düsseldorf, F.R.G.). Both modules are positioned in the lower section of the reactor in a well areated region. They can be steam-sterilized at 121°C. A permeation rate of 0.5–1.0 ml/min could be maintained during fermentation. The filtrate was pumped continuously through capillary Teflon tubes into the injection valve of the HPLC system. For sampling, two tubes were used and alternated daily; one was connected with the sample loop and the other was flushed with formaldehyde and then with water in order to avoid microbial growth. The sampling dead time in the tubes, including the sampling module, was 10 min.

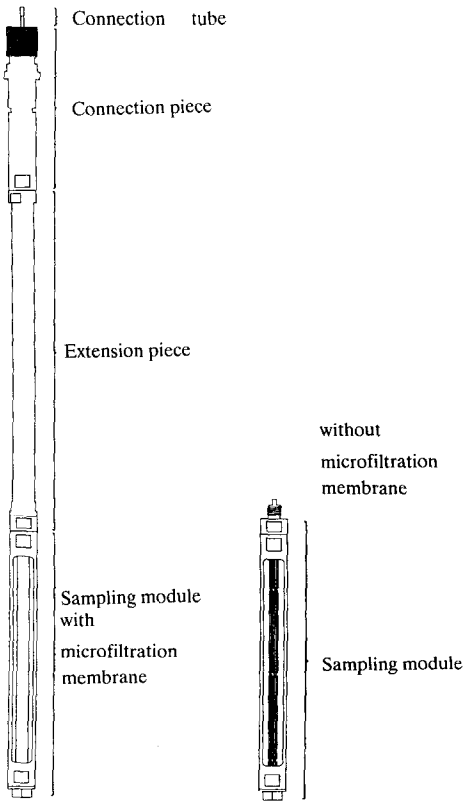

Fig. 2. Sampling module with tubular micromembrane.

HPLC equipment

The HPLC equipment used for cephalosporin analysis consisted of a peristaltic pump (Verder Deutschland, Düsseldorf, F.R.G.), a Spectroflow 400 HPLC pump (Applied Biosystems, Weiterstadt, F.R.G.), an electrical injection valve (VICI Valco Europe, Schenkon, Switzerland) with a 10-μl sample loop, a separation column, a Spectroflow 757 variable-wavelength UV detector (Applied Biosystems) and an SP4270 integrator. The analytical column (250 × 4.6 mm) was packed with Nucleosil 100-5C_{18} or Nucleosil 100-10C_{18} (Macherey-Nagel & Co., Düren, F.R.G.) and was protected by a guard column (50 × 4.6 mm I.D.) containing Vydac-201 SC reversed-phase material (Macherey-Nagel & Co.; 30–40 μm, dry packed). The columns were thermostated in a water-bath at 25–30°C.

The HPLC system was controlled by the integrator, which operated the injection valve, started automatically after a previously designated time (0.5–2 runs/h), set the auto-zero-point at the detector, started the pump before the injection (flow-rate 1.0 ml/min) and reduced the flow-rate after the end of the run (0.1 ml/min). Further, the integrator recorded the chromatograms, calculated the concentrations and transmitted them to a process computer (PDP-11/23, Digital Equipment, Munich, F.R.G.) or a PC (Epson, WINner software, Spectra-Physics) for on-line storage.

RESULTS AND DISCUSSION

During CPC fermentation, the following components are of special interest: CPC and its precursors DAC, DAOC, PEN N in the biosynthesis, MET and its degradation product MMBS. The cephalosporins show a UV maximum at 260 nm due to the cepham chromophore. This O=CNC=C group is not present in PEN N. MET and MMBS also did not show any absorbance at 260 nm. Consequently, the analytical wavelength must be lowered; the most suitable one for monitoring all compounds turned out to be 225 nm.

Different eluents were tested. A mobile phase consisting of 5–10% methanol–90–95% 14 m*M* phosphate buffer solution, containing 10.3 m*M* TBAHS (pH adjusted to 6.5), proved to be optimal for the separation of the six compounds. A 2-l volume of the mobile phase was prepared at a time and used during one fermentation by recycling the solvent. A constant flow-rate (1 ml/min) was maintained during the analysis. The methanol content depended on the age of the analytical column. For new separation columns, we started with 10% methanol in the eluent and, if the separation becomes unsatisfactory, the content was reduced to 5%. Further, it was possible to adjust the analysis time by controlling the temperature of the column; during one fermentation, however, the composition of the eluent and the temperature were kept constant. Over a period of 200 h, a 2-min reduction in the retention time for CPC and an increase in the back-pressure was observed. This was due to the complex medium containing proteins which were accumulated on the stationary phase. As the filling material is a highly efficient filter, it was advisable to change the guard column material before each fermentation. The lifetime of the separation column was about three fermentations, after which the selectivity of the column was insufficient so that the separation of the major components from other substances was not satisfactory for quantification.

At the beginning of a fermentation, the HPLC system was calibrated by mea-

suring the peak areas of a standard solution containing MET, DAC, DAOC, PEN N, CPC and MMBS. During the fermentation, there was no need for any additional calibration. The substances in the chromatograms of the fermentation samples were identified by the retention times of the peaks and by mixing a sample with a standard solution. The recovery for DAC and CPC in fermentation samples was nearly 100%. Fig. 3 shows selected chromatograms of a standard solution and a sample after 75 h of a *C. acremonium* fermentation. It can be seen that the separation of the standard compounds is excellent, but the chromatogram of the fermentation sample shows many unknown substances, particularly at the beginning. Thus, for CPC, DAC and PEN N, the most important components, the resolution was good, but the quantification of MET became difficult at lower concentrations. The linearity of the assay system was established by examining the response of CPC as a function of concentration, and a linear behaviour was obtained from 0.5 to 8.0 g/l. The relative standard deviations (R.S.D.) for the calibration solution (ten runs) are shown in Table I.

The results of typical on-line measurements for the main components involved in the biosynthesis are shown in Fig. 4. DAOC is not included because of its low concentration in the fermentation samples (below 0.5 g/l). After 200 h, a steady state

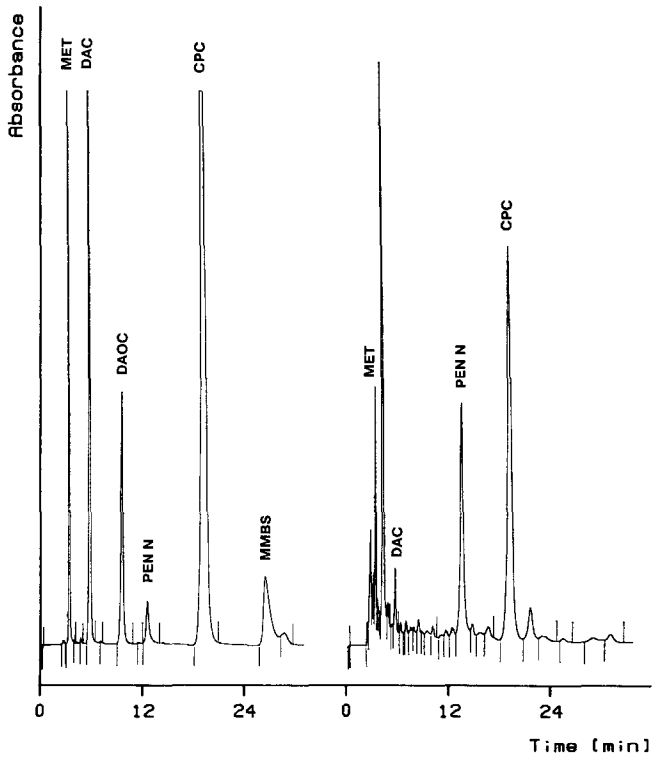

Fig. 3. Chromatograms of a calibration and a fermentation sample. Column, Nucleosil 100-5C_{18} (250 × 4.6 mm I.D.); temperature, 30°C; eluent, methanol–14 m*M* phosphate buffer containing 3.5 g/l TBAHS (pH 6.5) (5:95); flow-rate, 1.0 ml/min; range, 0.1 a.u.f.s. (A) Chromatogram of a standard solution: MET, 2.38 g/l; DAC, 0.4 g/l; DAOC, 0.2 g/l; PEN N, 0.26 g/l; CPC, 1.297 g/l; MMBS, 1.32 g/l. (B) Chromatogram of a CPC fermentation sample.

TABLE I

MEAN VALUES AND STANDARD DEVIATIONS FOR A CALIBRATION SOLUTION

Results calculated from the integrator for ten chromatograms. Concentrations in the standard solution: MET, 2.09 g/l; DAC, 0.50 g/l; DAOC, 0.20 g/l; PEN N, 0.25 g/l; CPC, 1.29 g/l; MMBS, 1.01 g/l.

Component	*Mean concentration (g/l)*	*S.D. (g/l)*	*R.S.D. (%)*
MET	2.17	0.039	1.8
DAC	0.51	0.005	1.1
DAOC	0.21	0.004	1.8
PEN N	0.26	0.006	2.4
CPC	1.29	0.015	1.1
MMBS	1.09	0.033	3.0

between CPC formation and decomposition was reached, and it was impossible to increase the CPC concentration by longer fermentation times. The main reason is probably the enzymatic degradation of the CPC to DAC by an acetylhydrolase, produced by the fungi, or repression of the last step of the biosynthesis. Therefore, the DAC and PEN N concentrations increased only slightly while the CPC production decreased.

Fig. 4 also shows a comparison of on-line and off-line measurements during fermentation. There is good agreement for the CPC and DAC analytical data. At the end of the production phase, the off-line values for PEN N differ from the on-line measurements, probably owing to inaccurate integration of the small peaks. This demonstrates the advantage of on-line determinations compared with a few off-line data.

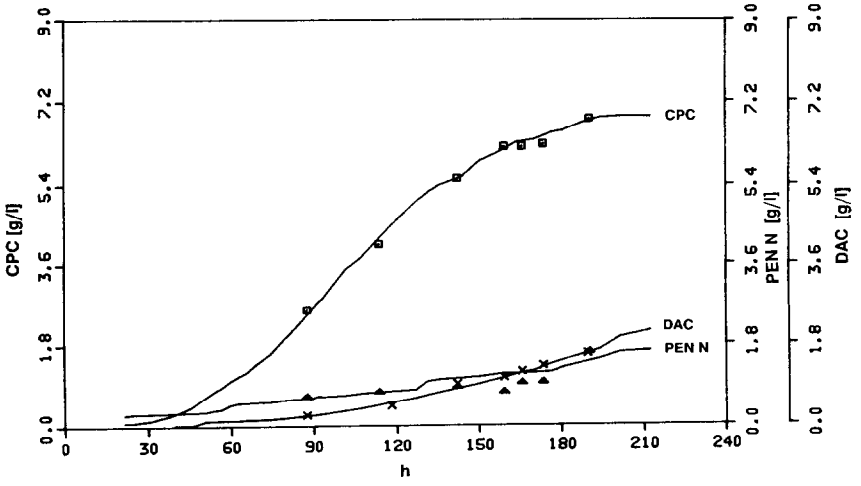

Fig. 4. Cephalosporin C production: comparison of on-line (solid lines) and off-line (points) determinations. Fresh samples: ⊡ = CPC; △ = PEN N; × = DAC.

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