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Review

Monitoring of the concentration of β-lactam antibiotics and their precursors in complex cultivation media by high-performance liquid chromatography

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Abstract

Monitoring the concentrations of β-lactam antibiotics and their precursors is required for the optimization of their production. It allows the identification of the bottlenecks in their biosynthesis and the optimisation of process control. Because of the complex cultivation media with high amounts of protein and the presence of a second liquid phase, such as soy oil, and solid particles, such as peanut flour, etc., aseptic sampling is a difficult task. Careful preconditioning of the sample is necessary to avoid falsification of the sample composition and impairment of the analysis of key components by the matrix. Monitoring of the production of the most important β-lactam antibiotics, penicillin G and V produced by *Penicillium chrysogenum*, and cephalosporin C produced by *Acremonium chrysogenum* is considered. The analytical methods and special requirements for monitoring the concentrations of final products and precursors during the entire cultivation process are discussed. The HPLC methods used in biotechnology are compared with those used for on-line analysis of β-lactam antibiotics in biotechnology.

Keywords: Reviews; Process monitoring; Antibiotics; Lactams; Cephalosporins; Penicillins

Contents

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1. Introduction

High-performance liquid chromatography (HPLC) is one of the most important analytical techniques for the determination of the concentration of nonvolatile and temperature-sensitive compounds, like antibiotics [1,2]. On account of the monoseptic production of the most important β-lactam antibiotics, such as penicillin G and V as well as cephalosporin C, aseptic sampling of the cultivation medium is used. A careful preconditioning is necessary, because of the presence of several matrix components which impair the analysis [3]. After separation of the matrix components, the concentrations of β-lactam compounds are measured with a UV detector. Some other components are converted into a fluorophore compound by pre- or postcolumn derivatization and their concentration is determined with fluorescence spectrometry.

During production, the concentrations of the key components are usually monitored on-line and the information used for process control. Therefore, the time lag of the analysis caused by sampling and preconditioning must be short enough to detect the actual state of the cultivation. On-line analysis by HPLC indicates the time when the substrates are exhausted and the microorganisms reach the production phase.

Cultivations of *Penicillium chrysogenum* and *Acremonium chrysogenum* take about 1 week. With an analysis frequency of two per hour more than 330 analyses are carried out without replacement of parts of the sampling–preconditioning–analysis system. These special requirements can be fulfilled with careful preparation of the samples.

2. Sampling of complex biological media and preconditioning of the samples

All the important industrial processes for production of secondary metabolites use complex cultivation media, which consists of agricultural by-products (molasses, pharma medium, cornsteep liquor, peanut flour, etc.) and other components, such as yeast extract, soy oil, etc. It is difficult to take a representative sample from such a multiphase system consisting of liquid phase(s), cells, and solid particles (for review see [3]). In recent years it is increasingly important to sample very rapidly, especially for the measurement of intracellular components of the biosynthesis or the central metabolisms. It is necessary to stop chemical and enzymatic reactions in the off-line sample very quickly [4,5]. A coaxial catheter with a chemical sterile barrier was recommended for aseptic sampling of cell containing media (Fig. 1) [4]. Another very fast sampling system was developed by Theobald et al. [5] for monitoring of intracellular metabolites (Fig. 2). However, they are used only in laboratories for special investigations. There are a lot of other methods and several papers have been published on sampling systems (for review see [6]).

2.1. Aseptic sampling

Nearly all antibiotics produced by microorganisms are transported actively or passively out of the cells. Since the products are excreted into the aqueous phase, cell- and plant-fragment-free samples are used for the analysis of antibiotics. Crossed flow hydrophilic micro- or ultrafiltration membranes integrated into an external medium recirculation loop are used for the sampling of the aqueous phase. Another sampling module is shown in Fig. 3, which allows in situ aseptic sampling during more then 2 weeks of cultivation of fungi in complex media as used for penicillin and cephalosporin production. A hydrophilic microfiltration membrane tubing mounted on a suitable holder is immersed into the cultivation medium and installed before sterilisation. In small reactors, the sample is withdrawn with a peristaltic pump. In large reactors no pump is necessary, because the overpressure in the reactor is high enough for a sampling rate of 0.5–1 ml min^{-1}. In order to avoid membrane fouling, high liquid velocity in the medium recirculation loop and high turbulence in the direct vicinity of the immersed membrane tubing, have to be maintained. Under

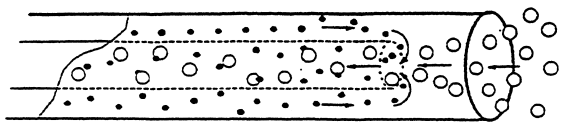

Fig. 1. Schematic diagram of the coaxial catheter used for sampling. The open circles represent the biological sample and the filled circles represent the inhibitor (e.g. for fermentations samples 10 m*M* potassium chloride was tested) [4].

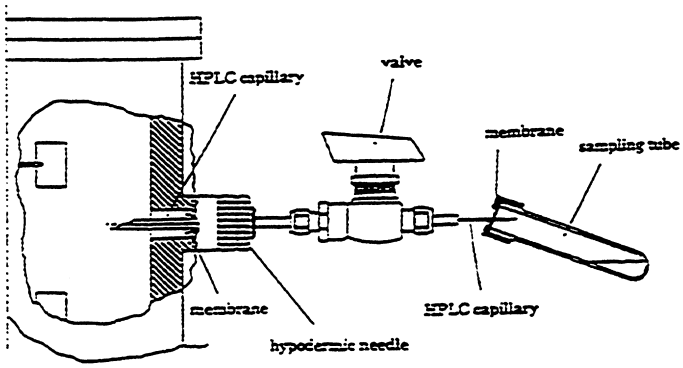

Fig. 2. Schematic representation of the sampling device connected to the mixing zone of the fermenter. The problem of this device is the small diameter of the HPLC capillary so that complex cultivation media are difficult to sample.

these conditions on-line sampling during the entire cultivation time is possible [3,7].

2.2. Preconditioning

The concentrations of antibiotics are high in industrial cultivation media, therefore the samples have to be diluted, in order to keep the analyte concentration in the linear range of the detector.

The composition of such cultivation medium can be changed within a short period of time by the dissolved enzymes of the cells and plant fragments, which are still present in the cell- and plant-fragment-free medium. Therefore, either the analysis has to be taken on-line, immediately after the sampling, or the sample has to be quickly cooled to −20°C. The best way is to combine these two preventive steps.

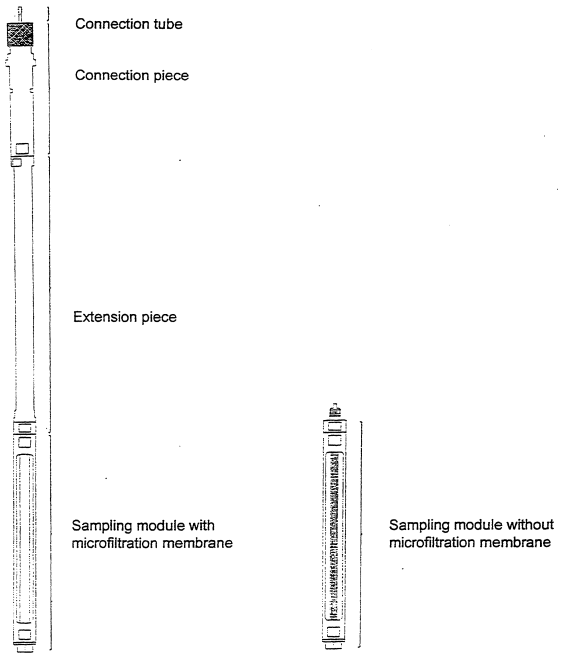

Fig. 3. Sampling module with tubular micromembrane as developed in our laboratory [7].

In order to protect the analytical column, proteins are removed completely from the sample. The solution is desalted on e.g. Sephadex G-25 and stored at −60°C [8].

The HPLC system is used in the nonaseptic zone. Therefore, microorganisms can infect the sample, change its composition and clog the analysis system. Growth inhibitors can be used to avoid these disturbances.

Across the membrane the pressure is decreasing, which can cause gas desorption in the downstream section. Degassing of the sample by bubbling helium through it for 15 min in an ultrasound bath can avoid bubble formation [9].

The sample may also contain a nonaqueous phase (e.g. soy oil and fatty acid droplets) in the cultivation medium. This must be removed by extraction e.g. with *n*-hexane for soy oil, to avoid fouling of the analytical column.

When using high production industrial strains, antibiotic concentration increases with time and at the end of the cultivation high concentrations are obtained. To keep the analyte concentration in the linear range of a detector, sample dilution is necessary, which can be a serious source of error. Finally, the optimal separation conditions (temperature, pH,

Table 1
Overview of common pre-, guard- and analytical columns which are used for HPLC-monitoring of β-lactam antibiotics and their precursors

Precolumns (for enrichment of the analyte)	Guard columns (for protection of the analytical column	Analytical column (separation)
SAX-Bond Elut	Nova Pack C_8	Shodex C_{18} 5A
Analytchem Internet, USA	Waters, USA	Showa Denko, Japan
LiCrosorb RP-8	Vydac-201 SC	Cosmosil 5 C_{18}-AR
Merck, Germany	Macherey–Nagel, Germany	Nacalai Tesque, Japan
Sep-Pak C_{18}	Guard Pack μBondapak C_{18}	Ultracarb 5 ODS-30
Millipore-Waters, USA	Waters, USA	Phenomex, USA
Kromasil 5 C_8	Perisorb RP 18	Ultrasphere C_{18}
Phenomex, USA	Merck, Germany	Beckman Instruments, USA
–	–	Kromasil 5 C_8 Phenomex, USA
–	–	RP-18 cartridge Pierce, Rockford, USA
–	–	Nucleosil 5 C_{18} Macherey–Nagel, Germany
–	–	Nova-Pak C_{18} Waters, USA
–	–	Hibar LiChrosorb C_{18} Merck, Germany
–	–	RP C_{18} LKB-Pharmacia, Sweden
–	–	Nucleosil 100-5 C_{18} Macherey–Nagel, Germany
–	–	Nucleosil 10 C_{18} Macherey–Nagel, Germany
–	–	Nucleosil 5 C_{18} Macherey–Nagel, Germany

buffer capacity) have to be established and maintained during the analysis.

3. HPLC systems and their operation

In general, HPLC systems for process monitoring consist of a guard column, an analytical column and a detector, a six-port switching valve for precolumn/waste exchange, injection valve and two high precision HPLC pumps for guard and analytical column operation with gradient elution, and a computing integrator for peak evaluation and control of the system (Table 1).

Fig. 4 shows a typical HPLC system as used in our laboratory for the detection of cephalosporin and amino acids in fermentation broth.

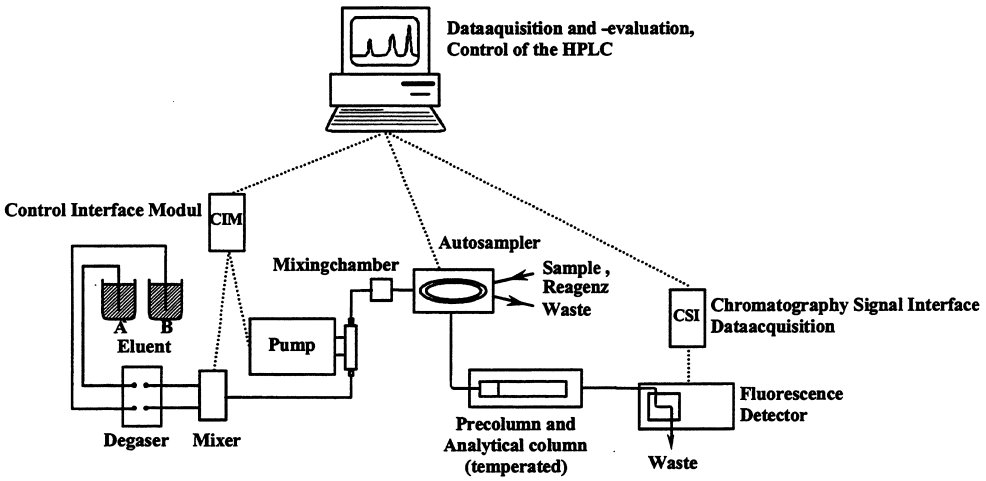

Fig. 4. HPLC for detection and monitoring amino acids and antibiotic compounds in the samples. There is no gradient and mixing chamber necessary for the antibiotics [24–26].

3.1. Guard column

In spite of the complete removal of the proteins, other contaminants (e. g. traces of soy oil) can impair the resolution of the analytical column. The guard column protects the analytical column from these contaminants [7,9,10,12–16]. A fairly frequent replacement of the guard column is recommended if soy oil is present.

3.2. Analytical column

The analytical column is the heart of the HPLC system and the key to obtaining a good separation of the compounds in the fermentation broth. There are many analytical columns and it is often difficult to get the appropriate column. For antibiotics reversed-phase HPLC columns are most often used with e.g. Shodex C_{18} 5A or other packings [7,9,12–18].

The particle size is usually 5 μm and the sample flow 1.0–1.5 ml min^{-1}. The mobile phases consist of various mixtures of acetonitrile and (usually) phosphate buffer [11–19] or the mixtures of tetrabutylammonium hydrogensulfate and methanol [7,9] or tetrabutylammonium dihydrogenephosphate and acetonitrile [22]. Gradient elution is necessary, if chemically similar analytes are to be separated.

3.3. Detection

The β-lactam antibiotics have measurable absorbance in the UV wavelength range, therefore, their concentration can be measured by UV detection [7,9–17,19,20]. Pre- or postcolumn derivatization can be used to improve the detection sensitivity by the formation of fluorophore products. In this case, fluorescence detectors are used, e.g. Model RF 530 variable wavelength fluorescence detector (Shimadzu, Japan) with excitation wavelength of 346 nm and emission wavelength of 422 for ampicillin [18]. Postcolumn derivatization was used and measurement were performed with Model UV 1000-UV detector (Spectra Physics, N. Ireland) at 320 nm [14].

3.4. On-line monitoring of antibiotics and precursors during fermentation by HPLC [7,9]

HPLC can be used for on-line monitoring of concentrations of products during the cultivation. A totally automated HPLC was used to monitor the product and by-product concentrations during the cultivation of *Penicillium chrysogenum* and *Acremonium chrysogenum*. For on-line process control of the cultivation, a PDP-11/23 computer (Digital) was used.

Based on the well known realtime/multiuser/multitask system, RSX-11m, on the PDP-11, all measurements, data evaluation, and data documentation of the process are controlled by a program called CASFA (computer automated system for fermentation plants). After testing the system successfully during penicillin production the on-line HPLC was then used for the optimisation of cephalosporin C production.

The system was used for a cultivation time of about 250 h and the separation of the main products as well. A reduction in the retention time was observed with increasing time; this was due to the complex medium containing contamination which accumulated on the stationary phase. The off-line and on-line HPLC analyses agreed well. Minor deviations can be caused by the slightly different off-line sampling preparation. By on-line monitoring of the process by HPLC, it is easy to observe the start and end of the production phase, so that the process could be optimized by feeding carbon or nitrogen sources and stopped at the right time to avoid wasting time and materials.

4. Monitoring of the concentrations of penicillin G and V [15,23]

Penicillin G and V are produced by *Penicillium chrysogenum* with phenylacetic acid and phenoxyacetic acid side chain precursors, respectively. A small amount of phenylacetic acid and phenoxyacetic acid side-chain precursors can be oxidized to *o*-hydroxyphenylacetic acid and hydroxyphenylacetic acid, which yields *p*-hydroxy penicillin G and *p*-hydroxy penicillin V, respectively.

For the analysis of these compounds, the sample must be completely deproteinated by methanol and microfiltrated. Reversed-phase gradient liquid chromatographic methods are used for the analysis of these compounds. Different analytical columns were used, e.g. Hibar LiChrosorb C_{18}, 250×4 mm I.D.

with 5 μm packing (Merck, Germany) or Nova-Pak C_{18}, 300×3.9 mm I.D. with 4 μm packing (Waters, USA). A Guard-pack μBondapak C_{18} guard column (Waters) or a Nova-Pak C_{18} guard column (Waters) were used for protection of the analytical column.

A 10-μl volume of sample was injected onto the column and the chromatogram was developed with degassed eluents. Eluent A consisted of 0.2 *M* Na_2SO_4, 0.04 *M* H_3PO_4 and 1.3–5.0% acetonitrile. The pH was adjusted to 5.5 by 10 *M* NaOH or ethanol amine. Eluent B consisted of a water–acetonitrile (50:50, v/v). The following gradient elution was used: 95% A, 5% B for 5 min, 95% A, 5% B ⇒60% A, 40% B, linear, over 15 min, 60% A, 40% B for 5 min, 60% A, 40% B⇒95% A, 5% B, linear, over 15 min and 95% A for 15 min and finally 95% A, 5% B constant. Detection was performed at 228 nm with a UV–vis spectrometer (Type 481, Waters), the data acquisition and pump control was by the 810 BASELINE software from Waters.

Another common technique was described by Adlard et al. [23]. The samples were filtered (Whatman filter No. 40) and immediately frozen. After coming to room temperature the samples were refiltered using a 0.22-μm Durapore filter (Millipore, Harrow, UK). The HPLC-system was a binary solvent gradient Gilson HPLC consisting of two pumps (model 303 and 302 with 10 SC and 5 SC head respectively), a dynamic mixer (model 811), an autosampler (model 231) and a diluter. The HPLC system was controlled by an Apple computer (Model IIC, Anachem, Luton, UK). Detection and data handling were performed by Shimadzu SPD-2am variable wavelength UV detector (Dyson, Houghton-Le-Spring, UK) and a SpectraPhysics SP 4290 integrator plotter (St. Albans, UK). All analyses were carried out using a Spherisorb 5-μm reversed-phase column C_{18}(S5) protected by a guard column. The same stationary phase was used. Two eluents were used: eluent A was a solution of KH_2PO_4–K_2HPO_4 (30 m*M*, pH 5.5) which was degassed and filtered before use. Eluent B was HPLC grade acetonitrile. When the eluents were mixed, no salt precipitation could be observed during the analysis. Solvents A and B were combined according to a gradient programme which gave a total running time of 45 min; 100% A for 15 min, then linear over 15 min to 80% A and 20% B. This composition was held for 5 min, then linear over 5 min to 100% A and this was held for at least 5 min. Sample injection volumes were 10 μl, a flow-rate of 1.5 ml min^{-1}, and a detection wavelength of 220 nm were employed for all measurements.

5. Monitoring of the concentrations of amino acid and tripeptide precursors of cephalosporin C

In Fig. 5 the generally accepted mechanism for the biosynthesis of cephalosporin C as observed in *Acremonium chrysogenum* is shown. The first step of the cephalosporin C synthesis is the formation of the tripeptide δ-(L-α-aminoadipyl-L-cysteinyl-D-valine (LLD-ACV) from L-α-aminoadipinic acid, L-cystein and L-valine by the LLD-ACV synthetase (enzyme 1). There are still discussions about the mechanism and the sequence of the addition of the amino acids.

The determination of the concentrations of L-α-aminoadipic acid and L-valine were performed after precipitation of proteins with methanol at 0°C for 10 min and dilution with borate buffer (pH 10) with OPA–MCE [54 mg *o*-phthaldialdehyde (OPA) in 1 ml ethanol solution and 40 μl mercaptoethanol (MCE) made up to 10 ml with 0.4 *M* borate buffer, pH 9.5, and after 24 h stored at 4°C] precolumn derivatization and with autosampler (Promis II, Spark, Netherlands) control at 20°C for 1.5 min. Resolve C_{18}, 90 Å, 5 μm, 150×3.9 mm (Waters) was used as analytical column.

For the gradient elution eluents A and B were applied: eluent A consisted of 96% buffer (50 m*M* sodium acetate, 50 m*M* sodium dihydrogenphosphate, pH 7.0), 2% tetrahydrofuran and 2% methanol. Eluent B consisted of methanol–bidistilled water (54:46, v/v). The analysis was performed with 10-μl injection volume and 1 ml/min flow-rate at 30°C with following gradient: 0% B⇒50% B, linear over 55 min, 50% B for 5 min, 50% B⇒0% B, linear, over 5 min, 0% B for 2 min. Autochrom CIM interface was used for the gradient control. Fluorescence detection λ_{ex}=370 nm and λ_{em}=420 nm (Shimadzu RS 535) was used, Autochrom CIM interface for the data acquisition and APEX chromatography software for the evaluation of the measurements. The duration of the analysis was 67 min.

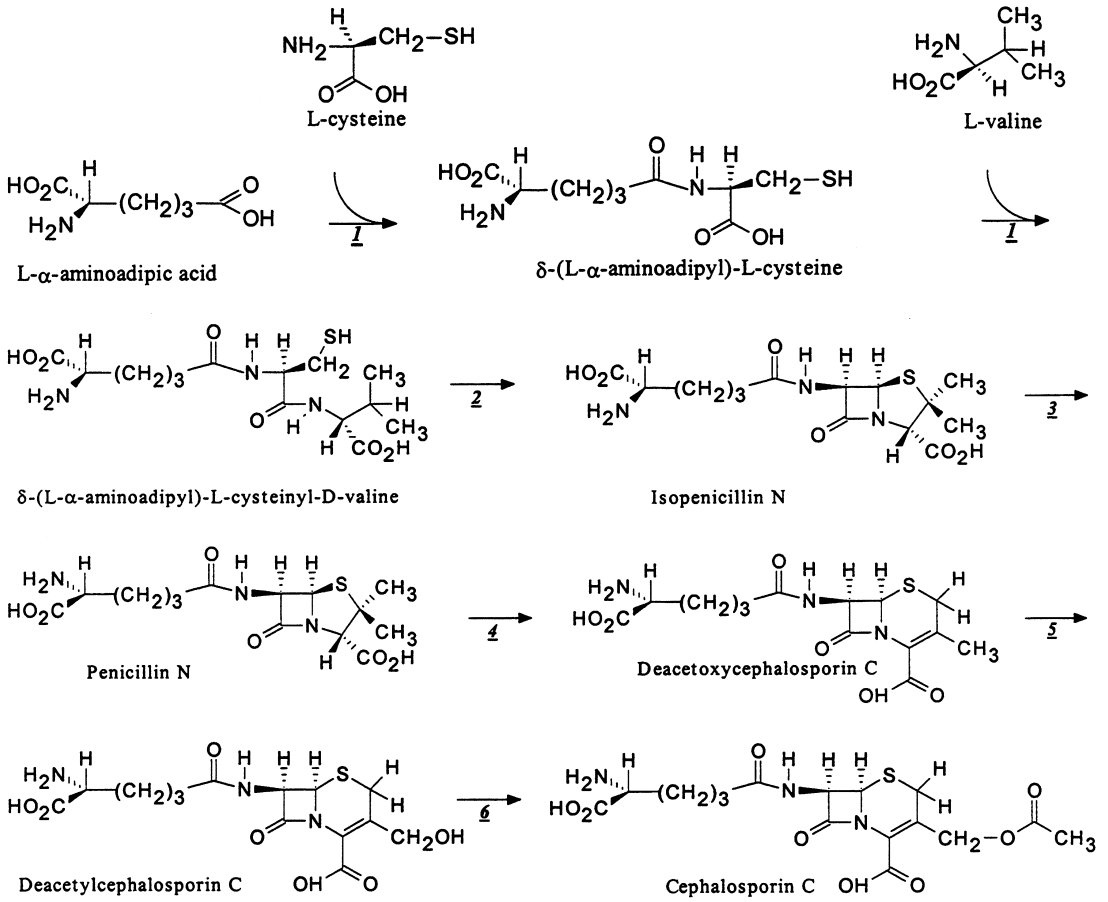

Fig. 5. Mechanisms of the biosynthesis of cephalosporin C [25,26].

For the determination of the concentrations of L-cystein and LLD-ACV a different method was used. A 100-μl volume of deproteinated sample, 250 μl borate buffer (pH 8.0), 20 μl 20 m*M* dithioerythritol (DTE), 50 μl 400 m*M* methyliodide in 50% methanol and 100 μl 4 *M* NaOH were mixed and incubated at 20°C for 10 min. Finally, 100 μl 4 *M* HCl and 350 μl borate buffer (pH 10.0) were added to this mixture. Fig. 6 shows the chromatogram of all amino acids which were detectable. All amino acids were very well separated.

Depending on the age and the preparation of the samples, there were sometimes double peaks, but it was possible to determine their concentrations by measuring the samples twice in different columns.

According to another method, the precolumn

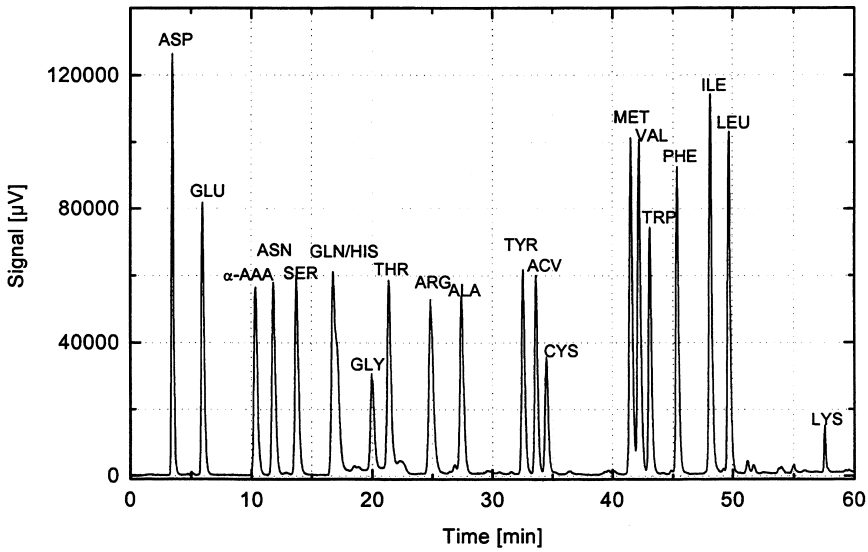

Fig. 6. Typical chromatogram of the analysis of amino acids after treating the samples with methlyliodid (necessary for the detection of ACV and CYS) [26].

derivatization was performed with MBB reagent (2 m*M* monobromobimane in acetonitrile) at 20°C for 20 min. ET 250/8/4 Nucleosil 100 5 C_{18} (Macherey–Nagel, Düren, Germany) was used as analytical column [21]. A 10-μl volume of sample was injected at a flow-rate of 1 ml/min at 30°C. For the gradient elution, eluent A (0.00025% acetic acid–NaOH, pH 5.0) and eluent B (100% methanol) were applied. Following gradient was used: 10% B⇒50% B, linear over 22 min, 50% B, for 8 min, 50% B⇒10% B, linear, over 10 min, with an analysis time of 40 min.

6. Monitoring of the concentrations of cephalosporin C and β-lactam precursers of cephalosporin C [25]

The concentration of cephalosporin C (CPC) and its β-lactam precursors: isopenicillin N (IPenN)/ penicillin N (PenN), deacetoxycephalosporin C (DAOC) and deacetylcephalosporin C (DAC) were determined without OPA–MCE-precolumn derivatization. The sample was taken directly from the reactor through two modules which are shown in Fig. 3. The modules were inserted in the reactor and connected via two peristaltic pumps with an autosampler (Gilson XLl222). The autosampler placed the samples in a refrigerated rack which allowed freezing of the samples immediately to −18°C). Due to this point there was no hydrolysis of the cephalosporin in the samples as in former studies. Even all important enzymatic reactions could be stopped at this temperature. The sample could be stored at −20°C for some weeks without a loss in the concentration. For measuring the sample was first deproteinated with methanol (1:4, v/v), stored on ice overnight, centrifuged and then analysed on an analytical column (ET 250/8/4 Nucleosil 100 5 C_{18} (Macherey–Nagel), which was protected by a guard column (50×4 mm, Vydac-C_{18} RP). The injection volume was 5 μl. A 1 ml/min eluent flow-rate with a composition of 14 m*M* phosphate buffer (pH 6.5), 10.3 m*M* tetrabutylammonium hydrogensulfate and 5% methanol was used. The measurements were carried out at 25°C. The detection was performed at 220 nm and 260 nm simultaneously with the UV-spectrophotometer Gynkotek. Autochrom CSI2 Interface was used for data acquisition and APEX chromatographic software for the evaluation of the chromatograms.

Newer experiments show that the same separation and detection were obtained with increased flow-rate to 1.2–1.4 ml min^{-1} or by a smaller analytical column with the same material or at a higher

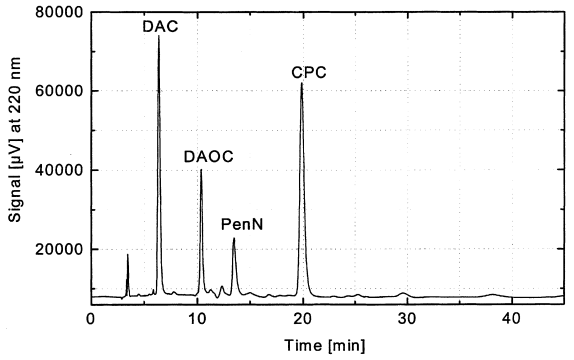

Fig. 7. Separation of the four main products appearing during the fermentation of *Acremonium chrysogenum* at 220 nm, the optimal wavelength for penicillin N detection [26].

temperature e.g. 30°C. By their combination, the measuring time was shortened from 45 min to 25 min per sample. Figs. 7 and 8 show a typical separations of the main product and the main precursors [26].

The precursors penicillin N and isopenicillin N have very similar structures only the conformation of the α-aminoadipyl chain is different, so it was not possible to separate the peaks with reversed-phase chromatography. Jensen et al. have shown that there is a possibility for separation by formation of the diastereomeres. A 20-µl volume of the samples were incubated for 1 h with tetraacetylglucoseisothiocyanate and N-acetylcysteine and injected. The eluent in the isocratic method was 0.01 *M* H_3PO_4–methanol–acetonitrile (57:36:7, v/v); the pH was adjusted to 4.0. The flow-rate was 2 ml min^{-1} and

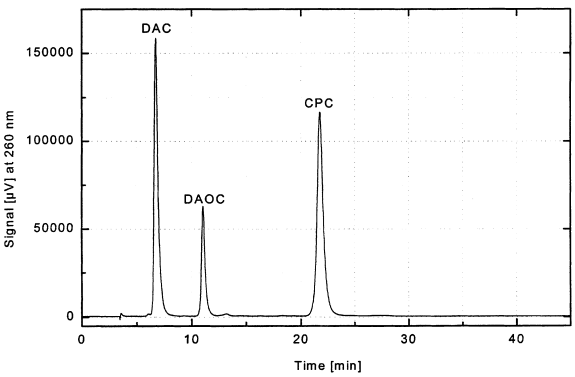

Fig. 8. Separation of the three main products appearing during the fermentation of *Acremonium chrysogenum* at 260 nm [26].

the detection was carried out at a wavelength of 220 nm [26].

7. Analysis of intracellular concentrations of cephalosporin C precursors [24]

To identify the bottlenecks of the biosynthesis of antibiotics, the extracellular and intracellular concentrations of the precursors are monitored as well. Penicillin and cephalosporin are synthesized by the fungi from three amino acids through tripeptides to β-lactam compounds. Therefore, their analysis in presence of all other amino acids, which were present in the cultivation medium and in the cells, are necessary.

This requires high resolution and reproducibility of the analysis. For the determination of the intracellular concentrations of the precursors, the cells have to be disintegrated. The proteases with hydrolase activity, which are released by the destruction of the cells, decompose the tripeptide and other precursors. Special precautions are necessary to reduce the change of the analyte concentrations by hydrolyses.

The cell suspension was diluted with 4°C tap water in proportion of 1:2 (v/v), centrifuged at 4°C and 3000 *g* for 10 min (Sorvall RC-5B Ultracentrifuge, Du Pont Instruments, USA), the sediment was washed with buffer solution (50 m*M* 3-morpholinopropansultonic acid (MOPS), pH 7.5+100 m*M* KCl), centrifuged at 4°C and 3000 *g* for 10 min again, the sediment was washed with buffer solution and centrifuged again at 4°C and 3000 *g* for 10 min. New research indicates that one washing step is sufficient [26].

The cells were disintegrated 4×30 s by ice cooling in 5 ml cell suspension in a buffer (100 m*M* MOPS–KOH 7.5+20 m*M* EDTA+50 vol% glycerol) with ultrasound (Branson Sonifier Cell disrupter B15, with 0.5 inch titanium nozzle, 6.3 mm in diameter) and then centrifuged at 4°C and 30000 *g* for 15 min. Another disintegration was lead with french-press (Hoechst, Frankfurt) two times with a pressure of 8000 p.s.i. (1 p.s.i.=6894.76 Pa). According to recent investigations the disintegration with a mixing mill or a mortar (using frozen and dried samples) were more careful for the intracellular enzymes.

The supernatant was treated with gel filtration

column (Sephadex G25 NAP-100, Pharmacia, Sweden) and eluted with an elution buffer (100 m*M* MOPS (pH 7.5)+20 vol% glycerol+50 m*M* KCl). The cell free eluate was stored at −80°C and used for the determination of the intracellular enzyme activities with suitable assays and the concentrations of the intracellular precursors according to Section 5 Section 6. The dilution was taken into account for the evaluation of the precursor concentrations.

Proteases with hydrolase activity were set free during the cell disintegration, which decomposed the tripeptide, ACV. Furthermore the activity of the isopenicillin N-synthetase, which converts ACV to isopenicillin N, was higher by a factor of 1000 than that of ACV synthetase, which catalyses the synthesis of ACV. Therefore, a serine protease inhibitor (PMSF) and ethylendiamintetraacetate (EDTA) were added to the solution. By inhibition of the hydrolyses and the isopenicillin N-synthetase, the rates of ACV-decomposition and its conversion to isopenicillin N were reduced and the ACV concentration was increased.

Latest experiments show that the inhibition of the proteases with hydrolase activity was absolutely necessary. The samples for the measurement of the intracellular concentration of ACV were treated with several protease inhibitors, for example Leupaptin, Pepstatin, Pefabloc, Bestatin, E64 and some combinations of these inhibitors with EDTA (all Boehringer Mannhein, Germany). The concentration of ACV and the other precursors was increased and it was possible to draw conclusions about the activity of the ACV synthetase and the other enzymes involved in the biosynthesis of cephalosporin C. Table 2 shows the relative concentration of the tripeptide ACV after treating the samples with various inhibitors [26].

Table 2
Relative peak areas (HPLC data evaluation) of the concentration of ACV after treating the samples with several protease inhibitors[a]

Protease inhibitor combination	Relative peak area (%)
EDTA/PMSF	100
EDTA/PEFABLOC	45.3
EDTA/Leupeptin	113.5
EDTA/Pepstatin	156.4
EDTA/Bestatin	118.7
Combination of all	47.34
Combination/E64	41
EDTA/PMSF/Pepstatin	74

[a] It is shown that the highest concentration could be reached with a combination of pepstatin and EDTA.

8. Comparison of various β-lactam antibiotic analyses

Specific enzymes are also applied for the monitoring of the concentration of β-lactam antibiotics. For the analysis of penicillin G and V immobilized penicillin G-amidase or β-lactamase (penicillinase) are used [27–30]. Penicillin G-amidase splits penicillin G and V into 6-aminopenicillanic acid (6-APA) and the side chain precursor (phenyl acetic acid and phenoxyacetic acid, respectively). β-lactamase splits the β-lactam bond and converts penicillin to penicilloic acid. β-lacatamase is not as specific as penicillin G-amidase, since the former also reacts with the β-lactam precursors.

Both of these enzymes form acids, the concentration of which was measured with pH sensitive field effect transistor (pH-FET) [27], with chemiluminescence [28], with pH-optode [29] or iodometrically [30]. These enzymes are immobilized and combined with an transducer, and the formed biosensor is integrated into a flow injection analysis (FIA) system [27–30]. All of these enzymatic analytical methods require a preconditioning (protein removal and setting the suitable pH and temperature values).

Penicillin G and V and hydroxylammonium chloride form hydroxamic acid, and with iron(III), iron(III)-hydroxamate in presence of Ni(II)-ions, which act as catalyst. The concentration of iron(III)-hydroxamate was measured at 480 nm. This system was integrated into an (air segmented) continuous flow analyzer (CFA) system (Scalar Analytika, Netherlands) for process monitoring [31].

The concentration of cephalosporin C can be measured by conversion to acid with cephalosporinase. The acid can be detected with various pH meters, similarly to penicilloic acid. (However, cephalosporinase is not available commercially). The concentration of cephalosporin C can be monitored without derivatization with UV photometer at 254–260 nm as well [32].

The advantage of the enzymatic methods is their inexpensive equipment and speed of analysis (only few minutes). Therefore, they are well suited for process monitoring in situ. However, enzymatic methods, like chemical analysis methods, cannot distinguish between similar β-lactam compounds.

9. Conclusion

HPLC analyses of the β-lactam antibiotics are performed with the same type of columns as process and conventional analyses. However, for process analysis a short analysis time is essential. The sample matrix is complex, especially when it contains large amount of proteins and hydrophobic contaminants (e.g. soy oil and lard). Proteins have to be removed completely by precipitation and the hydrophobic compounds by extraction. The analytical column is protected from the residual contaminants by a guard column.

Analysis of the β-lactam products is impaired with the β-lactam precursors, which are present in the cultivation media. Therefore, a high selectivity of the process analysis is necessary.

In case of the determination of the intracellular concentrations of peptide precursors and β-lactam compounds, the analysis is impeded by proteases with hydrolase activity, which are excluded during the disintegration of the cells and decompose the analytes. Therefore, the use of protease inhibitors are recommended.

References

[1] J.F. Kennedy, Z.S. Riviera, C.A. White, J. Biotechnol. 9 (1989) 83–106.
[2] S.E. Jensen, B. Altpeter, GIT Suppl.No. 1 (1990) 40.
[3] K. Schügerl, Bioreaction Engineering, Vol. 3. Bioprocess Monitoring, John Wiley, Chichester, 1967.
[4] H. Hakanson, M. Nielsson, B. Mattiasson, Anal. Chim. Acta 249 (1990) 61–65.
[5] U. Theobald, W. Maillingere, M. Reuss, M. Rizzi, Anal. Biochem. 214 (1993) 31–37.
[6] B. Mattiasson, H. Hakanson, TIBTECH 11 (1993) 136–142.
[7] K. Holzhauer-Rieger, W. Zhou, K. Schügerl, J. Chromatogr. 499 (1990) 609–615.
[8] A. Scheidegger, M.T. Küenzi, A. Fiechter, J. Nüesch, J. Biotechnol. 7 (1988) 131–140.
[9] J. Möller, R. Hiddessen, J. Niehoff, K. Schügerl, Anal. Chim. Acta 190 (1986) 195–203.
[10] M. Ishida, Y. Tsuda, Y. Onuki, T. Itoh, H. Shimada, H. Yamada, J. Chromatogr. B 652 (1994) 43–49.
[11] R.G. Aoyama, D.D. Kitts, H.M. Burt, K.M. McErlane, J. Chromatogr. 626 (1992) 271–276.
[12] H. Lee, J.S. Lee, H.S. Lee, J. Chromatogr. B 664 (1995) 335–340.
[13] S. Chlavatnatol, B.C. Charles, J. Chromatogr. 615 (1993) 91–96.
[14] J.A. Tarbin, W.H.H. Farrington, G. Shearer, Anal. Chim. Acta 318 (1995) 95–101.
[15] L.H. Christensen, G. Mandrup, J. Nielsen, J. Villadsen, Anal. Chim. Acta 296 (1994) 51–62.
[16] K. Borner, E. Borner, H. Lode, J. Chromatogr. B 615 (1993) 174–179.
[17] P. Hilgendorf, V. Heiser, H. Diekmann, M. Thoma, Appl. Microbiol. Biotechnol. 27 (1987) 247–251.
[18] J. Lal, J. Kumar Paliwal, P. Krishen Grover, R. Chandra Gupta, J. Chromatogr. B 655 (1994) 142–146.
[19] S.H. Eriksen, B. Jensen, I. Schneider, S. Kaasgaard, J. Olsen, Appl. Mirobiol. Biotechnol. 40 (1994) 883–887.
[20] W.J.J. Krauwinkel, N.J. Volkers-Kamermans, J. Chromatogr. B 679 (1996) 129–135.
[21] T. Yamazaki, T. Ishikawa, H. Nakai, M. Miyai, T. Tsubota, K. Asano, J. Chromatogr. B 615 (1993) 180–185.
[22] W.J.J. Kranwinkel, N.J. Volkers-Kamermans, J. Chromatogr. B 679 (1996) 129–135.
[23] W.E.M. Adlard, B.M. Gordon, T. Keshavarz, C. Bucke, M.D. Lilly, A.T. Bull, G. Holl, Biotechnol. Tech. 5 (1991) 121–126.
[24] M. Beyer, Dissertation, University of Hannover, 1996.
[25] C. Tollnick, Dissertation, University of Hannover, 1996.
[26] G. Seidel, Dissertation, University Hannover, in preparation.
[27] U. Brand, T. Scheper, K. Schügerl, Anal. Chim. Acta 266 (1989) 87–97.
[28] Rong Wei Min, J. Nielsen, J. Villadsen, Anal. Chim. Acta 320 (1996) 199–205.
[29] T. Scheper, W. Brandes, H. Maschke, F. Plötz, C. Müller, J. Biotechnol. 31 (1993) 345–356.
[30] R. Wolters, M.A.J. van Opstal, G. Kateman, Anal. Chim. Acta 233 (1990) 65–76.
[31] J. Niehoff, J. Möller, R. Hiddessen, K. Schügerl, Anal. Chim. Acta 190 (1986) 205–212.
[32] T. Bayer, T. Herold, R. Hiddessen, K. Schügerl, Anal. Chim. Acta 190 (1986) 213–219.