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Monitoring of selected metabolites and biotransformation products from fermentation broths by high-performance liquid chromatography

György Morovján[a,*], György Szakács[b], Jenö Fekete[c]

[a]*State Institute for Veterinary Biologicals, Drugs and Feeds, Szállás str. 8, 1107 Budapest, Hungary*

[b]*Department of Agricultural Chemical Technology, Technical University of Budapest, Gellért tér 4, 1111 Budapest, Hungary*

[c]*Department of Analytical Chemistry, Technical University of Budapest, Gellért tér 4, 1111 Budapest, Hungary*

Abstract

Reversed-phase high-performance liquid chromatographic methods with UV-photodiode array or multiwavelength detection were developed for the analysis of secondary fungal metabolites mevinolin (also known as monacolin K, lovastatin or Mevacor) and fumagillin in fermentation broths. Samples obtained from screening experiments of *Aspergillus fumigatus* and *Aspergillus terreus* strains were analyzed for fumagillin and mevinolin, respectively. Isocratic and gradient elution was used with acetonitrile–aqueous phosphoric acid or acetonitrile–phosphate buffer eluents. Sample preparation was accomplished by extraction of the whole fermentation broth with an acid–organic solvent mixture. Similarly, bacterial degradation products of 2,4,6-trichlorophenol obtained from microbial remediation processes were analyzed by reversed-phase HPLC with UV-photodiode array detection. Extraction of the analyte from solid samples was done by solvent extraction and solid-phase extraction clean-up using octadecyl-modified silica sample preparation cartridges.

Keywords: Fermentation broth; *Aspergillus* spp.; Mevinolin; Fumagillin; Trichlorophenol

1. Introduction

High-performance liquid chromatography (HPLC) plays an increasing role in the analysis of biotechnology products and in the study of biotechnological processes. It provides an essential tool in the analysis of thermally unstable or high molecular weight compounds in fermentation broths, especially when coupled with UV-photodiode array detection for compound identification [1]. In our screening program, microbial strains are being tested for the production of enzymes, for the production of a specific metabolite or an activity to degrade hazardous chemicals. Among these activities, a screening program was initiated to test 65 *Aspergillus fumigatus* and 70 *Aspergillus terreus* fungal strains for fumagillin and mevinolin production, respectively. During the search for bacteria capable of degrading 2,4,6-trichlorophenol (2,4,6-TCP), a highly toxic and persistent chemical, over a hundred bacteria isolated from sewage sludge and polluted soil of a chemical company were screened. These screenings required selective and sensitive sample preparation and analytical methods to handle and analyze a large number of fermentation samples.

1.1. Monitoring of production of mevinolin and fumagillin during fermentation

Mevinolin is a potent inhibitor of 3-hydroxy-

*Corresponding author.

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methyl-3-glutaryl-coenzyme A (HMG-CoA) reductase, the rate-controlling enzyme in cholesterol biosynthesis [2,3]. It has been proved to effectively lower the plasma cholesterol level in animals and humans [4]. Mevinolin is produced by submerged fermentation using filamentous fungi, such as *Monascus ruber* [2], or *Aspergillus terreus* [3,4]. It is present in the fermentation broth largely in the hydroxycarboxylate (mevinolinic acid) form but the lactone form (mevinolin) also can be found [3]. Minor metabolites with similar structure and biological activity to that of mevinolin were also isolated from *A. terreus* broths [5].

During the production of mevinolin, fermentations were monitored using a C_8 column with acetonitrile–aqueous phosphoric acid or acetonitrile–methanol–water eluent [6,7]. For detailed analysis of the fermentation product, impurity mycotoxins or other metabolites, elution from a C_{18} column with acetonitrile–aqueous trifluoroacetic acid gradient elution was used [8]. Reversed-phase HPLC determination of mevinolin with acetonitrile–aqueous phosphate buffer eluents in bile, faeces and plasma has also been described [9]. Separation of mevinolin, mevinolinic acid and its methyl, ethyl, and propyl esters with methanol, ethanol or propanol containing buffered mobile phases has been reported [10]. Use of an expert system for the planning of the reversed-phase separation of mevinolin and the corresponding hydroxy-acid has been published [11].

Fumagillin, which is an amebicid compound, was first isolated in the early 1950s by Eble and Hanson [12,13] from the fermentation broth of *Aspergillus fumigatus* and was initially tested for the treatment of dysentery. Up to the mid-1980s, the compound was only used in the veterinary practice for the treatment of disease in honey-bees caused by *Nosema apis* [14] or fish diseases caused by certain parasites [15]. Recently, fumagillin gained growing interest because of its angiostatic property [16] and tumor growth inhibitory effect [17]. Although fumagillin was discovered more than forty years ago, only a few publications deal with its liquid chromatographic determination. These are mainly used for the determination of the compound in honey because of its almost exclusive use in beekeeping practice [18,19]. Recently, determination of fumagillin in fish tissues has been reported [20]. In our paper a method for the determination of fumagillin in fermentation broths is presented.

1.2. Detection of the biodegradation of 2,4,6-TCP during remediation processes

Remediation of the sites polluted with industrial waste has increasing importance since extensive areas contain dangerous levels of hazardous compounds. Chlorophenols are a group of primary-pollutant compounds used for the production of biocides. Only a few microorganisms, such as *Pseudomonas* [21–24], *Alcaligenes* [25] or *Clostridium* strains [26] can mineralize these xenobiotics. Their microbial biodegradation involves oxidation, dechlorination and rearrangement processes [21,23,25,26]. Analysis of chlorophenols in fermentation media was carried out by capillary gas chromatography [27] or reversed-phase HPLC [28] with ultraviolet detection using methanol–aqueous acid solution as eluent. Chloride released from chlorinated phenols was measured by ion chromatography [27,28]. In our experiments 2,4,6-TCP was chosen as target compound for biodegradation studies. A method was developed for the liquid chromatographic determination of 2,4,6-TCP from fermentation liquids and from polluted/remediated soil.

2. Experimental

2.1. Reagents and disposables

Solvents acetonitrile, methanol and phosphoric acid were of HPLC grade (E. Merck, Darmstadt, Germany), potassium dihydrogenphosphate was analytical grade (Carlo Erba, Milan, Italy). Other chemicals were of reagent grade (unless otherwise specified). Solid-phase extraction cartridges (Sep-Pak C_{18}, 360 mg) were from Waters (Milford, USA). As a reference material, Mevacor tablets (MSD, Rahway, USA) with nominal content of 20 mg mevinolin per tablet were used. Fumagillin was kindly donated by Chinoin Pharmaceutical and Chemical Works (Budapest, Hungary). 2,4,6-TCP was obtained from E. Merck.

2.2. Microorganisms

Bacteria and fungi used in these studies were obtained from the TUB culture collection (Technical University, Budapest). The most important strains were: *Aspergillus terreus* TUB F-514 for mevinolin production, *Aspergillus fumigatus* TUB F-266 for fumagillin production and *Pseudomonas putida* TUB B-534-4 for biodegradation of 2,4,6-TCP.

2.3. Fermentation media

For screening experiments, the following shake flask media were used (concentrations are given in g/l). The medium for mevinolin fermentation contained: lactose 20, corn steep liquor 2.5, soybean meal 2, potassium dihydrogen phosphate 1, sodium chloride 1, magnesium sulphate 1, antifoam (Struktol SB 2023, Schill and Seilacher, Hamburg, Germany) 0.5. The medium for fumagillin fermentation consisted of: sucrose 50, corn steep liquor 10, potassium dihydrogen phosphate 1 sodium nitrate 1, potassium chloride 0.5, magnesium sulphate 0.5, antifoam (Struktol SB 2023) 0.5; ferrous sulphate 0.005. The pH of both media was adjusted to 6.5 by 20% sodium hydroxide solution before sterilization. The medium for biodegradation of 2,4,6-TCP biodegradation experiments was comprised of: sucrose 1.5, starch 1.5, calcium carbonate 1, ammonium sulphate 1, Bacto yeast extract (Difco Labs, Detroit, USA) 1, Bacto peptone (Difco) 1, potassium dihydrogenphosphate 0.5, sodium chloride 0.5, magnesium sulphate 0.5, 2,4,6-TCP 0.1. The pH was set to 6.8 by 20% sodium hydroxide solution before sterilization. All media were sterilized in an autoclave at 121°C for 30 min.

2.4. Fermentation

Shake flask fermentations were carried out in 750 ml Erlenmeyer-flasks containing 150 ml medium. Inoculated flasks were incubated on a rotary shaker at 220 rpm and 30°C for 5 or 7 days (for mevinolin and fumagillin fermentation, respectively). Bacterial cultures for biodegradation of 2,4,6-TCP were harvested after 14 days of propagation. During and at the end of fermentation, whole fermentation broth samples were used for HPLC analysis.

2.5. Instrumental

For the determination of mevinolin and fumagillin, the chromatographic system (System 1) consisted of a Waters 600 high pressure pump, U6K injector and a Waters 990 photodiode array detector (Waters, Milford, USA). For the determination of chlorophenols, a Perkin-Elmer TurboChrom HPLC system (System 2) was used comprising of a Perkin-Elmer LC Pump 200, a Perkin-Elmer ISS200 sample processor and a Perkin-Elmer 235C photodiode array detector (Perkin-Elmer, Norwalk, USA). Alternatively a Waters 990 photodiode array detector was used. For the determination of mevinolin by multiwavelength detection, the chromatographic system (System 3) comprised of two Waters 501 HPLC pumps, a WISP 717 autosampler and a Waters 490E multiwavelength detector interfaced with a Maxima 820 chromatographic data station.

2.6. Sample preparation

Fermentation samples (2 ml whole broth) containing fumagillin were mixed with 6 ml of methanol and 0.25 ml of 1 *M* phosphoric acid solution was added. The suspension was mixed thoroughly with a Vortex mixer and was left to stand for 15 min. After this period of time, samples were centrifuged (2000 *g*, 15 min). Aliquots of the clear upper layer were injected onto the chromatographic column. Samples were protected from light because of the sensitivity of fumagillin to light.

Mevinolinic acid containing fermentation broths (2 ml) were diluted with 2 ml of acetonitrile and 0.1 ml of concentrated phosphoric acid was added. Acid treatment facilitated the formation of mevinolin from mevinolinic acid which is largely present in the broth. Samples were mixed and incubated precisely for 60 min, then were centrifuged (2000 *g*, 15 min) and aliquots of the clear supernatant were injected.

Chlorophenol-containing fermentation broths were mixed with a five-fold volume of methanol, were incubated for 15 min and were filtered using a 0.45 μm pore size disposable filter. Filtrates were directly injected. When soils or solid-phase fermentation mixtures were analyzed, 15 g solid was extracted with 50 ml methanol. The extract was filtered and evaporated to about 2 ml, in vacuum. The concen-

trate was diluted with 30 ml 0.1% phosphoric acid solution and was applied to a Sep-Pak C_{18} cartridge, conditioned with 3 ml water and 3 ml methanol. After washing the cartridge with 5 ml water, adsorbed compounds were eluted with 2 ml methanol–water (80:20, v/v) and chromatographed. Recovery using this procedure was 94% for 2,4,6-TCP.

2.7. Chromatographic conditions

For the chromatographic determination of mevinolin, either a Beckmann Ultrasphere ODS (50×4.6 mm I.D., 5 μm) or a Waters NovaPak C_{18} (150×3.9 mm I.D., 4 μm) column was used. The eluent was acetonitrile–0.1% phosphoric acid (50:50, v/v) solution flowing at 1.5 ml/min for both columns. The detection wavelength was 235 nm. Using the Ultrasphere column and 10-μl injection volume, the detection limit for mevinolin was 50 ng/ml for the fermentation sample. Determination of fumagillin was carried out with a Spherisorb ODS-2 (250×4.6 mm I.D., 5 μm) column. The eluent was methanol–5 m*M* potassium dihydrogenphosphate (80:20, v/v) (pH 3.1, set with 1 *M* phosphoric acid). A Waters NovaPak C_{18} (150×4.6 mm I.D., 4 μm) column was also tested for the analysis of fumagillin (data not shown). In this case, the eluent was acetonitrile–0.1% phosphoric acid (50:50, v/v). The flow-rate was 1 ml/min with both columns. Detection limit for fumagillin was 75 ng/ml.

Chlorinated phenols were analyzed using a LiChrospher 100 RP-18 (125×3.9 mm I.D., 5 μm) column with an eluent comprising of acetonitrile–0.1% phosphoric acid (60:40, v/v) running at a flow-rate of 1 ml/min. Detection wavelength was 225 nm. The injection volume was 20 μl. Linear range of the determination was determined to be 0.25–200 μg/ml. Detection limit for 2,4,6-TCP was 8 ng/ml for fermentation broths and 45 ng/g for soil samples.

3. Results and discussion

Of sixty-five wild-type *A. fumigatus* cultures screened, 27 strains were found to produce more than 10 μg/ml fumagillin. Among them, five strains gave better fumagillin yield than 200 μg/ml. The most effective strain (TUB F-266) produced 244 μg/ml fumagillin in 5 days. Screening of 70 strains of *A. terreus* showed that only seven strains produced more than 1 μg/ml mevinolin in liquid culture. The best isolate (TUB F-514) produced 140 μg/ml mevinolin in 7 days. Over a hundred bacteria tolerating 100 ppm concentration of 2,4,6-TCP were tested for the biodegradation of this compound. The fermentation samples were monitored by HPLC for the disappearance of 2,4,6-TCP or for the decrease in its concentration and appearance of a new peak representing a degradation product having similar spectra to that of the mother compound.

Using methanol or acetonitrile for sample extraction, good recoveries (95–98%) were experienced. However, variation of recoveries in the case of fumagillin and mevinolin were decreased when the sample was acidified. Using an acidic treatment during the sample preparation of mevinolin fermentation broth, an equilibrium mixture of mevinolin and mevinolinic acid is formed from the mevinolinic acid which is largely present in the fermentation broth since in aqueous acidic solution lactone formation and lactone hydrolysis reactions lead to an equilibrium [29]. This treatment helped the identification of minute amounts of mevinolinic acid, because the spectrum of the more retained lactone was identified easily due to the absence of interfering compounds. Since ester formation of mevinolinic acid in alcoholic–acidic solution has been reported in the literature [10], acetonitrile was chosen for the sample preparation of mevinolinic acid-containing samples.

We found that screening of a large number of strains with different biochemical properties and using various fermentation media requires a further confirmation of the analyte identity besides its retention time. Since the target compounds have characteristic UV spectra, a photodiode array detector was used and the target analytes and structurally related compounds were identified by their ultraviolet spectra. Gradient elution (20–70% acetonitrile in 25 min) of a mevinolin-containing fermentation broth is shown on Fig. 1. This chromatogram represents the variety of fungal metabolites. Monitoring wavelengths were chosen so that almost all compounds (Fig. 1A), the analyte (Fig. 1B) and possibly polluting mycotoxins absorbing light at 280

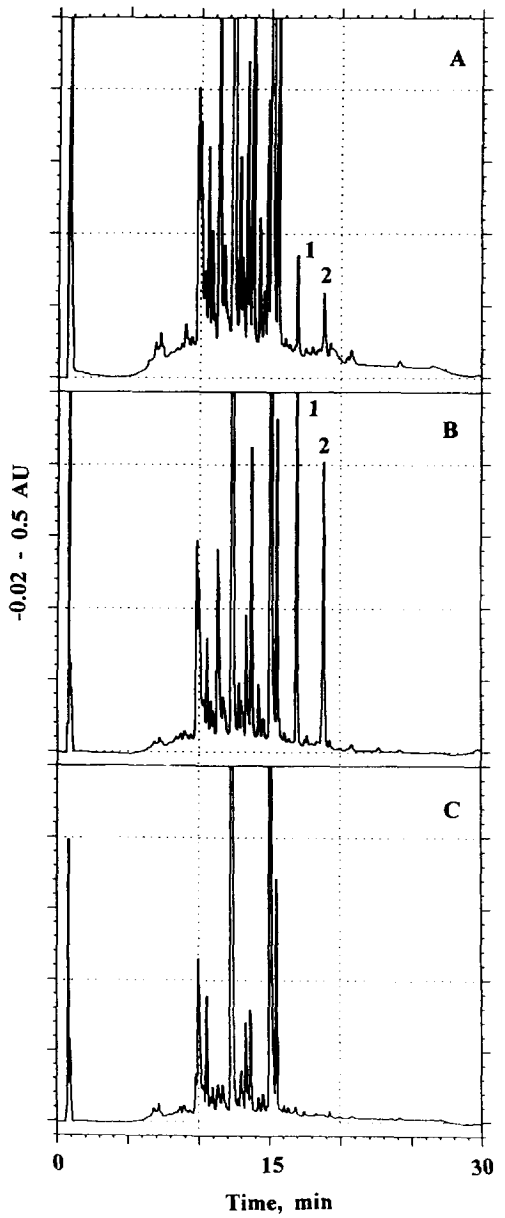

Fig. 1. Gradient separation of *Aspergillus terreus* TUB F-514 fermentation products (System 1). Column: NovaPak C_{18} (150× 3.9 mm I.D., 4 μm). Gradient, 20–70% (v/v) acetonitrile in 0.1% phosphoric acid solution; sample volume, 20 μl; flow-rate, 1.5 ml/min; detection wavelength, (A) 205 nm, (B) 235 nm, (C) 280 nm. Compounds: 1=mevinolinic acid, 2=mevinolin.

nm (Fig. 1C) could have been detected. Mevinolin and mevinolic acid were identified using their retention time and UV spectra. On the basis of this gradient separation, an isocratic method was developed using the same column (Figs. 2 and 3). From these figures it can be concluded that the fermentation broth contained the target analyte as

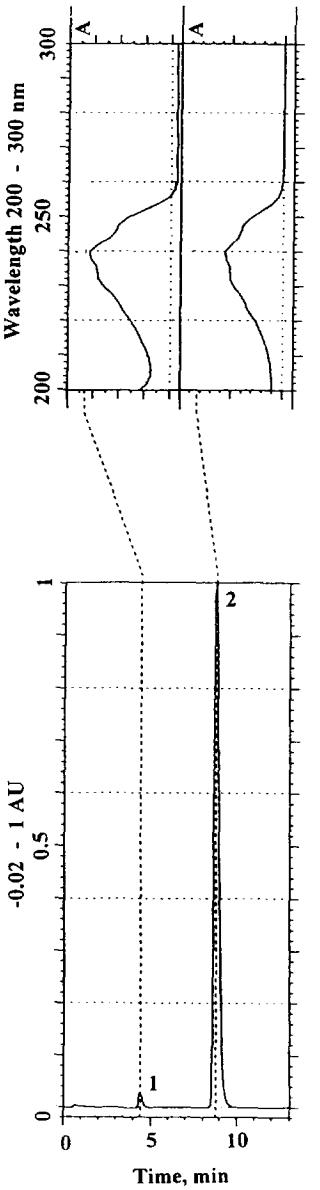

Fig. 2. Isocratic chromatogram (System 1) of a mevinolin reference solution (80 ppm) after 10 min acid treatment. Column, NovaPak C_{18} (150×3.9 mm I.D., 4 μm); eluent,, acetonitrile–0.1% aqueous phosphoric acid (50:50, v/v); flow-rate, 1.5 ml/min; injection volume, 10 μl; detection wavelength, 235 nm. Compounds: 1=mevinolinic acid, 2=mevinolin.

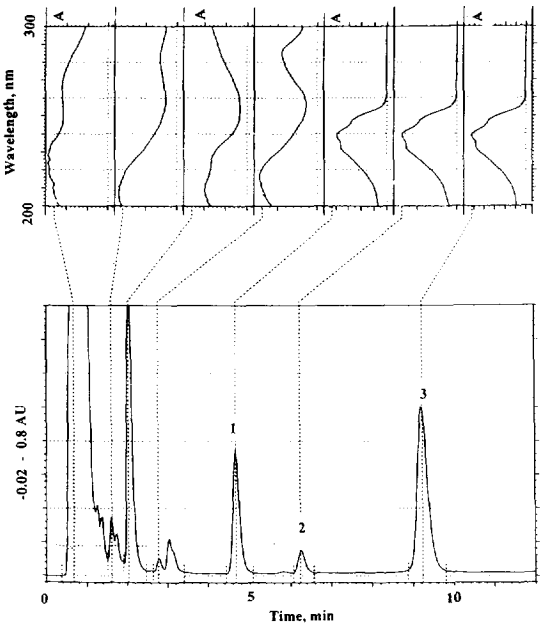

Fig. 3. Isocratic chromatogram of *Aspergillus terreus* TUB F-514 fermentation sample (System 1). For chromatographic conditions, see Fig. 2. Compounds: 1=mevinolinic acid, 2=unknown, 3=mevinolin. The unknown compound has similar UV absorption to that of mevinolin.

retention times and UV spectra were in good agreement.

Because of the great number of samples, a shorter (5 cm) column was chosen for the routine analysis of fermentation samples. The run time with this column could have been shortened to 6 min with the same eluent composition (Fig. 4) with adequate resolution. Detection for the routine analysis of samples from the best producing fungus was done by multiwavelength ultraviolet detection and peak identity was confirmed using the ratio of absorbances at 235 and 255 nm. Repeatability of analyses using the same homogenized sample was found to be about 2% ($n=5$) and the detection limit for mevinolin was 50 ng/ml. With these methods, more than 800 samples were analyzed. Sample preparation of broths containing fumagillin were done similarly to that of mevinolin except that methanol was used for sample preparation. A chromatogram of a fumagillin-containing fermentation broth is shown in Fig. 5. Short retention times allowed the analysis of a large number of samples.

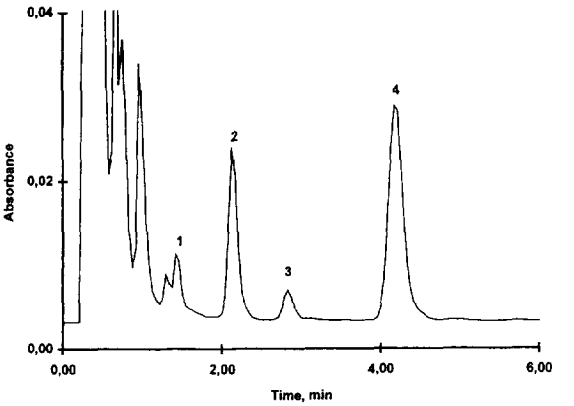

Fig. 4. Rapid isocratic analysis (System 3) of a mevinolin-containing fermentation broth. Column, Beckmann Ultrasphere ODS (50×4.6 mm I.D., 5 μm); eluent, acetonitrile–0.1% phosphoric acid (50:50, v/v); flow-rate, 1.5 ml/min; sample volume, 10 μl; detection wavelength, 235 nm. Compounds: 1=unknown, 2=mevinolinic acid, 3=unknown, 4=mevinolin.

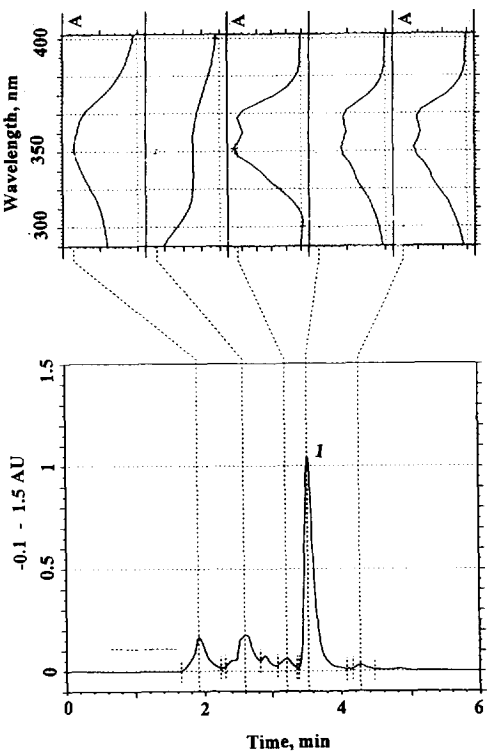

Fig. 5. Analysis of a fumagillin containing fermentation broth (System 1). Column, Spherisorb ODS 2 (250×4.6 mm I.D., 5 μm); eluent, methanol–phosphate buffer (80:20, v/v) (see text); flow-rate, 1 ml/min; sample volume, 10 μl; detection wavelength, 351 nm. Compounds: 1=fumagillin.

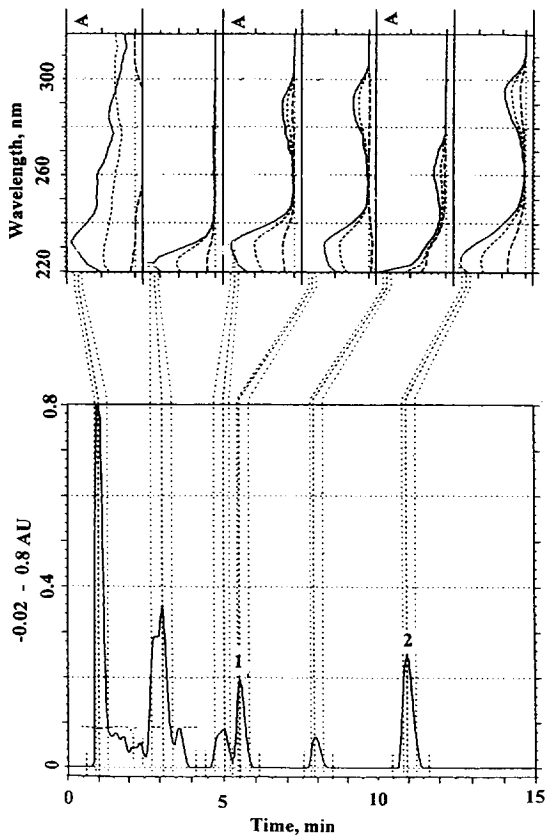

Fig. 6. Chromatogram of a 2,4,6-TCP-containing fermentation liquid after 14 days incubation with *Pseudomonas putida* (System 2). For chromatographic conditions, see text. Compounds: 1= biodegradation product, 2=2,4,6-TCP.

A typical chromatogram from the assay of 2,4,6-TCP in a biodegradation study is shown on Fig. 6. Decomposition products can be identified by their UV spectra being similar to that of 2,4,6-TCP. In this case, a compound with a retention time of 5.5 min was found to be a dichlorophenol isomer formed by dechlorination from its retention time and ultraviolet spectra.

4. Conclusions

These examples show that crucial factors for the successful HPLC analysis of fermentation products are simple sample preparation procedure, rapid and efficient chromatographic separation of the analytes, and a well-established method for the identification of the analyte. To ensure high throughput of the analytical method, speed and simplicity of operation may be the overriding factor because of the labile nature of the analyte and the great number of samples to be analyzed. At the time of harvesting, fermentation broth contains intact microorganisms, cell fragments, medium components and metabolic products including the analyte [30]. Precipitation of proteins, polysaccharides and cell components using a cheap solvent proved to be very efficient for the clean-up of the samples and solubilization of the analyte. For compounds having characteristic UV absorption, photodiode array detection is a reliable, robust and cheap alternative to more expensive LC–MS identification in the initial phase of the research. Further identification steps can be the analysis of the target compound in a separation system with different selectivity, application of a detection method providing structural information or the isolation and structural analysis of the compound under investigation. However, these can be applied only at the later stages of research because of their time, work and cost requirements.

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References

[1] H.P. Fiedler, Nat. Prod. Lett., 2 (1993) 119.
[2] A. Endo, J. Antibiot., 32 (1979) 852.
[3] R.L. Monaghan, A.W. Alberts, C.H. Hoffmann and G. Albers-Schonberg, US Pat., 4 231 938 (1980).
[4] A.W. Alberts et al., Proc. Natl. Acad. Sci. USA, 77 (1980) 3957.
[5] T.Y.K. Lam, US Pat., 4 76 63 (1983).
[6] G.S. Brenner, D.K. Ellison and M.J. Kaufman, in H.G.Brittain (Editor), Analytical Profiles of Drug Substances and Excipients, Vol. 21, Academic Press, New York, 1994, pp. 277–305.
[7] V.J. Gullo, R.T. Goegelman, I. Putter and Y.-K. Lam, J. Chromatogr. A, 212 (1981) 234.

[8] V.A. Vinci, T.D. Hoerner, A.D. Coffman, T.G. Schimmel, R.L. Dabora, A.C. Kirpekar, C.L. Ruby and R.W. Stieber, J. Indust. Microbiol., 8 (1991) 113.
[9] R.J. Stubbs, M. Schwartz and W.F. Bayne, J. Chromatogr. A, 383 (1986) 438.
[10] J. Friedrich, M. Žužek, M. Benčina, A. Cimerman, A. Štrancar and I. Radež, J. Chromatogr. A, 704 (1995), 363.
[11] J. Fekete, G. Morovján, F. Csizmadia and F. Darvas, J. Chromatogr. A, 660 (1994) 33.
[12] T.E. Eble and F.R. Hanson, Antibiot. Chemother., 1 (1951) 54.
[13] F.R. Hanson and T.E. Eble, US Pat., 2 52 56 (1953).
[14] M.C. McCowen and J.A. diPaolo, Science, 113 (1951) 202.
[15] J.F. Sövényi, J. Fish Dis., 15 (1990) 255.
[16] J. Folkman, D. Ingber, T. Fujita and T. Kanamura, Eur. Pat., Appl. 325 199 (1989).
[17] J. Folkman, Adv. Cancer Res., 43 (1985) 175.
[18] H.I. Assil and P. Sporns, J. Agric. Food Chem., 39 (1991) 2206.
[19] J.M. Brackett, M.D. Arguello and J.C. Schaar, J. Agric. Food Chem., 36 (1988) 762.
[20] J. Fekete, Z. Romvári, I. Szepesi and G. Morovján, Chromatogr. A, 712 (1995) 378.
[21] G. Bestetti et al., Appl. Microbiol. Biotechnol., 37 (1992) 260.
[22] S.Y. Dapaah and G.A. Hill, Biotechnol. Bioeng., 40 (1992) 1353.
[23] F.K. Higson and D.D. Focht, Appl. Environ. Microbiol., 58 (1992) 2501.
[24] E. Schmidt, M. Hellwig and H.J. Knackmuss, Appl. Environ. Microbiol., 46 (1983) 1038.
[25] B. Menke and H.J. Rehm, Appl. Microbiol. Biotechnol., 37 (1992) 655.
[26] X. Zhang and J. Wiegel, Appl. Environ. Microbiol., 58 (1992) 3580.
[27] L.R. Arcand and F.S. Archibald, Enzyme Microb. Technol., 13 (1991) 194.
[28] C. Kennes, W.-M. Wu, L. Bhatnagar and J.G. Zeikus, Appl. Microbiol. Biotechnol., 44 (1996) 801.
[29] J. March, Advanced Organic Chemistry – Reactions, Mechanisms, Structure, Wiley, New York, 4th ed., 1992.
[30] P.F. Stanbury, A. Whitaker and S.J. Hall, Principles of Fermentation Technology, Pergamon Press, Oxford, 2nd ed., 1995, pp. 277–281.