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Development and Validation of HPLC Methods for Analytical and Preparative Purposes

BY

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Abstract

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This thesis concerns the development and validation of high performance liquid chromatography (HPLC) methods aimed for two industrially important areas: (i) analysis of biotechnological synthesis and (ii) determination of adsorption isotherm parameters. There is today a lack of detailed recommendations for analytical procedures in the field of biotechnological production of drugs. Therefore, guidelines were given for analytical development and validation in this field; the production of 9α-hydroxyprogesterone was used as model. In addition, a rapid method using HPLC coupled with diode-array-detection (DAD) and mass spectrometry (MS), was developed for the preliminary identification and quantification of the product. In addition, requirements and recommendations were developed for the selection of the internal standard and for its inclusion in the process liquid. By using this approach the precision and accuracy of the quantitative method were considerably improved.

Preparative chromatography is a powerful separation method for the purification of pure compounds from more or less complex sample mixtures. One such mixture can be the process liquid from a fermentation, another example can be a racemic mixture of compounds whose enantiomeric constituents must be isolated. Computer-assisted modeling can be used to optimize preparative chromatography. However, competitive adsorption isotherm parameters are required as input data for the computer simulations. In this thesis, a new injection technique, based on a firm theoretical basis, was developed for the peak perturbation (PP) method allowing the determination of binary competitive adsorption isotherm parameters from a broad concentration range. With the new method the determination of adsorption isotherm parameters from a quaternary mixture could be done for the first time. The profiles simulated with these parameters showed excellent agreement with the corresponding experimental profiles, validating the accuracy of the adsorption isotherm parameters derived by the new method.

Keywords: High performance liquid chromatography (HPLC), Analytical biotechnology, Sample preparation, Diode-array-detection (DAD), Mass spectrometry (MS), Validation, Preparative chromatography, Competitive isotherm parameters, Perturbation peak (PP) method

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Till mina föräldrar och
min älskade Linnéa

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals I-VI:

I. **Use of liquid chromatography-diode-array detection and mass spectrometry for rapid product identification in biotechnological synthesis of a chiral steroid.**
Johan Lindholm, Douglas Westerlund, Karl-Erik Karlsson, Karin Caldwell and Torgny Fornstedt. Journal of Chromatography A, 992 (2003), 85-100.

II. **Guidelines for analytical method development and validation of biotechnological synthesis of drugs. Production of a chiral steroid as model.**
Johan Lindholm, Monika Johansson and Torgny Fornstedt. Journal of Chromatography B, 791 (2003), 323-336.

III. **Investigation of the adsorption behaviour of a chiral model compound on Kromasil CHI-TBB.**
Johan Lindholm and Torgny Fornstedt. Manuscript.

IV. **Theoretical and experimental study of binary pertubation peaks with focus on peculiar retention behaviour and vanishing peaks in chiral liquid chromatography.**
Patrik Forssén, Johan Lindholm and Torgny Fornstedt. Journal of Chromatography A, 991 (2003), 31-45.

V. **Validation of the accuracy of the perturbation peak method for determination of single and binary adsorption isotherm parameters in LC.**
Johan Lindholm, Patrik Forssén and Torgny Fornstedt. Accepted May 2004 in Analytical Chemistry.

VI. Validation of the accuracy of the perturbation peak method for determination of multi-component adsorption isotherm parameters in LC.
Johan Lindholm, Patrik Forssén and Torgny Fornstedt. Accepted June 2004 in Analytical Chemistry.

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Paper I was experimentally planned by me under supervision of Torgny Fornstedt and the experiments were done by me except for the following parts: fermentation NMR and MS. I wrote the article with the co-authors. **Paper II** was planned and performed experimentally by me. I wrote the article with the co-authors. **Paper III** was experimentally designed and written by me under supervision by Torgny Fornstedt. I planned **papers IV-VI** and I performed all experiments except some in **paper IV** (performed by Lars Lindbom). The writing of the **papers IV-VI** was done by me and Patrik Forssén in cooperation with Torgny Fornstedt; I contributed more with the experimental and result sections and Patrik more with the theoretical sections. The computer programs used in the papers were developed by Patrik Forssén in cooperation with Torgny Fornstedt. I performed most of the simulations.

Manuscript not included in the thesis

A biotechnological approach to synthesis of 9α-hydroxylated steroids.
Robert Arnell, Rickard Johannisson, Johan Lindholm, Bo Ersson, Andras Ballagi, Torgny Fornstedt, Karin Caldwell. To be submitted 2004.

Contents

Abbreviations

9α-OH-PS	9α-hydroxyprogesterone
11α-OH-PS	11α-hydroxyprogesterone
a	equilibrium constant at infinite dilution
a_I	nonselective equilibrium constant at infinite dilution
$a_{,II,i}$	enantioselective equilibrium constant at infinite dilution of component i
AD	4-androsten-3,17-dione
APCI	atmospheric pressure chemical ionization
b	binding coefficint (mM^{-1})
b_I	nonselective binding coefficient (mM^{-1})
$b_{II,i}$	enantioselective binding coefficient of component i (mM^{-1})
C	mobile phase concentration (mM)
CSP	chiral stationary phase
C.V.	coefficient of variation (%)
DAD	diode-array detection
ε	porosity
ED	equilibrium-dispersive
ESI	electrospray ionization
F	phase ratio
FFF	field flow fraction
FDA	the US food and drug administration
GC	gas chromatography
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
ICH	the international conference on harmonisation
IEC	ion-exchange chromatography
IMAC	immobilized metal-affinity chromatography
I.S.	internal standard
k	retention factor
L	column length (m)
LC	liquid chromatography
LLE	liquid-liquid extraction
LLOQ	lower limit of quantification
LOD	limit of detection
MALDI	matrix-assisted laser desorption
m/z	mass-to-charge

MS	mass spectrometry
N	number of theoretical plates
NMR	nuclear magnetic resonance
NPLC	normal-phase liquid chromatography
ODS	octadecyl-bonded silica
PP	peak perturbation
PS	progesterone
θ	surface coverage
QC	quality control
q	stationary phase concentration (mM)
q_s	monolayer saturation capacity (mM)
$q_{s\,I}$	nonselective saturation capacity (mM)
$q_{s\,II,\,i}$	enantioselective saturation capacity (mM) for component i
RI	refractive index
RPLC	reversed-phase liquid chromatography
RSD	relative standard deviation
SEC	size exclusion chromatography
SFC	supercritical fluid chromatography
SMB	simulated moving bed
SPE	solid-phase extraction
t	time (min)
TLC	thin layer chromatography
TOF	time-of-flight
t_0	column hold-up time (min)
TP	tracer-pulse
t_R	retention time (min)
μ	linear flow velocity (cm min^{-1})
USP	US pharmacopeia
UV	ultra violet
V_g	geometric volume of the column (ml)
V_m	column hold-up volume (ml)
V_0	dead volume of the column (ml)
V_S	stationary phase volume (ml)
V_T	total dead volume of the system (ml)
z	axial position in the column

1 Introduction

Liquid chromatography (LC) is a separation method of great importance to the chemical, pharmaceutical and biotechnological industry. The principle is that a sample of a solution of the substances is injected into a column of an porous material (stationary phase) and a liquid (mobile phase) is pumped through the column. The separation of substances is based on differences in rates of migration through the column arising from different partition of the substances between the stationary and the mobile phase. Depending on the partition behavior of the different types of substances, these will elute at different times from the column outlet. The technique was originally developed by the Russian botanist M.S. Tswett in 1903 [1, 2] and since then there has been an enormous development of this technique. The definite breakthrough for liquid chromatography of low molecular weight compounds was the introduction of chemically modified small diameter particles (3 to 10 μm) e.g., octadecyl groups bound to silica in the late 1960s [3]. The new technique became rapidly a powerful separation technique [3] and is today called high performance liquid chromatography (HPLC). Theoretical contributions to linear chromatography during this time period were summarized by Giddings [4].

For biological samples where the analytes often are non-volatile and/or occur in an aqueous matrix, the reversed-phase mode, using a hydrophobic stationary phase and an aqueous mobile phase is extremely useful. The usefulness and popularity of HPLC was further increased by the possibility to automate and computerize the systems providing unattended operations and high sample capacities. Many Nobel Prize awards have been based upon work in which chromatography played an important role [5]. Most recently, the 2002 Nobel Prize in chemistry was awarded to “the development of methods for identification and structure analyses of biological macromolecules" in which HPLC and Mass spectrometry (MS) were used [6].

In *analytical chromatography* the aim of the separation is to obtain quantitative and qualitative information about the compounds of interest (analytes) in a sample. Analytical chemists have to analyze a variety of complex samples often originating in difficult matrices to answer questions about the quality and quantity of different analytes. A complex sample often contains a wide range of components with varying solubility’s. Therefore the sample preparation and the separation methods must be highly selective and sensitive. These requirements are satisfied with HPLC especially if combined

with an advanced detection technique such as diode-array detection (DAD) or mass spectrometry (MS). A very large number of reports have been published in the analytical and bioanalytical (analysis of drugs in biological fluids) areas using these techniques. Also in the field of biotechnology where desired compounds are produced through fermentation, HPLC is commonly used [7]. This thesis describes the use of modern analytical methods, notably HPLC-DAD and MS for the rapid identification (**paper I**) and validated quantification (**paper II**) of the fermentation product, 9α-hydroxyprogesterone from a complex fermentation media.

Another important application area than for analytical purposes is the purification of products in large scale, called preparative chromatography which is mostly applied in nonlinear mode. The theory of nonlinear chromatography was developed already during the 1940s [8-10]. Among many important early contributions, the Nobel Laurate of 1948, professor Arne Tiselius of Uppsala University introduced a clear and systematic definition of the three operational modes: (1) elution (2) frontal analysis and (3) displacement chromatography [11]. Theoretical contributions in the multi-component case were summarized by Helfferich 1970 [12]. During the 1990s Guiochon adapted the multi-component theory to a format suitable for computer simulations [13, 14] allowing systematic investigations of fundamental issues as well as computer-assisted optimisations of practical problems.

In *preparative chromatography* the goal is to isolate as much as possible of the desired component(s) in a complex sample mixture. Thus, high sample concentrations and/or large volumes are injected to the column. Preparative chromatography is an important tool for the purification of the fine chemicals and drug products from biotechnological synthesis or from chiral asymmetric synthesis; one example being the fermentation product Cyclosporine A [15]. Sometimes preparative chromatography must be applied also when the primary goal is analysis; one such example is when traditional nuclear magnetic resonance (NMR) spectroscopy is used for identification of the product in biotechnological synthesis requiring mg-amounts (**paper I**). When the goal is purification of enantiomers, stationary chiral phases (CSPs) with high loading capacity are highly desirable. For this type of work, the determination of the adsorption isotherm parameters of the actual enantiomers on the selected CPSs is especially important (**paper III).**

Because of the limited surface capacity of the stationary phase, the column often operates under overloaded conditions. This is why a further increased sample load results in a smaller amount of the fraction adsorbed. Thus, nonlinear conditions prevail. A further complication in the preparative case is that the different types of components compete with each other for the same binding site, an effect that ultimately results in strong band interactions and band contaminations. The functions describing this complex behavior are called competitive adsorption isotherm parameters. Because of

this complication computer simulations should be used for proper optimization of the operational conditions in preparative separations and especially for the complex situations that occur e.g., in simulated moving bed (SMB) applications.

The purpose of computer simulation is to optimize the resolution of a desired compound. The essential input data are the competitive adsorption isotherm parameters. Single component adsorption isotherm parameters are easy to measure. However, in all chromatographic processes of practical importance multi-component situations prevail, and thus competitive adsorption isotherms have to be measured. Existing methods do not give much help and this has hampered the use of computer-assisted optimization. The issue of determining competitive adsorption isotherm parameters is therefore one of the most important remaining research topics today in the field of chromatography. An interesting approach is the use of the peak perturbation (PP) method in the competitive (binary) mode in **paper IV-V**. Adsorption isotherm parameters were for the first time acquired from a quaternary mixture in paper **VI**.

Validation issues are especially important in the analytical field when quantifications are made. However, it is also important to validate the adsorption isotherm model and its estimated parameters used in preparative chromatography. In contrast to the situation for the bioanalytical area in the pharmaceutical industry, there are few published reports on validation in the analytical biotechnological and in the isotherm parameter determination field, and there exists no detailed validation guidelines [16, 17]. This is why much effort in this thesis is focused on the development of validated methods in both these fields.

The first section of this thesis deals with development and validation of analytical biotechnological methods for qualitative and quantitative analysis (**papers I-II**). The second section (**papers III-VI)** concerns the issue of isotherm parameters determination for preparative purposes. More particularly, this section deals with the validated characterization of phase systems through the determination of isotherm parameters and computer simulations (**paper III**) and with the development and validation of methods to determinate adsorption isotherm parameters directly from component mixtures (**papers IV-VI**). The two sections together have one important feature in common; the development and validation of chromatographic methods for analytical and preparative purposes (**papers I-VI)**. The intention of this summary is to give readers who are new in the area a general introduction to the fields described above. For a more detailed discussion, see **papers I-VI**.

1.1 Aims of Study

The aims of the present studies were:

- Analysis of Biotechnological Synthesis

- To develop a strategy for rapid identification of the product in a fermentation process where reference sample is not available **(Paper I)**.
- To develop and validate a method for analytical quantification of both the substrate and the product in a fermentation process (**Paper II**).
- To give general guidelines for development and validation of methods aimed for quantification of drugs produced through fermentations **(Paper II)**.

- Determination of Adsorption Isotherm Parameters

- To characterize a new CSP (Kromasil CHI-TBB) aimed for preparative chromatograpy through the determination of its validated isotherm parameters for a chiral model compound **(Paper III)**.
- To develop a new injection technique for the perturbation peak method in order to determine adsorption isotherm parameters directly from binary (**Paper IV-V**) or quaternary mixtures (**Paper VI**).
- To validate the newly developed injection techniques for the binary (**Paper V)** and quaternary cases (**Paper VI**).

2 Analysis of Biotechnological Synthesis

This section is intended to give a short overview of method development and validation of analytical methods - for qualitative and quantitative analysis of small (Mw < 1000) drugs - from process liquid such as fermentation medium. The same principles can be applied for any low-molecular weight compounds in biotechnological or organic synthesis.

2.1 Biotechnology

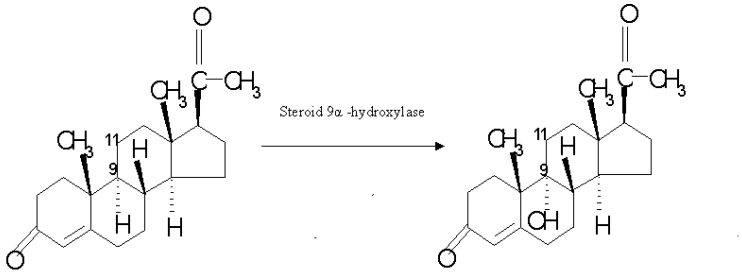
Biotechnology is a discipline concerned with the commercial production and isolation of specific molecules from plant, animal or microbial sources. Fermentation is often used in the production of specific molecules that are hard to synthesize. Many biotechnological processes have been developed used, and perfected over a number of millennia. Such processes include brewing, wine-making and cheese-making. Although these processes are dependent upon the enzymatic conversion of suitable substrates into desired products, those who first developed the methodologies were ignorant of this fact. It was during the 1940s to 1960s that many large-scale industrial processes dependent upon enzyme-catalyzed bio-transformations were first developed [18].

Much research has been done in steroid chemistry to find ways to synthesize important components and cortisones in particular [19]. Very often, an early step in these transformations is a site- and stereo specific hydroxylation of the steroid core. Using traditional chemistry to introduce hydroxyl functionalities is difficult, since a typical steroid presents 19 to 29 carbon atoms for attack. From the beginning of the 1950s this problem has been approached by using the high specificity of enzymes in microorganisms [19].

A significant break-through was the process for 11α-hydroxylation developed in the beginning of the 1950s. To date, several different reactions (e.g., hydroxylation) involving the various positions on the steroid skeleton can be achieved using microbial cultures [19]. The main advantage of using microbial hydroxylation is that the enzymes involved are both regio- and stereo-specific. Using traditional synthetic methods, production is complicated by the existence of several easily hydroxylated carbons, which may accept the OH-group through either α- or β- attachment. In addition, the

likelihood of introducing more than one hydroxyl per steroid skeleton is high.

The steroid used as substrate in **paper I** and **II** was progesterone (PS) and the enzyme used to produce 9α-hydroxyprogesterone (9α-OH-PS) was steroid 9α-hydroxylase. The hydroxylation is shown in Figure 1.



Progesterone 9α-hydroxyprogesterone

Figure 1. Structures of substrate and product. Position 9 and 11 is marked in the figure.

2.2 Bioprocess Monitoring

2.2.1 Traditional Bioprocess Monitoring

The rapid growth of biotechnological research the last decades has emphasized the need for proper analytical techniques for bioprocess monitoring. Rapid identification and quantification of essential components in biotechnological processes is essential for process development and optimization. It has been demonstrated that improved monitoring will result in better control and thus improved productivity [20]. While most bioprocesses still are only monitored by the measurement of traditional parameters, such as CO_2, pH and O_2, there is an increasing need to also follow the concentrations of fermentation substrates and products [21].

The control of biotechnological processes can be specified in three groups:

I Off-line control with distance: a sample takes out from the process and a central laboratory carry out the analysis. This is often the case when the investment and/or running costs of expensive equipments have impact on the technology, such as HPLC, gas chromatography (GC) or MS, etc. The response time is often 24-48 hours, which give opportunity only for subsequent measures.

II Off -line control by local equipment: also here the analysis is made on a sample, which is taken out from the process, but the response time can be much lesser, which ensure more reliable adjustments on the process. The advantage compared to the distance control is obvious. The analysis methods can be expensive and complicated, but it can be as simple as thin layer chromatography (TLC) [22, 23]. Chromatographic methods such as HPLC and GC have been commonly used as off line methods for bioprocess monitoring [24, 25] and HPLC is described in section 2.5.

III On-line control: the samples are taken automatically from the process and the measurement carried out immediately. The computer calculates the results and the computer make the necessary decision. Depending on the time requirement of the measurement compared to the rates of the process (growth rate, product formation rate), the control can be termed as real-time control. From the process control point of view this is the most desirable approach. However, it is hampered by the fact that it requires sterilized equipment. Furthermore, the recalibration of the equipment is limited. To take automatically sterile samples from processes is not always reliable.

Most of the time the industry processes are using off line methods. However, there are some trials to couple HPLC, GC, MS or biosensors on-line to the processes. One commonly used method in analyzing fermentations has been flow injection analysis; (FIA). If desired, in FIA the sample can be mixed with reagents (e.g., enzymes) and the reaction can take place during the flow. It can be characterized by high sampling frequency, low reagent consumption, and it is simple and inexpensive [25, 21], but these features are depending on the reaction used. One major drawback with FIA is that it is rare that the product can be detected with sufficient selectivity and sensitivity, the product is instead quantified by the detection of co-substrates or co-products, e.g., the consumption of O_2 or the formation of H_2O_2 [24]. When the reaction is based on an enzyme reaction (most of them does), the stability of the enzyme is often cause difficulties. Furthermore, without selectivity compared to chromatography only one analyte can be analyzed at a time.

It is possible to circumvent the drawbacks caused by the sample treatment by using non-invasive method, such as near infrared spectroscopy (NIR) [26]. However, this and such methods are generating indirect information from the process with a lot of noises. The evaluation of such methods is difficult and seldom reliable.

2.2.2 New Approaches in Bioprocess Monitoring

Mass spectrometry (MS) is one sophisticated technique that has been applied relatively recently for monitoring biotechnological processes, but mainly for the on-line detection and quantification of gases [27], MS is described in section 2.10.2. One drawback with MS is that is requires expensive equipment and is not as easy to handle as HPLC coupled to an UV-detector. Diode-array detectors (DAD) have most recently begins to be to be used for monitoring of the fermentation of wines and ethanol [28-30].

Field flow fraction (FFF) is an elution technique suitable for molecules with a molecular weight > 1000 and large molecules are beyond the scope of this thesis. However, reports on investigated substances with FFF are widespread and cover applications such as the separation and characterization of proteins [31] and enzymes [32], the separation of human and animal cells [33] and the molecular weight and particle size distribution of polymers [34, 35]. FFF is a relatively new approach in biotechnology; therefore practical experiences are not yet abundant.

2.2.3 Product Identification

HPLC-UV diode-array detection (DAD) or HPLC-MS techniques take advantage of chromatography as a separation method and DAD or MS as identification and/or quantification methods. Both DAD and MS can rapidly provide on-line UV and MS information for each individual peak in a chromatogram. In most cases, the identification of the peaks can be made directly on-line by comparison with literature data or with standard compounds. However, when no standard compounds are available, the rapid preliminary identification process becomes significantly more complex. The general identification problem in bioprocess monitoring described below exists in all cases when a new biotechnological process is initiated and when the product is not available as a reference substance.

An important step in the design of a biotechnological production protocol is to produce reference material of the product for further use in the optimization and validation of the process for the pilot- scale (100-300 L) and the production-scale (~m^3) levels. The reference material may be obtained in a lab scale synthesis (~10 L size), which produces a sufficient quantity of material for unambiguous chemical identification by NMR. However, it may be important to have access to a fast and preliminary identification method of the formed product without *a priori* need of reference material combined with a preliminary quantitative monitoring. This is because a fermentation process may be time-consuming requiring a week or more, and it is important already at the initial biotechnological process step to judge whether it should be continued or not. It is clearly of large economical interest to stop the process as quickly as possible if the production gives undesirable results.

Rapid analysis of the products of a biotechnological process at an early stage is essential to clearly distinguish the product peak from other growing peaks. It is a general pattern in biotechnological processes that the composition of the culture medium changes depending on the microbiological metabolism during the fermentation. The highly complex fermentation broth contains a broad range of breakdown products and intermediates. If there is more than one peak growing in the process it is important to be able to identify the desired product among the growing peaks, in order to save time and effort and to avoid unnecessary fraction collections. At this stage there may be no time to purify the significant amounts of product required in order to make a proper NMR identification as may be necessary for final product confirmation.

An adequate route for preliminary product identification, in cases when reference sample is not available, could involve HPLC combined with DAD or MS. The goal of **paper I** was to investigate and compare the use of HPLC-DAD and electrospray ionization (ESI) MS to provide rapid and preliminary identification of the product without any reference material available at an initial stage of a synthesis.

2.2.4 Product Quantification

Rapid quantification of products and substrates in a fermentation process is essential for process development and optimization. Most fermentation laboratories have access to HPLC equipment with possibilities to couple them to quite inexpensive diode-array-detectors, and this equipment could be used for quantitative monitoring of the process. Because HPLC can allow multicomponent analyses, i.e., several analytes in the same sample can be determined virtually simultaneously, and since it is often necessary to monitor more than one substance at a time, this technique is an important tool for bioprocess monitoring. HPLC coupled to expensive MS does not represent standard equipment at fermentation laboratories. Even if mass spectrometers are available, DAD is often sufficient for quantification because product concentrations are relatively high, so the MS could be used for other issues. In **paper II** the goal was to develop and validate a method for analytical quantification of both the product and the substrate to enable the proper characterization of the kinetics of the process; i.e., the determination of the values of substrate conversion and product formation.

2.3 Steroids

Steroids are lipid compounds of major interest to the pharmaceutical industry, due to their high impact in biological systems. They are important regulators that usually give dramatic physiological effects. Examples are male

and female sex hormones, adrenocortical hormones and D vitamins. Since the 1940s corticosteroids have been employed successfully to treat inflammatory diseases and injuries, which has created a great demand for ways to synthesize these compounds. 9α-hydroxy steroids are useful intermediates in the production of a number of pharmaceutically active compounds [36, 37].

2.3.1 Steriod Analysis

Much effort has been made to detect steroids in biological fluids. Even simple TLC methods have been used for qualitative analysis [38]. One method that been used for quantification involves an immunoassay, but several problems exist with that method, most notably cross-reactions and interference with other substances [39]. On the other hand, a number of chromatographic methods have been developed to overcome these problems. The majority of analytical methods involved GC, which has good detection limits, but requires previous derivatization [40] of the steroids to accomplish volatilization. Many methods have also been reported using HPLC with UV detection or LC-MS [40, 41]. Previously used stationary phases for LC was e.g., Sephadex LH-20, Celite and Lipidex, but they could not be operated with high pressure [42]. These columns were therefore slow to run and the separation of steroids was very time-consuming [43]. Nowadays applications mainly use HPLC as a separation method with both normal-phase and reversed-phase chromatography.

2.4 HPLC

A modern HPLC system is shown schematically in Figure 2. The equipment consists of a high-pressure solvent delivery system, a sample auto injector, a separation column, a detector (often an UV or a DAD) a computer to control the system and display results. Many systems include an oven for temperature control of the column and a pre-column that protects the analytical column from impurities. The actual separation takes place in the column, which is packed with chemically modified 3.5-10 μm (often silica) particles. A mobile phase is pumped through the column with the high-pressure pump and the analytes in the injected sample are separated depending on their degree of interaction with the particles. A proper choice of stationary and mobile phase is essential to reach a desired separation.

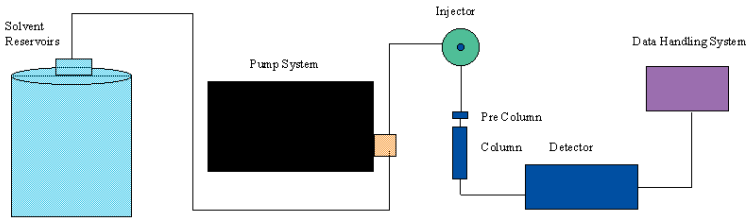


Figure 2. Block diagram of a general LC-system.

The possibility to extract information (both qualitative and quantitative) about several important compounds in one single analytical run, makes HPLC to a strong tool for bioprocess monitoring.

2.5 Method Development

Analytical chemistry deals with methods for determining the chemical composition of samples. A compound can often be measured by several methods. The choice of analytical methodology is based on many considerations, such as: chemical properties of the analyte and its concentration, sample matrix, the speed and cost of the analysis, type of measurements i.e., quantitative or qualitative and the number of samples. A qualitative method yields information of the chemical identity of the species in the sample. A quantitative method provides numerical information regarding the relative amounts of one or more of the species (the analytes) in the sample. Qualitative information is required before a quantitative analysis can be performed. A separation step is usually a necessary part of both a qualitative and a quantitative analysis.

Early in a project the method development should be kept at a minimum and for a small number of samples a tedious work up procedure can be afforded. Further on in a project it may be worthwhile to make efforts towards automation and convenience in the method. Each analytical method contains a number of steps; a simplified flow diagram for an analytical chromatographic method procedure is given in Figure 3. This figure shows chemical analysis as a combination of information and operations with the typical structure: input → process → output.

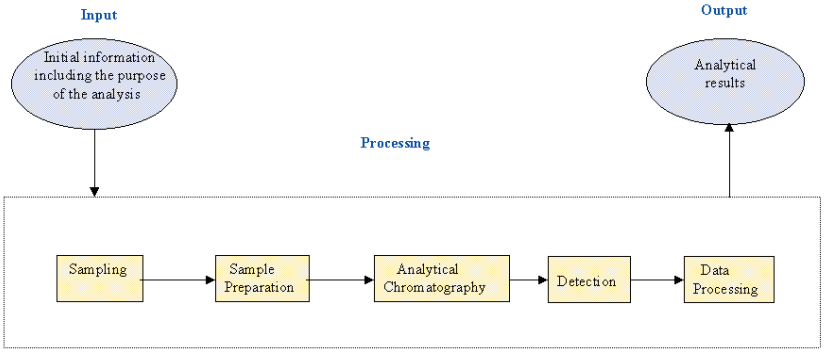


Figure 3. Simplified block diagram of an analytical chromatographic method.

Before beginning the method development, we need to review what is known about the sample; also the goal of the analysis should be defined at this point and considerations must be given regarding how many samples will be analyzed and what HPLC equipment are available. The nature of the sample (e.g., whether it is hydrophilic or hydrophobic, whether it contains protolytic functions etc.) determines the best approach to HPLC method development. The steroids used in **papers I** and **II** are neutral compounds. In **paper I** the desired product was not available as a standard, which made method development more difficult.

Generally, a chemical analysis is performed on only a small fraction of the material whose composition being sought. Obvious the composition of this sampled fraction must reflect as closely as possible the average composition of the bulk of the material if the results are to have value. The process by which a representative fraction is being acquired is termed sampling. Some samples require a pre-treatment prior to HPLC because of the need to remove interferences or to concentrate sample analytes. The sample pre-treatment development can at times be more complex than the HPLC separation itself. The goals of the separation should be specified at the beginning of the method development. It may, for instance be required to resolve all components or it may be required to separate the analytes from impurities and degradation products without the need to further separate these impurities and degradations product from each other. Before the sample is injected during the HPLC method development, the detector should be selected to be sensitive to all sample components of interest. Variable-wavelength ultraviolet detectors are often the first choice, because of their convenience and applicability for most samples. The final procedure should meet all the goals that have been defined at the beginning of the method development and when the HPLC method for quantitative use is finalized it should be validated.

The aim in **paper I** was to develop a method capable of separating the product and the substrate from each other and from degradation products and impurities from the fermentation medium to enable rapid product identification. The aim in **paper II** was to develop a method for accurate quantitative analysis of both products and substrate with high precision. It was also desired that the strategies used in **paper I** and guidelines given in **paper II** should be useful for ordinary fermentation laboratories.

2.6 Qualitative Analysis

To obtain structural information of an analyte and to be able to identify components in unknown samples, qualitative methods are required.

2.6.1 Reference Substance Available

The simplest qualitative analysis involves a comparison of the retention times between a chromatographic peak containing an unknown compound and peaks obtained for reference samples using more than one stationary phase.

2.6.2 Without Reference Substance

Often there is a need for structural identification of unknowns without available reference compounds and the identification can be done in connection with the chromatographic separation. One approach is to run measurements directly on-line using HPLC as the separation technique with UV-detection and monitoring at several wavelengths, but this is often not enough for safety identification. The last 15 years have seen a rapid development of combined liquid chromatography-mass spectrometry instrumentation, and this technique is the most valuable tool in qualitative analysis today (se below). In the absence of a reference compound some unknown substances e.g., isomers of the desired compound may require NMR for their definitive identification.

2.7 Quantitative Analysis

In quantitive analysis the goal is to determine the exact amount of analyte molecules in a sample. Most often two different analytes of equal concentration give different detector responses in chromatography, therefore the detector responses must be measured for known concentrations of each analyte. A standard curve is a graph showing the detector response as a function of the analyte concentration in the sample. For quantification analysis, three

methods of calibration are common: (i) *external standard calibration* (ii) *the internal standard method* and (iii) *the standard addition method* [44].

The external standard calibration method is a simple but less precise method and should only be used when the sample preparation is simple and small or no instrumental variations are observed. The method is not suitable for use with complicated matrices but is often used in pharmaceutical product analysis characterized by simple matrices and easy sample preparation. To construct a standard curve, standard solutions containing known concentrations of the analyte must be prepared and a fixed volume injected into the column. The resulting areas or heights of the peaks in the chromatogram are measured and plotted versus the amount injected. Unknown samples are then prepared, injected and analyzed in exactly the same manner, and their concentrations are determined from the calibration plot. The term "external standard calibration" implies that the standards are analyzed in chromatographic runs that are separate from those of the unknown sample.

The internal standard (I.S.) method is a more accurate method. The I.S. technique can compensate for both instrumental and sample preparation errors and variations (e.g., dilution and extraction) [45, 46]. Sample pretreatment steps such as extraction often result in sample losses, and a proper I.S. standard should be chosen to mimic the variations in these steps. Thus, both the accuracy and precision of quantitative data increase if an I.S. is included in the procedure. The I.S. should be similar but not identical to the analyte, and the two should be well resolved in the chromatographic step. The standard curves are obtained from standards of blank samples spiked with different known concentrations of the analyte of interest and addition of an I.S. at constant concentration. Also to the unknown samples the same constant concentration of the I.S. is added. The standard samples are processed in parallel with the unknown samples. In the calibration curve, the ratios of analyte to I.S. peak area (or height) are plotted versus the concentration of the analyte. A proper I.S. in a bioanalytical chromatographic method should fulfill the following requirements [44]:

- Be well resolved from the compound of interest and other peaks
- Not be present in the sample
- Be similar in retention to the analyte
- Be available in high purity (not contaminated with the analyte)
- Be stable
- Should resemble the analyte in all sample preparation steps
- Be of similar structure as the analyte
- Be of similar concentration as the analyte

Most often a compound with similar structure is selected. The internal standard method has become a very popular technique not only in chromatography, but also in quantitative HPLC-MS methods [47].

During the method development in **paper II** we desired to perform quantification of both the fermentation product and the substrate. Both the external and the internal standard method were evaluated, respectively. The internal standard method was chosen since it resulted in a higher precision. Initially, structurally related compounds were tested as I.S. in the quantitative part described in **paper II**, according to the above I.S.-protocol for bioanalytical approaches. However for biotechnological analysis no recommendations exist for the selection of internal standard. Thus in **paper II** an investigation was performed to see if the I.S.-protocol for bioanalytical methods could be applied also for biotechnological analysis.

The standard addition method is often used in cases when it is not possible to obtain suitable blank matrices; one example is the analysis of endogenous compounds in body fluids. The approach is to add different weights of analyte to the unknown sample, which initially contains an unknown concentration of the analyte. After the chromatographic analysis, peak areas (or heights) are plotted versus the added concentration. Extrapolation of the calibration plot provides the original unknown concentration of the analyte. A standard addition method that possesses even greater accuracy and precision is obtained if one incorporates an internal standard [48].

2.8 Sample Preparation

The purpose of sample preparation is to create a processed sample that leads to better analytical results compared with the initial sample. The prepared sample should be an aliquot relatively free of interferences that is compatible with the HPLC method and that will not damage the column. The whole advanced analytical process can be wasted if an unsuitable preparation method has been employed before the sample reaches the chromatograph. Specifically, analytical work with samples from fermentation processes require a sample pre-treatment that eliminates the fermentation broth before the analytes can be injected into the chromatographic columns. This is primarily to remove macromolecular sample constituents, which easily clog the columns. Complex matrices often require a more selective sample preparation than for instance pharmaceutical solutions. In practice the choice of sample-preparation procedure is dependent on both the nature and size of the sample and on the selectivity of the separation and detection systems employed. Sample pre-treatment may includes a large number of methodologies. Ideally, sample preparation techniques should be fast, easy to use and inexpensive. In **papers I** and **II** careful sample pre-treatment was performed before all injections.

2.8.1 Extraction

The main sample-preparation techniques are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). In these methods the analyst aims to separate the analyte of interest from sample matrix, so that as few potentially interfering species as possible are carried through to the analytical separation stage.

LLE of an analyte is based on its partition between an aqueous phase and an immiscible organic phase [48]. The distribution of the analyte between the two phases is affected by the pH and ionic strength of the aqueous solution, and also of the type of the organic solvent used. By adjusting the pH of the aqueous phase (for proteolytic analytes), the analyte may be extracted into the organic phase with good recovery. The distribution ratio can be increased by proper choice of the organic solvent. The volume ratio between the aqueous phase and the organic phase also affects the recovery. Hansch and coworkers have presented a method for prediction of distribution constants [49, 50] and the method gives fairly good estimates [51]. LLE is a well-established purification method for bioseparations [52]. LLE has recently been developed both into a semi-automated [53-55], and also into a fully automated method for preparing samples with the help of a 96-well LLE plate and a 96-channel robotic liquid-handling workstation [56] which significantly reduces the sample preparation time and increases sample throughput.

Solid-phase extraction (SPE) is an alternative to LLE. In SPE the analytes are partioned between a solid and a liquid [57, 58]. Generally, interfering compounds are rinsed off the solid adsorbent and the analytes are then desorbed with an eluting solvent [58]. A range (e.g., normal-phase, reversed-phase, ion exchange, restricted access) of sorbents and formats are available for SPE and the SPE systems are easy to automate [59, 60]. In order to accomplish the isolation of the products from the fermentation matrix, both SPE and LLE were evaluated for use in **papers I** and **II**.

2.9 Separation

Analytes in a mixture should preferably be separated prior to detection. Chromatography in different forms is today the leading analytical method for separation of components in a mixture. The chromatographic procedure for the separation of substances is based on differences in rates of migration through the column arising from different partition of the compounds between a stationary phase (column packing) and a mobile phase transported through the system. Chromatographic methods can be classified according to the physical state of the mobile phase into the following basic categories: gas chromatography (GC), supercritical fluid chromatography (SFC) and liquid

chromatography (LC). The stationary phases can be of widely different character and their main properties are often the basics for further classification of the methods.

LC is today the main tool for analysis of various substances in different matrices. LC can be categorized on the basis of the mechanism of interaction of the solute with the stationary phase as: adsorption chromatography (liquid-solid chromatography), partition chromatography (liquid-liquid chromatography), ion-exchange chromatography (IEC), size exclusion chromatography (SEC) and affinity chromatography.

Early work in liquid chromatography was based on highly polar stationary phases, and nonpolar solvents served as mobile phases. For historic reasons, this type of chromatography is now referred to normal-phase liquid chromatography (NPLC) [61]. Chromatography on bare silica is an example of normal-phase chromatography. In reversed-phase liquid chromatography (RPLC), the stationary phase is nonpolar, often a hydrocarbon, and the mobile phase is relatively polar [62]. In RPLC, the most polar component is eluted first, because it is relatively most soluble in the mobile phase. RP is the most common LC mode today [62]. The column can consist of many different kinds of stationary phases; in RPLC the most frequently used is octadecyl-bonded silica (ODS) (often referred to as C_{18}) or only an octyl-bonded silica (referred as C_8). The mechanism by which these surfaces retain solute molecules is not entirely clear [62-65]. Retention of solutes in LC systems is a very complicated process, affected by various different types of intermolecular interactions between the molecules of solute, and of stationary and mobile phase [66]. Some scientists believe that the mechanism of adsorption and partitioning are the two principal mechanisms that determine the retention for a compound passing through a C_{18} column [66].

Figure 4 shows schematically how the two substances A and B are chromatographically separated on a column. A single portion of the sample mixture dissolved in the mobile phase is injected at the top of the column packed with solid particles and filled with mobile phase. The components *A* and *B* distribute themselves between the two phases. The continuous flow of mobile phase down the column forces a part of the sample down the column where further distribution between the mobile phase and the stationary phase occurs. If solute *B* is more strongly distributed than solute *A* to the stationary phase, then solute *B* spend a smaller fraction of the time in the flowing mobile phase, so that solute *B* moves down the column more slowly than solute *A*. Then solute *B* elutes from the column after solute *A* and a chromatographic separation of the mixture has occurred. If a detector is placed after the column that responds to the presence of analyte and its signal is plotted versus time, a plot called “chromatogram” is achieved.

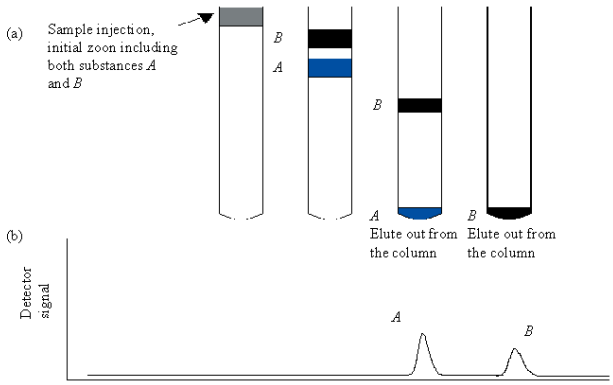


Figure 4. (a) Illustration of the chromatographic separation of a mixture of components *A* and *B* in a column. **(b)** The chromatogram of the separation of the mixtures.

Successful chromatography requires a proper balance of intermolecular forces between the three active parts in the separation process, the solute, the mobile phase, and the stationary phase; the polarities for these three parts should be carefully blended for a good separation to be realized in a reasonable time.

Commercial columns can differ widely among suppliers; different columns can vary in plate number, band symmetry, and retention. In **paper I**, an investigation of a proper HPLC column for separation of different steroids was investigated. After finding the best column the mobile phase must be optimized. The mobile phase composition is readily changed in HPLC separations and the retention is preferably adjusted by changing mobile phase composition or solvent strength. In **paper II** two different organic solvents were evaluated as modifiers in the mobile phase for the separation of a set of steroids on a C_{18} stationary phase.

2.10 Detection

There exist several different detectors suitable for detecting the analytes after the chromatographic separation. Some commercial detectors used in LC are: ultraviolet (UV) detectors, fluorescence detektors, electrochemical detectors, refractive index (RI) detectors and mass spectrometry (MS) detectors. The choice of detector depends on the sample and the purpose of the analysis.

2.10.1 UV and Diode-Array Detection

The UV detectors are the most common HPLC detectors since they are robust, cheap, easy to handle, and since many solutes absorb light in this frequency range. The ordinary UV detector measures the absorbance at one

single wavelength at the time. To change wavelength the monochromator must be moved, thereby introducing the problems of mechanical irreproducibility into the measurements A diode-array detector (DAD) can measure several wavelengths at the same time, and since no parts are moved to change wavelength or to scan, there are no mechanical errors or drift with time. Figure 5 illustrates the difference in operational mode between an ordinary single wavelength detector and a multi-wavelength detector.

Following one chromatographic run with a DAD enables the operator to display a chromatogram for any desired wavelength (usually between 190 and 400 nm), and in addition the UV spectra of each eluting peak can be displayed. DADs therefore provide more information on sample composition than is provided by a single wavelength run. While single wavelengths detectors are commonly used for quantitative analysis, a DAD may be used both for quantitative and qualitative information of the samples. If the peaks in a chromatogram are resolved, UV spectra can be collected for each peak with help of a DAD, and peak identification and peak purity can be carried out with the recorded signals. Peak identification is made by comparing the UV spectra for a standard with a sample peak by overlapping the two spectra. Even if a standard is not available it can reveal if a components represented by a peak in a chromatogram is related to another compound, e.g. a small chemical modification resulting from a fermentation process, a degradation product or a metabolite. This can be done because the software of the DAD instrument often has a tool to compare acquired spectra and describe their degree of overlap with a so-called matching factor. In **paper I** HPLC-DAD was used for fast preliminary qualitative analysis of the fermentation process.

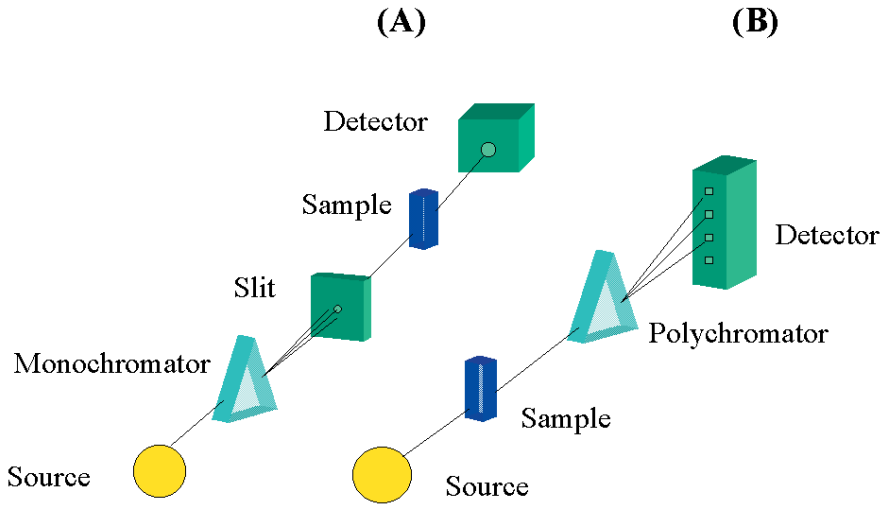


Figure 5. Simplified diagram of: **(A)** a conventional single wavelength detector and **(B)** a multi wavelength detector.

The modern DADs are suitable for numerous different kinds of applications. DADs are frequently used in the production control of drugs where rapid screening and identification of unknown impurities is necessary. This is because of the possibility to match an unknown high resolution UV spectrum with spectra corresponding to known components in libraries. DAD detectors have been proposed for various applications, such as preliminary identification of a steroidal glycoside in seed [67], peptide mapping [68], assay of sulfamethazine in animal tissues [69], or identification of pesticides in human biological fluids [14]. After an literature search, only one paper was found describing the use of DAD in monitoring a biotransformation until the late 1990s [71]. A method reported by analytical chemists. During the last five years HPLC-DAD has begun to be used for the fermentation of wines and production of ethanol [28-30]. The main advantage of DADs is the rapid availability of product information, but the main limitation for identification purposes is the general lack of detailed structural information provided by a UV-spectrum compared with a mass- or NMR-spectrum.

2.10.2 Mass Spectrometry

Mass spectrometry (MS) is a widely used detection technique that provides quantitative and qualitative information about the components in a mixture. In qualitative analysis it is very important to determine the molecular weight

of an unknown compound and MS is a technique capable of that. MS is also generally more sensitive than an UV-detector for quantification.

An MS detector consists of three main parts: the ionization source (interface) where the ions are generated, the mass analyzer (separation), which separates the ions according to their mass-to-charge ration (m/z), and the electron multiplier (detector). There are several types of ion sources, which utilize different ionization techniques for creating charged species.

Three popular ionization techniques are: electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and matrix-assisted laser desorption (MALDI). Electrospray is the most widely used ionization technique when performing LC-MS, and has proved to be a most versatile tool for soft ionization [72] of a large variety of analytes such as them described in **paper I**. Figure 6 shows the principle of the ESI.

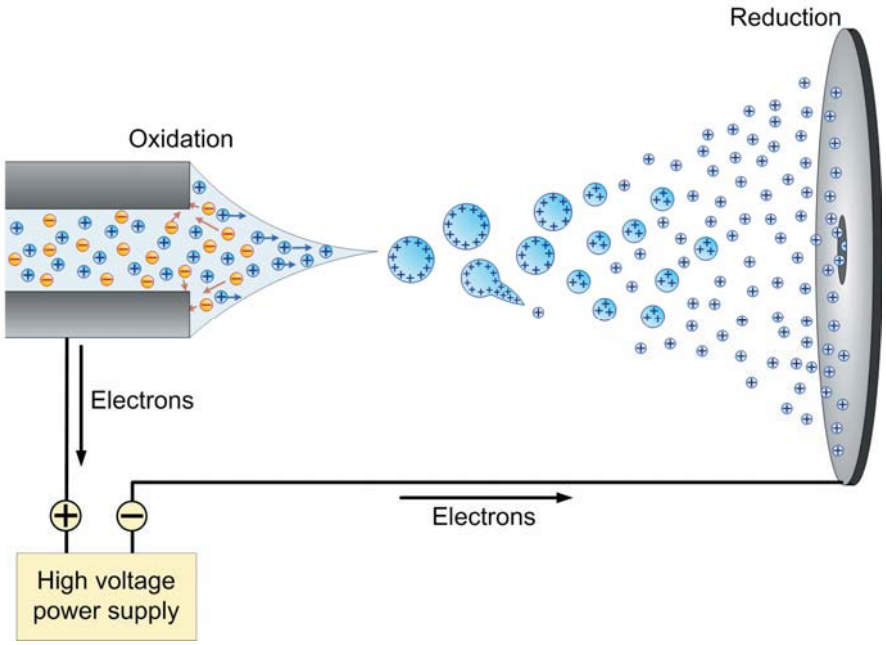


Fig 6. Illustration of the electrospray process in positive ion mode. The picture was kindly provided by Andreas Pettersson, Depertment of Analytical Chemistry, Uppsala University.

In ESI the formation of ions from the liquid to the gas-phase is achieved by applying an electric field over the liquid phase to create charged droplets. The solvent evaporation decreases the size of the droplets while the charge remains constant, thus the charge to volume ratio increases, and will eventually form gas-phase ions [73]. The formation of gas-phase ions from the very small droplets is still not definitely clear [74, 75], but the gas formation has little if any influence on the use of ESI with LC-MS.

Common mass analyzers that are commercially available are: quadrupole, ion-trap, time-of-flight (TOF) and magnetic sector analyzers. In **paper I** an ion-trap mass analyzer was used. MS is an excellent technique for identification purposes in all areas of chemistry, biochemistry and biotechnology. Ion trap MS is especially useful for advanced qualitative analysis due to the ability of the technique to provide scans up to the MS^5 range. The power of an ion trap MS stems from the fact that several generations of daughter ions can be generated providing MS^n spectra. This capability provides a rich source of structural information. A common mass spectrometric approach for identification is to compare the first and second-generation product ion spectra of known reference compounds with the spectra obtained for the unknown sample. In **paper I** up to MS^5 spectra were investigated for different steroids. Because the product 9α-OH-PS was not available as a reference substance, 11α-hydroxyprogesterone (11α-OH-PS) was used as a qualitative reference whose MS spectra showed convincing similarities (but not identical) with the spectra for our fermentation-derived product.

2.11 NMR

Nuclear magnetic resonance (NMR) spectroscopy is a very powerful tool for chemists to determine the structure of compounds. This nondestructive spectroscopic analysis can reveal the number of atoms (generally carbons and protons) and their connectivity's, and thus the conformations of the molecules [76]. The necessary information to determine the structures of the molecules is obtained by measuring, analyzing and interpreting NMR spectra recorded on liquids. Protons provide the highest sensitivity for NMR observations; therefore the acquisition of proton spectra can be accomplished with quantities in the range of 1-10 mg. Carbon NMR has much lower sensitivity and about of 20 mg sample may be required [77] for this type of analysis. Such quantities are not always available following a laboratory scale synthesis in biotechnological synthesis.

2.12 Validation

When a method has been developed it is important to validate it to confirm that it is suitable for its intended purpose. The validation tells how good the methods are, specifically whether it is good enough for the intended application. The method validation is today an essential concern in the activity of analytical chemistry laboratories. It is already well implemented in pharmaceutical industry. However, in other fields like food, petrol chemistry or in the biotechnological field, regulations have not reached such a level of requirement. The US Food and Drug Administration (FDA) have edited draft

guidelines with detailed recommendations for method validation of bioanalytical methods [16] in the pharmaceutical industry. The International Conference on Harmonisation (ICH) has provided definitions of validation issues included in "analytical procedures" for the fields of bioanalytical methodology, pharmaceutical and biotechnological procedures [78-80]. Likewise the US Pharmacopeia (USP) has published guidelines for method validation for analytical methods for pharmaceutical products [81]. However the guidelines from ICH and USP are not as detailed as those from the FDA, and in the analytical biotechnology area there exists no detailed validation guidelines. The most common validation parameters will be briefly described below.

2.12.1 Precision and Accuracy.

The precision of an analytical method is the closeness of a series of individual measurements of an analyte when the analytical procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix [16]. The precision is calculated as coefficient of variation (C.V.), i.e., relative standard deviation (RSD). The measured RSD can be subdivided into three categories: repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (between laboratories precision) [16, 78, 79, 81].

Repeatability is obtained when the analysis is performed in one laboratory by one analyst using the same equipment at the same day. Repeatability should be tested by the analysis of a minimum of five determinations at three different concentrations (low, medium and high) in the range of expected concentrations, according to FDA [16]. However according to the ICH [79] repeatability could be measured by the analysis of three determinations at three different concentrations or through six determinations at 100 % of the test concentration. The latter one is for analysis when the concentration is supposed to be constant for all samples, e.g., pharmaceutical products. The acceptance criteria for precision depends much on the type of analysis. For compound analysis in pharmaceutical quality control, precision should be better than 2 % [82]. For bioanalytical applications the precision values at each concentration level should be better than 15 % except for the lower limit of quantification (LLOQ) where is should not exceed 20 % [16]. The intermediate precision shows the variations affected in day-to-day analysis, by different analysts, different instruments etc. Reproducibility, as above, represents the precision obtained between different laboratories.

The accuracy of an analytical method is the degree of closeness between the true value of analytes in the sample and the value determined by the method and is sometimes called trueness [78]. Accuracy can be measured by analyzing samples with known concentrations and comparing the measured values with the true values. According to FDA [16] the accuracy for bioanalysis should be determined by a minimum of five determinations for at least

three concentrations (low, medium and high) in the range of expected concentrations. The mean value should be within 15 % from the true value except for the LLOQ where is should be within 20 % [16]. Both precision and accuracy can be calculated from the same analytical experiments. In **paper II** both the intra-day and the inter-day precision and accuracy were obtained for six replicates of 9α-OH-PS, which were analyzed at three different days at three different concentrations.

2.12.2 Selectivity/Specificity

Sometimes there is some confusion over which term that should be used in characterizing a method, selectivity or specificity. Vessman [83] pointed out the differences between the two terms. Selectivity refers to a method that gives responses for a number of substances and can distinguish the analyte(s) response from all other responses. Specificity refers to a method that gives response for only one single analyte. In chromatography with UV-detectors it is unusual that a method responds only to one analyte and therefore the term selectivity is appropriate. The selectivity of the method should be evaluated by processing blank samples with and without the addition of analytes and inject them to test for interferences. The selectivity of the method is very important to enable accurate analyte quantification.

In **paper II**, the selectivity of the chosen system was investigated by comparing chromatograms of pure matrix solution with matrix solution to which analytes had been added.

2.12.3 Limit of Detection and Quantification

A clear distinction should be made between the limit of detection, LOD, and the lower limit of quantification, LLOQ. The LOD is defined as the concentration of analyte that results in a peak height three times the noise when injected into the chromatographic system, i.e., the point at which a measured value is greater than the uncertainty associated with it. LOD is the lowest concentration of the analyte in a sample that can be detected but not necessarily quantified. The LLOQ is the lowest concentration of the analyte in a sample that can be quantified with acceptable accuracy and precision. The LLOQ should have an accuracy of 80-120 % and a precision of maximum 20 % [16]. Both the LLOQ and LOD were investigated in **paper II**.

2.12.4 Linearity and Range

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in a sample within a given range [81]. Linearity should be evaluated in the concentration span 80-120 % of the expected con-

centration range. The calibration standards should be prepared in the same matrix as the samples in the intended study [16]. The calibration curve should consist of six to eight non-zero standard samples [16]. The blank sample (matrix sampled processed without internal standard) and the zero sample (matrix sample processed with internal standard) should not be used in the calibration curve. Standard samples prepared independently make it easier to discover an error in any of the samples compared to if the standards were prepared by a serial dilution. A serial dilution gives better correlation coefficient but could give a false regression coefficient if an error has occurred at the preparation of the highest standard sample. An acceptable standard curve with a linear regression equation should have an intercept not significantly different from zero. The linear correlation coefficient (r) should in bioanalysis be 0.95 or greater accordingly to FDA [16], but according to Causey et al. [84] it is generally agreed that it should exceed 0.99.

According to Bildlingmeyer [85], a good linear correlation coefficient alone does not necessarily indicate a linear standard curve, because the standards in the lowest range can deviate from the linearity although the r is high. Instead the linear coefficient should be accompanied with a graph were the response/sample concentration is plotted versus the logarithmic sample concentrations and the deviation in the y-axes should not exceed 5% [85]. The range of an analytical method is the concentration interval that has been validated according to accuracy, precision and linearity as described above. In **paper II** the linearity was evaluated by both the r-values and with the logarithmic linearity plot approach.

2.12.5 Recovery

High recovery of the analyte(s) from the matrix is a desirable outcome of sample preparation, and is therefore an important characteristic of the extraction procedure. The absolute recovery is the ratio of the response measured for a spiked sample (in matrix) treated according to the whole analytical procedure to that of a non-biological sample spiked (in aqueous solution) with the same quantity of the analyte substance and directly injected into the chromatographic system [81]. The relative recovery is the ratio of the responses between extracted spiked samples (in matrix) and extracted spiked pure samples (in aqueous solution). The relative recovery can be used, together with the absolute recovery, to reveal whether sample losses in the extraction are due to matrix effects or to bad extraction.

The recovery should be determined by a minimum of six determinations for at least three concentrations (low, medium and high) in the range of expected concentrations. The absolute recovery should preferably exceed 90 % and the relative recovery 95 %. However, sometimes it may be necessary to sacrifice high recovery in order to achieve better selectivity, but it is important that the sensitivity, precision and accuracy still are acceptable [86]. The

internal standard should have a similar recovery as the analyte(s), and it should be within 15 % of that determined for the analyte [86]. Both the absolute and relative recoveries were investigated for the product, substrate and internal standard in different fermentation media after different time intervals in **paper II**. Also the hypothesis that ultrasonication of samples should increase the recovery was investigated.

2.12.6 Stability

The stability of the analyte under various conditions should also be studied in method validation. Degradation is not unusual, it is therefore important to verify that the analyte is stable between the collection of a set of samples and their analysis. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis [16]. The following five stability conditions are advisable to investigate for the analytes and the I.S.:

- Short-Term Temperature Stability
  The stability of the analyte in matrix at ambient temperature should be evaluated. At least three aliquots of each of the low and high concentration should be investigated [16], and it is sometimes called the "benchtop stabilliy".

- Post-Preparative Stability
  The stability of the analyte in the final extract during the expected maximum analysis time, which for automatic injections can be up to 48 h, should be assessed.

- Freeze and Thaw Stability
  The stability of the analyte after at least three thaw-and-freeze cycles should be determined.

- Long-Term Stability
  The stability of frozen samples prior to the analysis should be evaluated for a time period that exceeds the time between the sample collection and the last sample analysis.

- Stock Solution Stability
  The stability of the stock solution of the standard should be evaluated at room temperature.

In **paper II** the stability of the stock solutions of PS, 9α-OH-PS, 11α OH-PS and binaphtol were investigated; also the short-term temperature stability,

post-preparative stability and long term stability were investigated for the product and the internal standard, binaphtol.

2.12.7 Robustness

Robustness tests examine the effect that small changes in operational parameters have on the analysis results. The robustness is not mentioned in the FDA guidelines [16] and is not considered in most validation guidelines. However, for methods that are used for a long time period and in different laboratories, testing of the robustness is important. Only effects of small changes in the experimental conditions need to be tested. Some parameters that could be tested are slight changes in the pH of the buffer, in the concentration of the organic modifier in the mobile phase, in ambient temperature and in the detection wavelength [87, 88].

2.12.8 Quality Control

When the analytical method has been validated for routine use, its accuracy and precision should be controlled regularly to ensure that the method continues to work satisfactorily. For this purpose, a number of separately prepared (from different weightings than the ones used for the standard curve) quality control (QC) samples should be analyzed in each run [16]. The QC samples are often duplicates at three concentrations (low, medium and high) within the range. At least four of the six QC samples should be within 20% of there respectively nominal value, and at least one at each concentration level [16, 89]. Also a standard curve should be processed during each run [89].

2.12.9 Validation of Biotechnological Synthesis.

The suggested tolerances for the validation parameters in the FDA recommendations for bioanalytical methods [16] are rather wide, with C.V. values < 15%. For pharmaceutical product analysis the requirements are tighter with C.V. values < 2% [82]. This is possible, since in the latter case the matrix is typically much simpler and the analyte concentration can be chosen freely, so that extremely low/high concentrations can be avoided.

There are no detailed recommendations for analytical procedures in the field of biotechnological production of drugs, in contrast to the recommendations made by the FDA for bioanalytical methods [16]. The absence of detailed guidelines for analytical method validation in this field is surprising. It is important to validate the quantification of both substrate and product during the process, at definite time intervals, to ensure proper calculation of the kinetics of the process; i.e., the coefficients of substrate conversion, and production rates must be adequately calculated. The aim of the study in **pa-**

per II was to investigate whether the detailed guidelines and validation rules given by the FDA for bioanalytical methods can be used also in the field of biotechnological synthesis and, if not, to determine what modifications would be required.

3 Determination of Adsorption Isotherm Parameters

In this section a short overview is given of preparative chromatography and the determination of adsorption isotherm parameters - single and competitive - to be used for computer-assisted optimization of separations.

3.1 Preparative Chromatography

Preparative chromatography has become an increasingly important separation process for the purification and isolation of pure substances in the pharmaceutical industry, in biotechnology and in the production of fine chemicals [90]. The use of preparative chromatography is not surprising since, by its very nature, chromatography seems to be an ideal tool for purification and collection of fractions [91]. In many cases, the required purity criteria can only be achieved with chromatographic techniques, e.g., in the resolution of many racemates [90]. The pharmaceutical industry is not free to adjust the specifications (e.g., purity) of its products to achieve maximum profit; these specifications must be accepted by the regulatory agencies, e.g., the FDA [92]. In many cases, preparative chromatography is the method needed to satisfy the purity specifications required [92].

There are two main areas in which preparative chromatography is used [13]. The first area is to obtain purified components in order to collect data and analyze them further; the second area is the production of the final material [13]. In the first area, the purified chemicals are needed as intermediates in the process generating the desired information. This is the case when relatively small amounts of material are needed for identification and characterization, like the case in **paper II**, or for the acquisition of toxicological or pharmacological data. Time is important in such cases and very little time is available for optimization [13]. In such cases the time spent by the person developing and executing the separation is the essential component of cost, and that time should be minimized. The contribution to the cost from solvents, stationary phases, chemicals and instruments will be small in comparison, due to the small amounts required. In such cases, there is no time available nor much purpose for isotherm measurements and modeling [13]. In the second area, a purified compound is needed to obtain a final product,

and the cost of the production of the compound is the important factor, which have to be minimized. The production process will last a long period of time, whether it is run continuously or periodically in batch mode, and the operation is in comparison a routine procedure [13]. The cost of components, equipment, solvent, packing material and downstream processing become very important and must be taken into account. In these cases a significant investment is required to design the separation process through careful optimization of the experimental conditions.

3.1.1 Nonlinear Chromatography

In linear chromatography the adsorption isotherm is linear and the amount adsorbed on the stationary phase at equilibrium is proportional to the solute concentration in the mobile phase. Thus, the adsorption isotherm is a straight line starting out at the origin [13] and the retention time and individual band shapes are independent of the sample composition and amount. In analytical chromatography linear conditions often prevail.

In nonlinear chromatography the concentration of a component in the stationary phase at equilibrium is no longer proportional to its concentration in the mobile phase [13]. Under such conditions, the adsorption isotherms also depend on the concentrations of all other components in the sample mixture. Thus, the retention time, peak height and band shape depend both on the sample composition and amount [13]. This is the situation found in practically all preparative applications [13]. Nonlinear chromatography is extremely complex due to the interdependence of the individual band profiles that are caused by the dependence of the amount of any component adsorbed on the stationary phase on all the components in solution [13].

Preparative chromatography is generally carried out under mass and/or volume overloaded conditions in order to increase the product throughput. In volume overloading, the sample concentrations are maintained in the linear region of the isotherm and the volume is increased until the throughput is optimized [93]. A fundamental problem with this technique is the underutilization of the column and the corresponding low throughputs. In mass overloading, the sample concentration is increased beyond the linear adsorption region [93] resulting in asymmetric band profiles with self-sharpening fronts and tailing rear boundaries for the most common adsorption isotherm type, the “Langmuirian” type, see 3.2.3. A combination of volume and mass overloading is normally employed to maximize throughput in preparative chromatography [13]. Chromatography on large scale consumes large volumes of expensive solvents, which is one of several reasons why optimization of the process is essential.

3.1.2 Simulated Moving Bed

When amounts from a few mg up to about 100 g of some pure substance are needed, batch chromatography is usually employed. When larger amounts are needed (several hundred grams to kilograms), the simulated moving bed (SMB) process is often a more efficient alternative [94]. Continuous SMB systems are of increasing interest for the purification of pharmaceuticals or specialty chemicals, such as enantiomers and proteins. This is particularly due to the typical advantages of SMB-systems, such as reduction of solvent consumption, increase in the productivity and purity as well as in investment costs in comparison to conventional batch elution chromatography [95]. One disadvantage with SMB-chromatography is that the purification is limited into only two fractions.

An SMB system consists of several identical columns (usually 8-12), which are placed in series, and between each of them four valves are placed which can be individually opened and closed. A recycling pump inside the column circle delivers the mobile phase flow through all columns. Two additional pumps constantly inject the feed and fresh eluent, and two pumps withdraw the raffinate and extract flows.

To operate SMB chromatography a lot of parameters (column diameter, column length, total column number and number of columns per section, eluent, feed, raffinate, extract and recycle fluid flow and switch time interval) have to be chosen correctly. Therefore, design and process optimization should be done by computer simulations. It is much more difficult to optimize SMB during nonlinear conditions as compared to linear conditions. In fact, empirical approaches for optimization during overloaded and non-linear conditions are in most cases even impossible [96, 97]. Computer-assisted optimization is therefore especially important for chiral separations since these CSPs have in general lower saturation capacities compared to non-chiral columns (**see paper III**).

3.1.3 Preparative Chromatography in Biotechnology

Substances produced by biotechnologically methods for therapeutic purposes are subjected to extensive downstream processing in order to assure a high degree of purity. Preparative chromatography is the dominant purification technique in modern biotechnological industries when producing biological compounds, such as high-value proteins from complex mixtures [98]. The development of efficient stationary phase materials with unique selectivity has expanded the scope of large-scale chromatographic bioseparations significantly.

In biotechnology several different preparative chromatographic separation techniques are common, the most common ones being: ion-exchange chro-

matography (IEC), hydrophobic interaction chromatography (HIC), reversed-phase liquid chromatography (RPLC), size exclusion chromatography (SEC), immobilized metal-affinity chromatography (IMAC), lectin-affinity chromatography, biospecific-affinity chromatography and dye-affinity chromatography.

RPLC is the most common technique for small organic molecules. It is also a powerful tool for preparative peptide separations, but is less common for protein purification, because it is often associated with protein denaturation. Nevertheless, there are successful examples of large-scale protein purification by RPLC [99]. In this thesis the substances used and produced in the biotechnological synthesis, in **papers I** and **II**, were small molecules (Mw < 1000) and therefore belong to a sample class for which RPLC is the dominant separation technique.

3.1.4 Preparative Chromatography of Enantiomers

Many pharmaceutically active compounds are chiral in nature. For these it is essential to generate the pair of pure enantiomers since they often exhibit marked differences in biological activity. Thus, the individual enantiomers have to undergo evaluation of their pharmacokinetic, metabolic, physiological and toxicological properties in order to identify which enantiomer is most suitable for development into a therapeutic drug. These data are also required by different regulatory agencies [100, 101]. As single enantiomer chiral synthesis is often more difficult to carry out than making a racemic mixture, therefore it may be easier to develop HPLC methods for isolating the enantiomers from the racemic mixture. The chromatographic separation of enantiomers does not basically differ from the classical methods of chromatography in that it takes advantage of the difference in interaction energies between two different solutes and a stationary phase; for steric reasons enantiomers may interact differently with a chiral surrounding.

Before starting a preparative chiral separation it is essential to identify a chiral stationary phase (CSP) exhibiting good chiral recognition ability. This is usually done with an analytical column because it is less substance- and time-consuming. A stationary phase mostly composed of silica gel with only a few chiral elements will be rapidly overloaded. In this event, even if the phase exhibits useful properties for analytical purposes, it will not be appropriate for preparative applications; this is the case for protein-based phases [102, 103]. Most chiral stationary phases have relatively low saturation capacity, so the enantiomer separations are usually done under strongly nonlinear conditions [103]. Accordingly, the accurate determination of the adsorption isotherms of the two enantiomers on a CSP is of fundamental importance to allow computer-assisted optimization to scale up the process.

At very large scales non-chromatographic methods, such as chiral synthesis, should also be considered. Often the best method involves a combination

of a non-chromatographic method with a chromatographic one. Independent of which non-chromatographic method used; chromatography is often also used to provide "starting building blocks" or for final “polishing”.

3.1.5 Optimization of Preparative Chromatography

Since it is difficult and costly to optimize preparative chromatography empirically, computer-assisted optimization has grown in importance. However, chromatography is still often based on “trial and error” methods, and this approach might be more or less successful for classic elution chromatography. In more sophisticated chromatographic technologies such as the SMB concepts, computer simulations are an important part of the design. In order to simulate preparative separations one mainly need to know the thermodynamic equilibrium between the mobile phase, the stationary phase and the components of the mixture to be separated, i.e., the adsorption isotherm parameters [13, 104-106]. No prediction of column performance can be made without first measuring or estimating adsorption isotherm parameters for the components of interest and the accuracy of the prediction is strongly dependent on the accuracy of this data [107].

By using a computer the calculation of the optimum conditions can be done by using accurate models of nonlinear chromatography, and the competitive adsorption isotherm parameters for the mixtures of interest as well as the efficiency parameter. This allows rapid calculation of the band profiles of each component, the determination of the position of suitable cut points, the production rate, and the recovery yield. By combining the algorithm that performs these tasks with an optimization algorithm it is possible to determine the experimental conditions that maximize the relevant objective function. Finally, the experimental conditions obtained by the computer calculations can be fine-tuned experimentally. By using the step above in the optimization process it is possible to significantly reduce both time and cost [13]. An overloaded chromatogram with cut points is shown in Figure 7. The cut points are the points where fraction collection begins or ends, and the first fraction contains the first purified component (zone A), a mixed fraction follows (zone B), and the third fraction contains the purified second component (zone C). The mixed fraction B is either recycled or sent to waste.

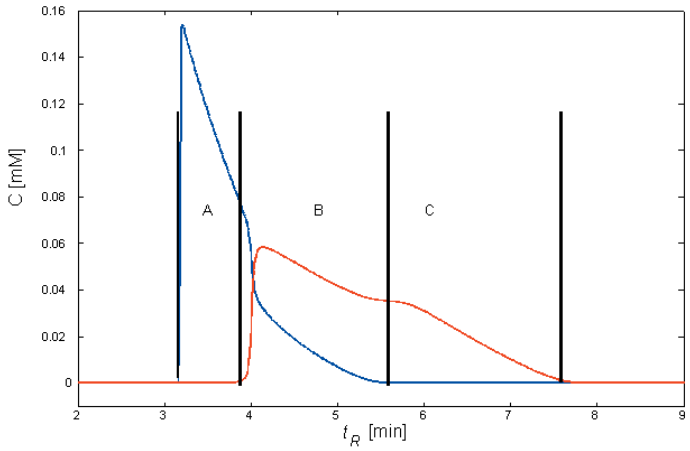


Figure 7. Illustration of cut points and where fractions should be taken in an overloaded chromatogram. Zone A is the first pure component, zone B is the mixed zone, and zone C is the second purified component.

3.2 Adsorption Model

An adsorption isotherm relates, at a constant given temperature, the equilibrium concentrations of a compound in the solid phase (stationary phase) and in the liquid phase (mobile phase or solvent). There are a number of nonlinear isotherm equations [13, 108] and some commonly used are, the Langmuir isotherm, the bi-Langmuir isotherm and the Freundlich isotherm [13]. There exist both single and competitive isotherms. When a single component is present in a solution, and is allowed to be adsorbed on the stationary phase the isotherm is called a single-component isotherm. However, in all cases of practical importance in liquid chromatography we are dealing with multicomponent mixtures [109]. In that case the solid phase concentration of any compound in the solution is a function of all the components also present in the solution (additives and all other sample components) and the isotherms are called competitive isotherms [13, 109].

3.2.1 Competition

Figures 8a and 8b show the simulated elution profiles of two compounds using the simple Langmuir model (eq. 1 below), after injection of 135 µl of a solution (solute 1 = 20 mM, solute 2 = 60 mM). Figure 8a shows the situation when there is no competition between the two solutes. Since competition always exists, such a situation can only be achieved experimentally by overlaying the chromatograms resulting from separate injections. The second

component has a stronger adsorption (a_2 = 10 and b_2 = 200 M^{-1}) than the first one (a_1 = 20; b_1 = 400 M^{-1}). Thus, in Figure 8a we can see that the low concentration region (the rear) of solute 2 is more retained than the rear of solute 1. However, the front of solute 2 strives harder to lower retention than solute 1 does. This is due to the higher sample concentration of solute 2, resulting in a larger degree of overloading. With this particular set of parameters and of sample concentrations, the two solute fronts coelute. If the sample concentration of the stronger adsorbed solute 2 was further increased, its front eluted even earlier than solute 1. In the situation in Figure 8a it would be impossible to collect any pure fraction of solute 1 and less than 50% of solute 2 could be collected. However, in reality there exist always competition between components (injected together). Figure 8b shows a more realistic simulated chromatogram for the same experimental conditions, using the Langmuir model accounting for the competitive effects (eq. 3 below). The band of solute 2 displaces that of solute 1 whose maximum concentration is nearly doubled. The profile of solute 2 changes less than that of solute 1 because its amount is larger and because it competes more strongly ($b_2 > b_1$). It is obvious from Figure 8a-b that the competitive effects can increase the production of pure fractions of both solutes. However, the selection of the best experimental conditions for the utilisation of these effects requires computer-assisted optimisations.

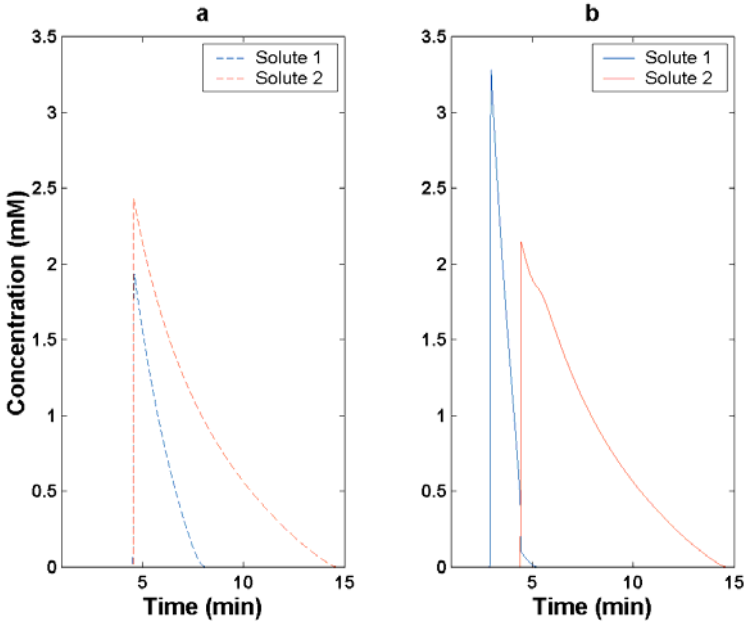


Figure 8. Visualization of elution profiles without and with competition. Profiles simulated **(a)** assuming independence of the isotherms (i.e., no competition) and **(b)** with competitive Langmuir isotherms. The Langmuir adsorption isotherms for the two compounds on a homogenous surface are; a_1 = 10; b_1 = 200 M^{-1}; a_2 = 20; b_2 = 400 M^{-1}; $q_{s,1} = q_{s,2}$ = 0.05 M. Column efficiency is 4000 plates.

3.2.2 General Properties of Chiral Stationary Phases

The surface of the adsorbent in a chiral stationary phase (CSP) often contains two different types of adsorption sites [103, 110, 111], type-I and type-II sites. In most chiral columns only type-II site is chiral selective, but the saturation capacity for most columns is very low resulting in that when the sample size is increased, the selective retention mechanism is rapidly overloaded and the chiral separation disappears. The type-II saturation capacity is also called the "true" chiral saturation capacity.

The type-I sites have, in protein based CSPs, identical behavior toward the two enantiomers, and cannot distinguish between them. Many columns contain mostly type-I sites. On type-I sites all possible molecular interactions, between the analyte molecules and atoms or groups of atoms belonging to the adsorbent surface, take place. These interactions can originate from the nonchiral parts of the protein and/or from the adsorbent (silica) matrix. The energies of each interaction on type-I sites are small. The other type of adsorption sites have, in protein based CSPs, much higher adsorption energy and are enantioselective (chiral). These sites, type-II sites, are responsible for the enantiomeric separations. On most CSPs the type-II sites are relatively few.

3.2.3 The Langmuir Isotherm

The isotherm that is most easily understood theoretically and widely applicable to experimental data is, known as the Langmuir isotherm. This is also the simplest model of a nonlinear isotherm. It accounts well for the adsorption of single components on homogenous surfaces at low to moderate concentrations, or for the adsorption to isolated type-I or type-II sites [103, 110, 111]. The Langmuir adsorption isotherm equation is written:

$$q = \frac{aC}{1 + bC} \tag{1}$$

Here a is the equilibrium or Henry constant at infinite dilution, a is also equal to the initial slope of the adsorption isotherm. The coefficient b is the equilibrium constant per unit of surface area, and hence this coefficient is related to the adsorption energy. C is the mobile phase concentration of the analyte in equilibrium with q, the concentration of the analyte in the stationary phase. The monolayer capacity, q_s ($q_s = a/b$) is the upper limit of concentration in the stationary phase (sometimes called specific saturation capacity of the stationary phase). The Langmuir equation can also be written as:

$$\theta = \frac{q}{q_s} = \frac{bC}{1 + bC} \tag{2}$$

Here θ is called the fractional surface coverage.

3.2.3.1 The Competitive Langmuir Isotherm Model

The Langmuir isotherm model can be extended to multi-component systems [109]. When several components are simultaneously present in a solution, the amount of each component adsorbed at equilibrium is smaller than if that component were alone [13] because the different components compete to be adsorbed on the stationary phase. The adsorption isotherm for the *i*:th component in a multicomponent system is written:

$$q_i = \frac{a_i C_i}{1 + \sum_{j=1}^{n} b_j C_j} \tag{3}$$

Here n is the number of components in the system, coefficients a_i and b_i are the coefficients of the single-component Langmuir adsorption isotherm for component i. The coefficient b_i is the ratio of the rate constants of adsorption and desorption, so it is a thermodynamic constant. The ratio a_i/b_i is the column saturation capacity of component i [13].

3.2.4 The bi-Langmuir Isotherm

In many cases the surface of the adsorbent used for chromatographic separations is not homogenous. The simplest model for a nonhomogeneous surface is a surface covered with two different kinds of adsorption sites or group of adsorption sites, e.g., this is the case in most columns for chiral separations. Here, the Langmuir equation is extended to an equation with two Langmuir terms, called the bi-Langmuir isotherm. The first term representing a large number of non-enantioselective interactions, the so-called type-I sites, and the second term representing a smaller number of enantioselective interactions, the so called type-II sites, these are the "true" chiral sites.

$$q_i = \frac{a_{I,i} C_i}{1 + b_{I,i} C_i} + \frac{a_{II,i} C_i}{1 + b_{II,i} C_i} \tag{4}$$

Here q_i is the solid phase concentration, C_i is the mobile phase concentration and $a_{\mathrm{I,i}}$ $a_{\mathrm{II,i}}$ $b_{\mathrm{I,i}}$ $b_{\mathrm{II,i}}$ are isotherm parameters for the *i*:th component. The column saturation capacity of the chiral site is defined as:

$$q_{s\,II,i} = \frac{a_{II,i}}{b_{II,i}} \tag{5}$$

It has been shown that the bi-Langmuir isotherm is a good model for many types of protein CSPs [103, 110-113]. The bi-Langmuir isotherm has been applied with success when using the theory of non-linear chromatography to characterise the adsorption of the enantiomers of β-blockers on an immobilized cellolase protein [103, 114, 121]. In these studies it were found that both the thermodynamics and the kinetics of the adsorption of β-blockers were heterogeneous [114]. In this study they determined the drug-protein stochiometry by using the saturation capacity for the chiral sites. Also for this system it was found that the thermodynamics of the adsorption was heterogeneous and the bi-Langmuir equation fitted best to the experimental data. The nonlinear theory and the bi-Langmuir model have also been used to study some chiral drugs on α_1-acid glycoprotein (AGP) [111].

In **papers III-VI** the adsorption data were fitted to both the Langmuir and the bi-Langmuir equation.

3.2.4.1 The Competitive bi-Langmuir Isotherm Model

Also for the bi-Langmuir isotherm, that describes a surface which is covered with two different kinds of sites, we can account for the competitive behavior of a mixed sample by using the bi-Langmuir competitive isotherm [109, 112]:

$$q_i = \frac{a_{I,i}\, C_i}{1 + \sum_{j=1}^{n} b_{I,j}\, C_j} + \frac{a_{II,i}\, C_i}{1 + \sum_{j=1}^{n} b_{II,j}\, C_j} \tag{6}$$

Here q_i is the solid phase concentration for the *i*:th component, C_i is the mobile phase concentration and $a_{I,i}$, $a_{II,i}$, $b_{I,i}$, $b_{I,i}$ are isotherm parameters.

3.3 Column Model

Peak profiles can be calculated with a proper column model, the differential mass balance equation of the compound(s), the adsorption isotherm, the mass transfer kinetics of the compound(s) and the boundary and initial conditions [13]. When a suitable column model has been chosen, the proper parameters (isotherm and mass transfer parameters and experimental conditions) are entered into the calculations. The results from these calculations can have great predictive value [13, 114]. The most important of the column models are “the ideal model”, “the equilibrium-dispersive (ED) model”, “the

lumped kinetic model", "the pore model", and "the general rate model" of chromatography [109].

3.3.1 The Equilibrium-Dispersive Model

The equilibrium-dispersive model is the most practical model for preparative chromatography when the column efficiency is high, as it is in RPLC of small molecules of moderate polarity, such as in **papers III-VI**. This model is valid when the band profile is more influenced by the nonlinear behavior of the adsorption isotherm than by kinetic effects [13]. In this model, the mass transfer resistance is assumed to be small enough to be neglected [109]. This situation is typically encountered in HPLC, when modern, highly efficient columns are used for small molecules [109]. As demonstrated by Giddings [115], the contribution of the mass transfer kinetics in these cases is equivalent to an increase in the axial dispersion coefficient. This model assumes constant equilibrium between the stationary and the mobile phase and uses an apparent dispersion term, D_a, to account for the band-broadening effects of both axial dispersion (molecular and eddy diffusion) and the contribution due to the mass transfer resistance, D_a is given by:

$$D_a = \frac{\mu L}{2N} \tag{7}$$

Here μ is the mobile phase velocity, L the column length and N the number of theoretical plates. The equilibrium-dispersive (ED) model [13, 109] was used to calculate the overloaded band profiles in **papers III-VI.**

3.3.2 Retention Factors

The classical retention factor is related to the numerical coefficients of the Langmuir isotherm by the following equation under linear conditions (i.e., at infinite dilution):

$$k = F \frac{\partial q}{\partial C} = F \; a \tag{8}$$

Here F is the phase ratio (V_s/V_m) and a is the equilibrium constant at infinite dilution which coincides with the initial slope of the isotherm. For a heterogeneous surface with two types of adsorption sites the retention factor k is the sum of two contributions, originating from type-I and type-II sites, and a general expression of the retention factors of the two enantiomers under linear conditions can be expressed as:

$$k = k_I + k_{II} = F(a_I + a_{II}) \tag{9}$$

3.3.3 The Mass Balance Equation

All mathematical models of chromatography consist of a differential mass balance equation for each component involved and the equation expresses mass conservation in the process [13, 109]. In the ED model the mass balance equation for a single component is expressed as follows:

$$\frac{\partial C_i}{\partial t} + F\frac{\partial q_i}{\partial t} + u\frac{\partial C_i}{\partial z} = D_a\frac{\partial^2 C_i}{\partial z^2} \tag{10}$$

Where t is time, z are the axial position in the column, q_i is the concentration of solute i in the stationary phase in equilibrium with C_i, the mobile phase concentration of solute i, u is the mobile phase velocity, D_a is the apparent dispersion coefficient, and F is the phase ratio (V_s/V_m). The equation describes that the difference between the amounts of component i that enters a slice of the column and the amount of the same component that leaves it is equal to the amount accumulated in the slice. The fist two terms on the left-hand side of Eq. 10 are the accumulation terms in the mobile and stationary phase, respectively [109]. The third term is the convective term and the term on the right-hand side of Eq. 10 is the diffusion term. For a multi component system there are as many mass balance equation, as there are active components in the system [13].

3.3.4 Initial and Boundary Conditions

As for all partial differential equations, it is also necessary to complement the mass balance equation with initial and boundary conditions, as explained in detail by Guiochon et al. [13]. The initial condition describes the state of the column when the experiment begins, i.e., at t = 0. In this case, the initial condition corresponds to a column empty of sample and containing only mobile and stationary phases in equilibrium:

$$C_i(t = 0, z) = 0 \qquad 0 \le z \le L \tag{11}$$

Where C_i (t , z) is the concentration of the the i:th component at position z and L is the column length. Boundary conditions characterize the injection and if the dispersion effects are neglected they can be described by rectangular pulses with duration t_p at the column inlet. Assuming that the sample concentration is $C_{0,i}$

$$C_i(t, z = 0) = C_{0,i} \qquad 0 \le t \le t_p \tag{12a}$$

$$C_i(t, z = 0) = 0 \qquad t > t_p \tag{12b}$$

The systems of mass balance equations with the proper isotherm equations are integrated numerically to obtain the concentration profiles at the column outlet.

3.4 Measurement of Adsorption Isotherm Parameters

In adsorption chromatography the relevant functions are the adsorption isotherm parameters. Since there are no theoretical tools available to predict isotherms from physico-chemical data of the solute, solvent and adsorbent, these adsorption isotherm parameters have to be determined experimentally. When measuring the data, it is important to use a broad concentration range, i.e., including both the linear part of the isotherm as well as concentration close to saturation of the stationary phase. Despite the fact that there are several methods available to obtain adsorption isotherm parameters, the experimental determination of the isotherms is still far from being routine work.

3.4.1 Single Component Adsorption Isotherm Parameters

The traditional approach in chromatographic modeling is to measure the adsorption isotherms for single solutes, and a convenient way to classify the experimental methods available is to distinguish between static and dynamic methods [13]. One example of a static method is the classic batch uptake method (BU) [116]. In the BU method, known volumes of adsorbent and solution are equilibrated in a vessel whereby the initial concentration of the solute is known and its concentration is measured in the supernatant after equilibrium is established. The application of the BU method requires a tedious work. The results are usually not very accurate, and further disadvantages are the large amounts of solute and adsorbent required. Although the BU method is hard to automate, this has recently been done successfully by T. Bergander et al. in GE Healthcare for single component isotherms of proteins [117].

The dynamic methods are based on direct chromatography and are popular because they are faster and easier to automate. Four direct chromatographic methods that are available for determination of adsorption isotherms are: frontal analysis (FA) [13, 109] frontal analysis by characteristic points (FACP) [109], elution by characteristic points (ECP) [109] and the perturbation peak (PP) method [118-121]. The FACP and ECP methods have

the serious drawback that they can only be used with high efficiency columns.

3.4.2 Frontal Analysis

Frontal analysis in the staircase mode requires two pumps, one connected to a bottle with pure mobile phase and one connected to a bottle with the analyte to be investigated dissolved in the pure mobile phase. Initially, only mobile phase is present in the column and the staircase is obtained by successive abrupt step changes at the inlet of the column. In each step the analyte concentration in the mobile phase is increased and the stationary phase will consequently adsorb successively more analyte [109], see Figure 9.

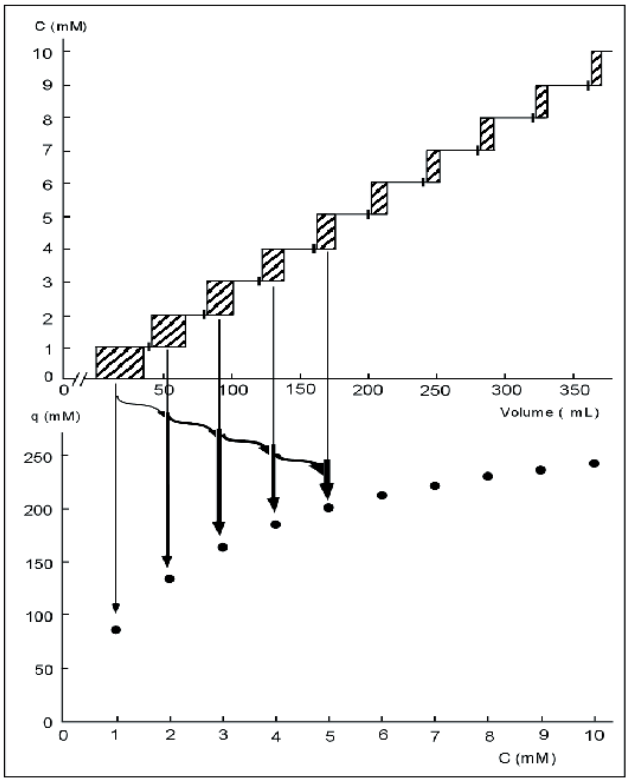


Figure 9. The principle of the frontal analysis technique. In the uper part of the figure a theoretic staircase is shown with 10 steps, showing the increment of solute in the stationary phase. A new step starts every 40 mL and is shown in the figure by a vertical line. In the lower part of the figure the corresponding isotherm is shown. The following values have been used: V_T = 2 mL, a = 120, b = 0.4 mM^{-1}, V_s = 0.4 mL. The illustration was used with kind permission from Gustaf Götmar [111].

The concentration in the stationary phase is:

$$q_{i+1} = \frac{Q_{i+1}}{V_S} = \frac{Q_i + (C_{i+1} - C_i)(V_{R,i+1} - V_T)}{V_S} \tag{13}$$

Here Q_i and Q_{i+1} are the amounts of compound adsorbed by the column packing after the i:th and the $(i + 1)$:th step, when in equilibrium with the concentrations C_i and C_{i+1}, respectively. $V_{R,i+1}$ is the retention volume of the inflection point of the $(i +1)$:th breakthrough curve, V_T is the total dead volume (including column void volume,V_0), and V_S is the volume of the stationary phase [109]. In frontal analysis two different dead volumes must be determined, namely the traditional column hold-up volume, V_0, which is used to calculate the volume of the stationary phase, V_S. The other dead volume is the total one, V_T, i.e., the entire volume after the T-connector (including V_0) and the FA raw-data should be corrected for V_T.

3.4.3 Competitive Adsorption Isotherm Parameters

The components in a mixture, injected into the column, compete for access to the limited number of adsorption sites. In adsorption chromatography, this behavior is expressed by competitive adsorption isotherm parameters. It is very important to be able to measure competitive adsorption isotherm parameters, because the single-component adsorption isotherm parameters determined from single-component experiments do not always give an accurate prediction of overloaded multi-component elution profiles when competition occurs [122, 123]. So, even if the components are available in pure form it is important to be able to measure competitive adsorption isotherm parameters. An additional problem when working with chiral components is that the pure enantiomers are not always accessible, especially during the exploratory stage in the development of a new drug in the pharmaceutical industry. Therefore, it is often necessary to determine adsorption isotherm parameters of components from a mixture.

The FACP and ECP methods cannot be used to determine adsorption isotherm parameters from multicomponent mixtures. By contrast FA can be used to determine multi-component adsorption data but it is a complex and time-consuming process [124, 125].

3.4.4 Pulse Methods

An alternative approach to isotherm determination is offered by the so called "Pulse methods" There are two types of pulse methods, the elution of a pulse

on a plateau, also called the perturbation peak (PP) method and the elution of an isotopic pulse on a plateau, also called the tracer-pulse (TP) method [12].

3.4.4.1 Perturbation Methods

The method of elution on a plateau was first suggested by Helfferich in Science more than forty years ago [126]. In the PP method, the chromatographic column is equilibrated with a constant stream of molecules in the mobile phase and a concentration plateau is established. A perturbation is then accomplished by injecting a sample containing an excess or a deficiency of the molecules as compared to the concentration at the plateau. [118-120, 127]. The response at the column outlet will be small peaks, known as perturbation peaks, and their retention times are used to determine the adsorption isotherm parameters. The retention time of the perturbation peak is related to the isotherm through the equation:

$$t_R(C_i) = t_0\left(1 + F\,\frac{dq}{dC}\Big|_{C=C_i}\right) \tag{14}$$

Here t_0 is the column hold-up time, F is the phase ratio and dq/dc is the derivative (slope) of the isotherm function that is evaluated at the plateau concentration C_i. The retention time of a small injection of a compound in a column equilibrated with pure mobile phase without the compound (such as for analytical chromatography) gives the retention time under linear conditions, through the equation:

$$t_R = t_0(1 + Fa) \tag{15}$$

Here a is the equilibrium constant at infinite dilution and also the initial slope of the isotherm. In a similar way, the set of retention times of perturbation peaks measured in a column equilibrated with streams of the mobile phase with increasing concentration allows the calculation of the slope of the isotherm at these different concentrations. This set of retention times is the fundamental data needed for determining the adsorption isotherm parameters. Figure 10 illustrates the principle of the perturbation method for a single component. At the first arrow a small sample containing the component is injected into the column that is only equilibrated with pure mobile phase lacking the component. In the following steps the column is equilibrated at different concentrations of the component and small amounts of pure mobile phase lacking the component are injected at the times marked with the arrows. At higher plateau concentrations the retention time is decreased caused by the non-linearity of the adsorption isotherm.

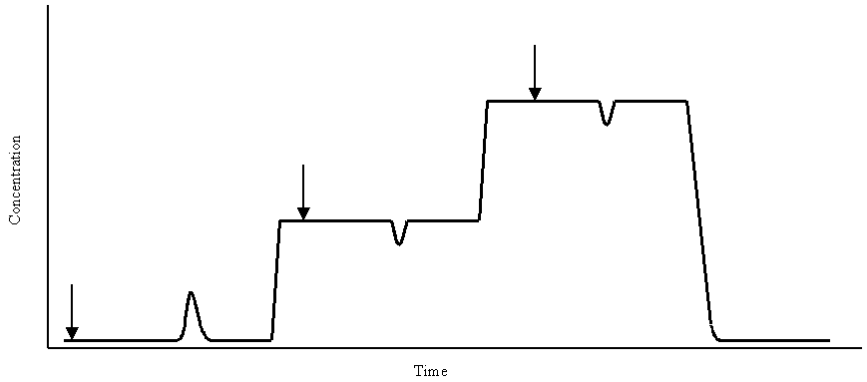


Figure 10. Principle of the perturbation peak method for the single component case with stepwise increased concentration plateaus. The arrows mark the injection times for small samples that perturb the equilibrium.

The perturbation procedure is repeated for different plateau concentrations, yielding peaks with different retention times, and the isotherm is constructed from its slope. Figure 11 shows the relationship between the isotherm and the retention time of perturbation peaks at different plateau concentrations.

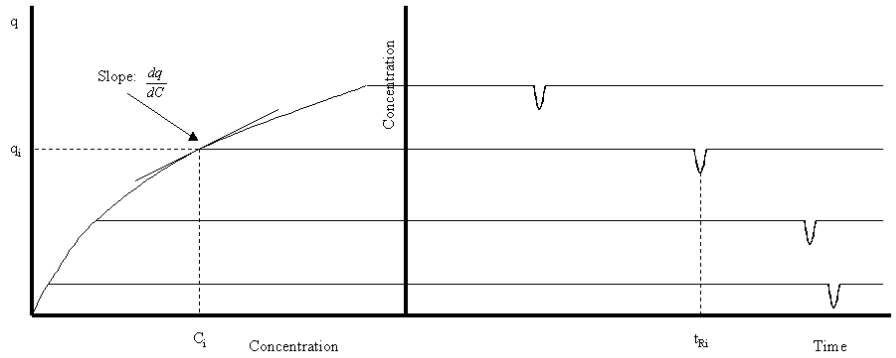


Figure 11. The principle for the perturbation method and its relationship between the isotherm, its slope and the retention time of perturbation peaks at different plateau concentrations.

A similar presentation as in Figure 10 for a single component is given in Figure 12 to illustrate the situation for the competitive binary mixture case. For each plateau concentration two perturbation peaks result from the injection marked by the arrows.

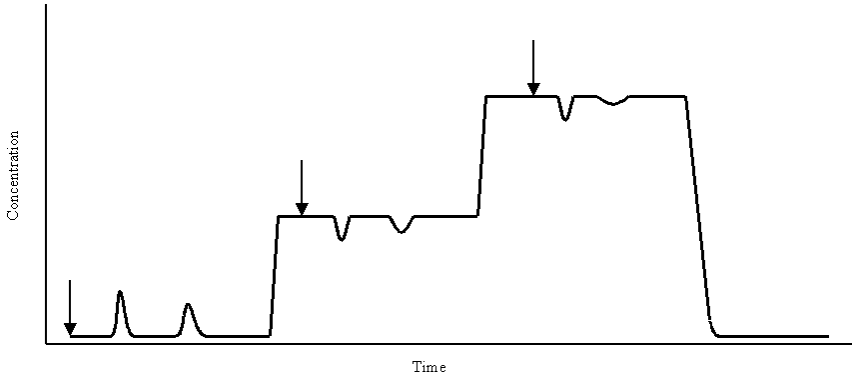


Figure 12. Principle of the perturbation peak method for a binary component case with stepwise increased concentration plateaus. The arrows mark the injection times for small samples that perturb the equilibrium.

The PP method is an alternative method to determine adsorption isotherm parameters from binary mixtures. It has been reported that the PP method works well under linear to weakly non-linear conditions when several enantiomer ratios are used [118, 120] and even when only racemic mixtures are used [119, 128]. One severe problem when using the PP method with the traditional blank injection technique is that one of the two perturbation peaks starts to vanish when the plateau concentration starts to deviates from the linear conditions. In **paper IV** we investigated this phenomenon and developed a new injection technique that should make both peaks detectable. The most practical and economical approach is to perform the PP method using only a mixture, but most often the PP method has been performed using several mixtures of various relative compositions to obtain high accuracy, [118, 120] the drawback being that this approach requires pure chemicals. No comparison and validation has been made of parameters determined by the PP method using only racemic mixtures compared to parameters determined by the PP method with several enantiomer ratios. In **paper V** the experimental errors in the PP method using different injection techniques were investigated and the new developed injection technique were used in **paper IV** for the determination of competitive adsorption isotherm parameters. The new injection technique aimed to considerably reduce the experimental errors and also make both perturbation peaks detectable. A further aim of the new injection technique was that is should be able to determine adsorption isotherm parameters directly from a racemic mixture without the need for large quantities of pure substances. In **paper VI** the aim was to develop an injection technique able to determine multicomponent adsorption isotherm parameters from mixtures without the need for large quantities of pure compounds and we therefore investigated different injection techniques.

3.4.4.2 The Tracer-Pulse (TP) Method

Fourty years ago, it was suggested by Helfferich, that if an excess of molecules are injected into a chromatographic column equilibrated with a constant stream of identical molecules, the injected sample molecules are not found in the observed perturbation peak [126]. The injected molecules should instead be found in a later-eluted, invisible peak, and the observed perturbation peak is just a wave phenomenon and only contains molecules from the stream. The injected molecules cannot generally be detected because they have a combined elution with a deficiency peak of the molecules originating from the stream. Helfferich considered it paradoxical that a single injection in a single-component system could cause the successive elution of two peaks [129]. In a recent study by Samuelsson et. al, [130] the paradox was experimentally proven for the first time, and also systematically investigated. Two different strategies were employed to visualize the second so called “tracer-peak”: (i) a radiochemical approach and (ii) a method based on the use of two enantiomers in a nonchiral separation system [130]. Among others, it could be verified that the retention time of tracer-peak is governed by the slope of the chord of the isotherm at the actual plateau concentration [130]. This is in contrast to the perturbation peak which has a retention governed by the tangential slope of the isotherm [130].

3.5 Validation of Isotherm Models

After the adsorption isotherm experiments have been completed, an isotherm equation must be chosen. This equation should fit the experimental data. Often are the experimental data (the experimental adsorption data acquired by the FA method or the perturbation retention times acquired by the PP method) only compared with the ones calculated using the adsorption isotherm parameters acquired from some adsorption isotherm models [131]. This is sometimes the only validation done in this field [131]. However, the adsorption isotherm parameters should preferably be validated in two step: (1) the different isotherm models should be compared using statistical calculations, e.g., an F-test, and (2) by using the parameters to computer simulate elution profiles and then compare them with experimental ones.

To conclude to which isotherm models the data fits best the values of the residuals should be used in an F-test [132]. In **paper III** an F-test comparing different adsorption isotherm parameters is done and clearly explained.

3.6 Validation of Simulated Elution Profiles

The accurate knowledge of the adsorption isotherm is necessary for accurate predictions of individual band profiles in all modes of chromatography and hence for process optimization [13]. Band profiles are sensitive to the shape of the isotherm, and thus the isotherm must be determined with accuracy and precision.

To validate the accuracy of the adsorption isotherm parameters determined experimentally, they could be used to computer simulate elution profiles, which then could be compared to experimental ones. So far, this has only been made by visual inspection [13, 118, 120, 128]. In order to quantify and validate how well the simulation fits the experimental data we defined in **papers III**, **V** and **VI**, the *overlap* to be:

$$\frac{\int_0^\infty \min\left[c_{sim}(t), c_{\exp}(t)\right] dt}{\int_0^\infty c_{sim}(t)dt} \tag{16}$$

Here $c_{exp}(t)$, $c_{sim}(t)$ are the experimental and simulated responses at time *t*. When the experimental and simulated elution profiles coincide perfectly the overlap is 100% and when they are totally separated the overlap is 0%. In **papers III-VI**, the accuracy of the parameters was validated by calculating the overlap. We suggest that an overlap of more than 90% could be considered as good. A high overlap validates that both right isotherm model and right column model have been chosen.

4 Results and Discussion

This section is intended to give a very brief overview of the results obtained. For more detailed results and discussions, see **papers I-VI**.

4.1 Analysis of Biotechnological Synthesis

4.1.1 Identification (**Paper I**)

In biotechnological process development the product must be rapidly identified. The aim of **paper I** was to investigate the usefulness of HPLC-DAD and MS for rapid preliminary product identification in a typical biotechnological process where reference material is lacking. The biotechnological synthesis involved the substrate Progesterone (PS) that was enzymaticaly derivized to 9α-hydroxyprogesterone (9α-OH-PS), see Figure 1 in section 2.1.

Because the product was not commercially available, a sample mixture containing a number of compounds similar to the product was injected into different columns in order to find the best separation system for such components. The Kromasil C_{18} 3.5 µm column had the best resolving properties and was used for the LC-DAD studies. The DAD and the selected software, made it possible to obtain spectra of all peaks in the mixture, save them in a library and compare them. The real process liquid was pre-treated by liquid-liquid extraction (LLE) before injection into the column, LLE resulted in higher recovery than solid phase extraction (SPE). The resulting single wavelength (245 nm) chromatogram of the pre-treated process liquid at the start of the fermentation process is shown in Figure 13a; only a single peak appears, the substrate (progesterone) peak. The corresponding DAD chromatogram can be seen in Figure 13b showing the typical spectral characteristics of progesterone when compared with the standard saved in the software library.

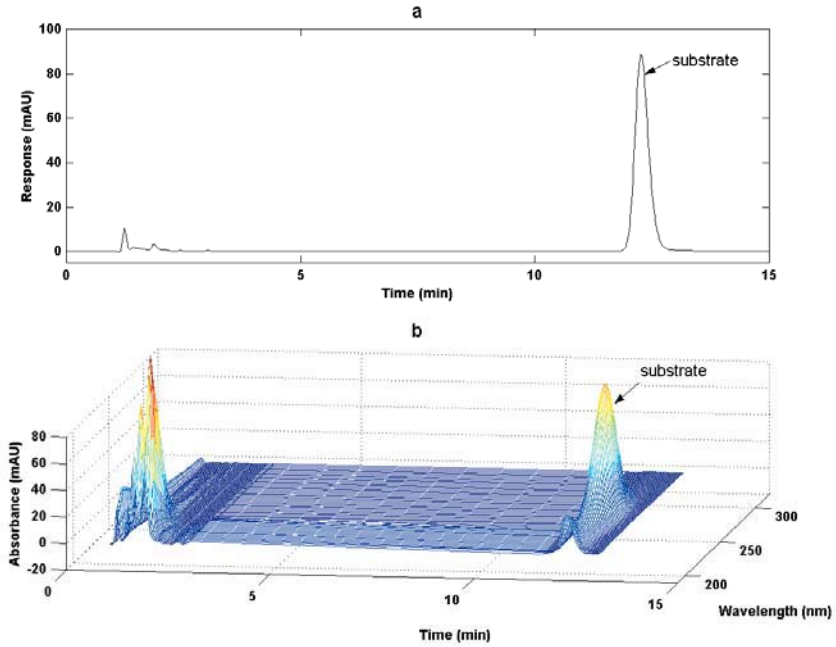


Figure 13 (a). Chromatogram obtained from injection of pre-treated process liquid from the beginning of the fermentation process, detected at wavelength 245 nm. **(b)** The three dimensional spectra-chromatogram obtained with the DAD from the same injection as (a).

Figure 14a shows the single wavelength chromatogram obtained after injection of a pre-treated sample taken at the end of the fermentation process (about 164 h from start). Two additional peaks appeared of comparable height and similar retention times, which were both less retained than the substrate. Since the product is assumed to be more hydrophilic than the substrate, any one of the peaks that appear might correspond to the product regarding their retention times. However, the corresponding DAD-chromatogram (see Figure 14b) reveals that only the peak with retention time about 4.5 min has a spectrum similar to the substrate. This demonstrates the potential of HPLC-DAD for rapid process control of biotechnological processes; a simple extraction is followed by a reasonably rapid separation and detection by DAD.

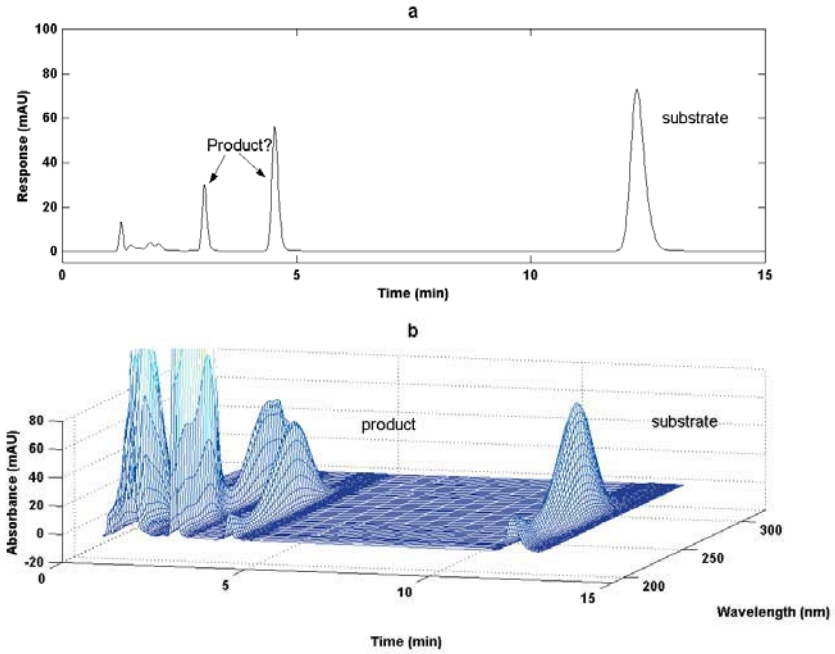


Figure 14 (a). Chromatogram obtained from injection of pre-treated process liquid from the end of the fermentation process, detected at wavelength 245 nm. **(b)** The three dimensional spectra-chromatogram obtained with the DAD from the same injection as (a)

The LC-DAD determination was compared with the corresponding information from mass spectrometry. Since, the product 9α-OH-PS was not available as a reference substance, full scan spectra were instead acquired by using 11α-hydroxyprogesterone (11α-OH-PS) as a reference. Figure 15a shows the full scan mass spectra of the fermentation product. In this system, quasi-molecular ions $[M+H]^+$ appear as base peaks for the fermentation product (m/z 331.3). A comparison with 11α-OH-PS shows that the fermentation product has the same molecular weight as a hydroxyprogesterone, which strongly indicates that the product has such a structure. According to Zaretskii et al. [133] it is possible to decide the epimeric configuration for substances such as our fermentation product by comparing relative intensities in the mass spectrum. Compared with the reference substances 11α-OH-PS and 11β-OH-PS, the fermentation product shows a fragmentation pattern more similar to the 11β-OH-PS, with the mono-axial hydroxyl, than to the 11α-OH-PS, with the mono equatorial hydroxyl. This indicates that the fermentation product really has an axial hydroxyl. Thus, it might be possible without access to the right reference substance to predict whether the hydroxyl group has an axial or equatorial position by MS.

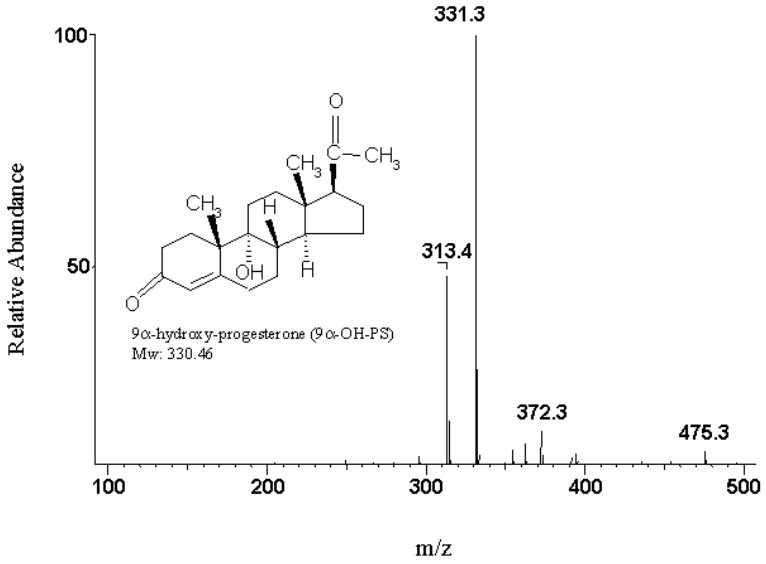


Figure 15. Full-scan mass spectrum of the fermentation product.

The product was finally identified as 9α-OH-PS by NMR, after isolation by semi-preparative chromatography. A two-step semi-preparative chromatographic separation was run in order to achieve high purity of the sample. First NPLC was performed to get rid of yellow strongly nonpolar compounds from the fermentation medium. The fraction from the NPLC eluate containing the product was further purified using a high resolution RPLC column.

4.1.2 Quantification and Validation (**Paper II**)

There are no detailed recommendations for analytical quantification procedures in the field of biotechnological production of drugs, in contrast to the recommendation made by the FDA [16] for bioanalytical methods. The aim of this paper was therefore to investigate whether the latter detailed guidelines (given by the FDA for bioanalytical methods) also could be used in the field of biotechnological synthesis. Validated methods for quantification are important also in biotechnological synthesis for the proper calculation of rate coefficients.

As mentioned in section 2.7, the internal standard (I.S.) method is useful for quantification when the matrices are complicated and sample pretreatment is necessary. However, it is generally difficult to choose an I.S. that meets all the necessary requirements (se above in section 2.7). Thus, 4-androsten-3,17-dione (AD) was chosen as I.S. because of its great structural similarity with both substrate and product. However, it soon appeared that

when an I.S. too similar to the substrate is added directly into the fermentation process, it will also be consumed. This is illustrated in Figure 16 showing the resulting chromatograms for samples taken at different times after start of the fermentation using two different types of I.S. One of the internal standards was 4-androsten-3,17-dione (AD) with a close structural similarity in to both substrate and product. The other I.S. was binaphthol, which is structurally much different from both the substrate and product.

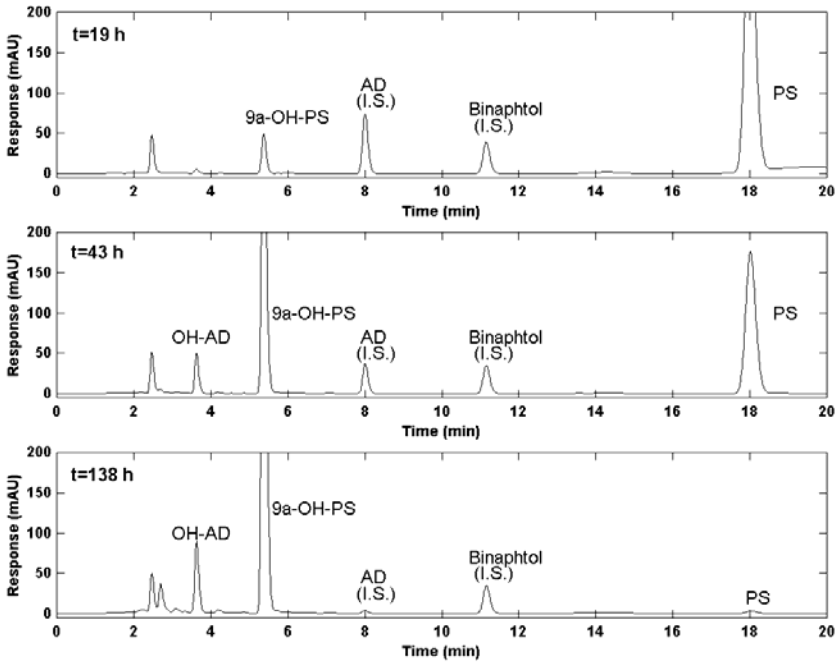


Figure 16. Chromatograms resulting from injections of worked-up fermentation liquid using two types of internal standards; the structurally similar AD and the structurally different binaphtol.

It was observed that the AD peak was decreased in height with time and also that a new peak was showing up with a smaller retention time (*cf.* t=19 h and 43 h in Figure 16). At the end of the process the AD peak had almost disappeared (*cf.* t=138 h in Figure 16). It was concluded that the disappearing peak contained AD was enzymatically consumed and that the appearing peak contained hydroxylated AD. The peak area of the bulky binaphthol was constant during the whole process (*cf.* Figure 16 t=19 h-138 h). These results demonstrate clearly the importance of choosing an I.S. which instead is structurally different from the substrate implying a big difference compared to ordinary analytical method development. The study showed that the recommendations and guidelines for method validation in the bioanalytical field

could also be used for quantification of biotechnological production of small drugs with the following exceptions:

1. The tolerances (C.V.-values) of the validation terms can be much narrower; in this work 10% is suggested. This value is between the accepted tolerances for bioanalytical methods and those for pharmaceutical product analysis.
2. The internal standard (I.S.) must be introduced in the process liquid at the start of the process together with the substrate.
3. The I.S. must be sufficiently different in structure from the substrate so that it will not participate in the enzymatic process.
4. The selectivity must be checked frequently during the process due to changes of the blank process liquid with time.

4.2 Determination of Single Component Adsorption Isotherm Parameters - Characterization of a New CSP (**Paper III**)

The accurate determination of the adsorption isotherm parameters of the two enantiomers on a CSP is of fundamental importance to do computer-assisted optimization to scale up the process. Such determinations are usually done with an analytical column and the most traditional method to determine the parameters and saturation capacity is by frontal analysis (see section 3.4.2). The aim of **paper III** was to investigate the adsorption behavior and the chiral capacity of the newly developed Kromasil CHI-TBB column using a typical model compound. Many of the previous studies from the group have been made on low-capacity protein columns; which has revealed interesting information about the separation mechanism [103, 110, 111]. For this reason a column really aimed for preparative chiral separations was chosen for investigation [134]. As solute the enantiomers of 2-phenylbutyric acid was chosen.

Figure 17 shows a section of two individual overlaid staircase chromatograms resulting from single component frontal analysis of (S) and (R)-2-phenylbutyric acid, respectively. At the first step up to 30.5 mM the enantiomers are clearly separated from each other, at the second step up to 61.0 mM they are still separated and even at the third step up, to 91.5 mM it is still a very small tendency for separation. This figure indicates that the chiral capacity is somewhat higher than 90 mM.

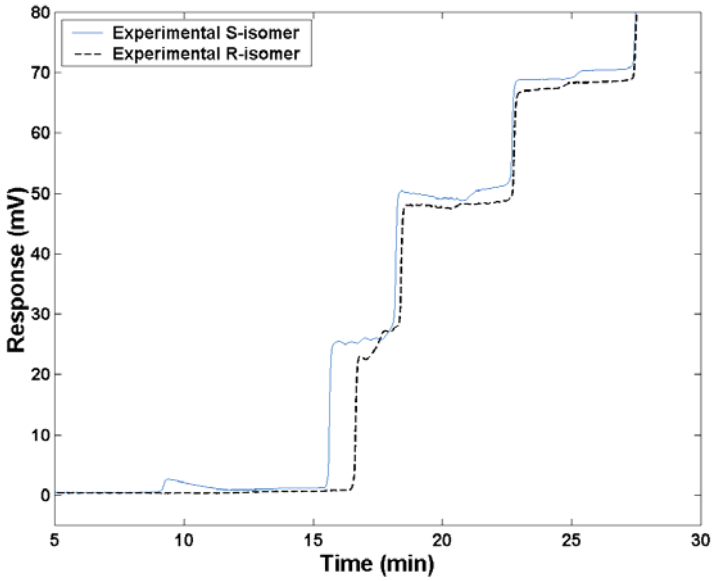


Figure 17. Part of two individual overlaid staircase chromatogram for (S) and (R)-2-phenylbutyric demonstrating the extremely high chiral capacity of the column. The first step is 30.5 mM, the second step is up to 61.0 mM and the third step is up to 91.5 mM.

The isotherm data acquired from frontal analysis over a broad concentration range fitted well to the bi-Langmuir model, see Figure 18, demonstrating that the adsorption on Kromasil CHI-TBB is heterogeneous with two types of sites. The saturation capacity of site II obtained from the bi-Langmuir isotherm parameters were $q_{s,II}$ = 130 mM for (R)-(-)-2-phenylbutyric acid and $q_{s,II}$ = 123 mM for (S)-(+)-2-phenylbutyric.

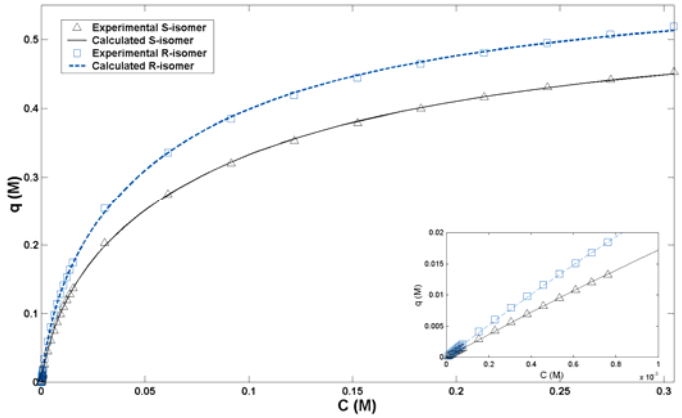


Figure 18. Isotherms for (R) and (S)-2-phenylbutyric acid, experimentally acquired by frontal analysis and compared with their fits with bi-Langmuir adsorption isotherm parameters. The lines are calculated data using the best single bi-Langmuir isotherm parameters.

The results reported in **paper III** showed that the chiral Kromasil column has the highest "true" chiral capacity measured so far, about 130 times larger than columns packed with immobilized proteins. Thus, the column is indeed very suitable for preparative separations.

4.3 Determination of Competitive Adsorption Isotherm Parameters

4.3.1 Development of a New Injection Technique (**Paper IV**)

Single-component isotherm parameters cannot always predict elution profiles with satisfied accuracy [122, 123]. Therefore, to be able to predict accurate overloaded multi-component elution profiles where competition occurs competitive adsorption isotherm parameters are often necessary. Measurement of isotherms from a mixture is also often necessary because the pure enantiomers are not always accessible in large quantities. However, there exist only a small number of reports on the determination of multi-component adsorption isotherm parameters. FA can be used to determine binary isotherm data but it is time-consuming. The PP method is an alternative method to determine isotherm parameters from binary mixtures. It has been reported that the PP method works well up to weakly non-linear conditions [118, 119].

In **paper IV** investigations were made with the traditional PP method, involving small injections of pure mobile phase at different binary plateau concentrations. The experiments in Figure 19a, show that two negative peaks appear after injection at very low binary plateau concentration. At higher plateau concentrations the second peak vanishes and it is thus impossible to measure any retention time for the second peak (see Figure 19b).

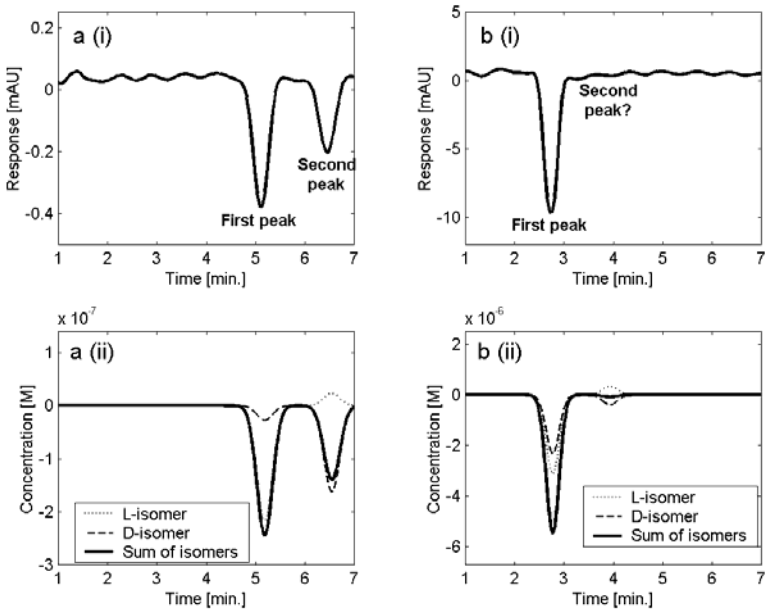


Figure 19. Vanishing perturbation peaks in chiral LC at **(a)** weak and **(b)** moderate non-linear levels, **(i)** experimental chromatograms and **(ii)** simulated showing the sum and the individual concentrations of the isomers

Because of this severe problem when using the PP method with the traditional blank injection technique we developed a new injection technique, called the "Lindholm-technique". In this technique the injections should be done with the same excess of one of the compounds as deficiency of the other component: $C_{sample\ 1} = C_{plateau\ 1} - \mu$ and $C_{sample\ 2} = C_{plateau\ 2} + \mu$ for some number μ chosen so that the concentrations always are equal to or greater than zero. This will always yield two perturbation peaks with the same area but of opposite signs, i.e., one positive and the other negative. By this technique the retentions can be measured for both peaks also in the high concentration range. In Figure 20 the injection technique is demonstrated and compared with the traditional blank injection technique, where the second peak has begun to vanish. In Figure 20a it can be seen that the experimental chromatogram shows a deep negative first peak and a small negative second one when using the traditional injection technique. In Figure 20b it can be seen that area are close to 50 % for both peaks using this new injection technique.

If the PP method is used in a multi-component case it should also be noted that the determined isotherm parameters could not be assigned to specific components without additional information, e.g., by comparing computer simulations with an experimental chromatogram where the peaks can be identified.

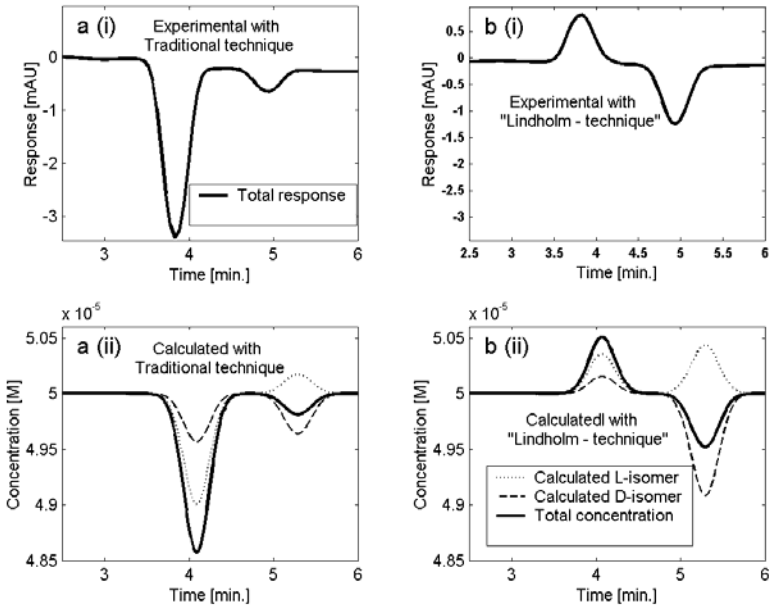


Figure 20. Visualisation of both peaks with experimentally verified **(i)** and calculated **(ii)** chromatogram after perturbation of a moderately nonlinear racemic plateau using the **(a)** blank and **(b)** optimal "Lindholm-technique".

4.3.2 The Binary Case (**Paper V**)

The most practical and economical approach is to perform the PP method using only one mixture, but most often the PP method has been performed using several mixtures of various relative compositions to obtain high accuracy, the drawback being that this approach requires pure chemicals. No comparision and validation has been made of parameters determined by the PP method using only racemic mixtures compared to the PP method using several different enantiomer ratios. Hence, in **paper V** a comparison was done between adsorption isotherm parameters obtained from the PP method using different various relative compositions and from only racemic mixtures. The "Lindholm-injection- technique" developed in **paper IV** was used.

The adsorption isotherm parameters obtained in **paper IV** were validated in two step: (1) the experimental perturbation retention data were compared with the calculated retention data using the adsorption isotherm parameters, and (2) by using them with computer simulation programs to calculate elution profiles. The simulated profiles were compared with the experimental profiles using the calculations of the overlap (see section 3.6) as evaluation

tool. Figure 21 shows comparison between simulations and experiments with parameters obtained from the PP method using all plateau ratios, and the overlap is excellent.

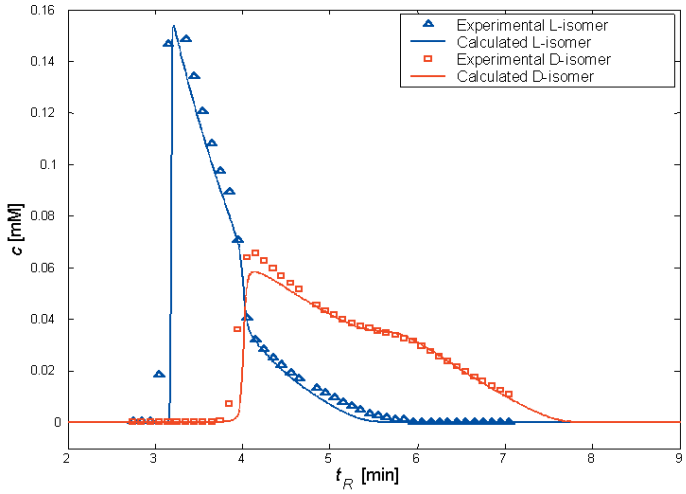


Figure 21. Experimental and simulated elution profiles for different sample compositions. The symbols are experimental data, the lines are simulated elution profiles using the competitive bi-Langmuir adsorption parameters determined by the PP method. Sample: 20 µL 5.0 mM L-enantiomer and 5.0 mM D-enantiomer.

The overlap using only parameters from racemic mixtures was good (> 90 %) and this validates the accuracy of the parameters. In fact the overlap was almost as high as when parameters from all plateau ratios were used. This is an important result, allowing major savings in time and money when determining competitive adsorption isotherm parameters. In this paper investigations were also done that show that too large disturbances or injected volume with the PP method result in parameters with lower accuracy.

4.3.3 The Quaternary Case (**Paper VI**)

There are, so far, only two reports on the determination of isotherm data from mixtures containing more than two components. In both cases, the FA method was used for ternary mixtures [135, 136]. There are no reports on the experimental determination of adsorption isotherms for quaternary mixtures using any chromatographic method. The competitive quaternary adsorption isotherm parameters could be very valuable for the separations of the four isomers of a compound with two chiral centers, or for the preparative separation of two compounds in the presence of one or two impurities.

The purpose of this paper was twofold: (i) to investigate how to visualize all four perturbation peaks on a quaternary concentration plateau and to (ii) validate the accuracy of the PP method for determination of isotherm parameters directly from quaternary mixtures of 1/1/1/1 compositions. For this purpose the technique developed and validated for the binary case in **papers IV** and **V** (the "Lindholm-technique") was extended to the multi-component case. The approach is to inject the same excess as deficiency for every second solute. Thus, in a quaternary system the excess of components 1 and 3 is the same as the deficiency of components 2 and 4, or the converse. The concentrations of the components in the sample can be calculated according to: $C_{sample\ i} = C_{plateau\ i} - (-1)^i \mu$, for some number μ chosen so that the injected concentrations always are equal to or greater than zero. This technique made all perturbation peaks clearly detectable, although the area were not the same for all of them, see Figure 22.

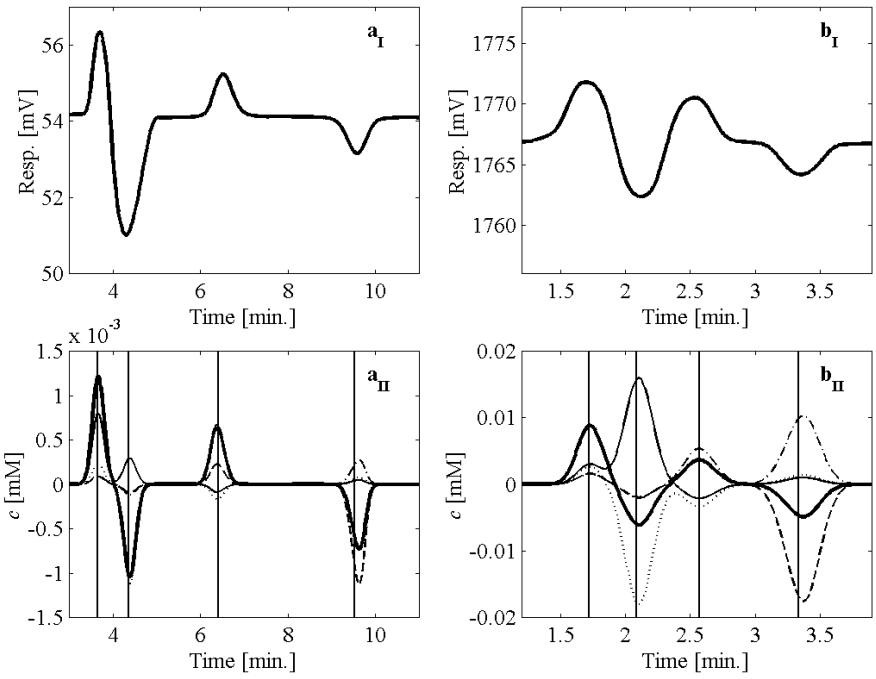


Figure 22. Experimental **(I)** and simulated **(II)** chromatograms using the new injection technique applied on a quaternary plateau of (**a**) 0.01875 mM and (**b**) 0.250 mM of each component. The simulated chromatograms show the concentrations of component 1 (thin solid line), component 2 (dotted line), component 3 (dash-dotted line), component 4 (dashed line) and the total response (fat solid line).

The accuracy of the adsorption isotherm parameters were validated in two step: (1) the experimental perturbation retention data were compared with the calculated retention data using the adsorption isotherm parameters, and (2) by comparing simulated multi-component elution profiles with experimental ones. Figure 23 (a)-(d) shows the experimental (dashed lines) elution

profiles with the corresponding simulated (solid lines) ones, as single, binary, ternary and quaternary-component elution profiles. When comparing this series of chromatograms going successively from (a)- to (d), it is clearly observed that the retention times of the less retained components decreases further when further one component is added. This is due to the increasing degree of competition by the new components(s) added to the sample-mixture. Figure 23d shows the injection of 60 µL 1.250 mM of all components.

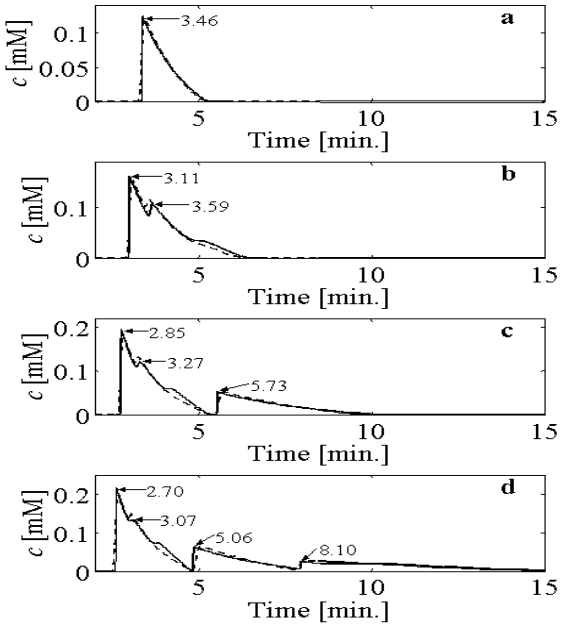


Figure 23. Comparison between single- and multi-component elution profiles (dotted lines) with calculated ones (solid lines). The sample injected was 60 µL 1.250 mM of **(a)** component 1, **(b)** component 1-2, **(c)** component 1-3 and **(d)** component 1-4.

The agreement between the experimental and calculated profiles was great in all cases and the overlaps between simulated and experimental data were all between 91 to 96 %.

To conclude, in **paper VI** were for the first time ever the adsorption isotherm parameters successfully determined for a quaternary mixture. This was done using the newly developed injection technique with the PP method. Accurate adsorption isotherm parameters were determined without the need of pure components in large quantities. With our injection technique only about 0.3 mg of two of the components have to be available in

their pure form to be able to measure the competitive isotherm parameter. This should be compared with the about 80 mg of pure components of each type if single-component isotherm parameters are to be measured. This is an important result, allowing major savings in time and money in the determination of the competitive adsorption isotherm parameters.

5 Concluding Remarks and Future Perspectives

The biotechnological field is growing fast and has generated a cumulative demand for analytical methods and analytical thinking. **Paper I** in this thesis demonstrates rapid and simple methods for preliminary identification in biotechnological synthesis without a priori reference material. The possibility to stop a synthesis immediately if the results are not satisfied, also in cases when the product is not available, is of large economical interest.

Rapid and accurate quantification of the substrate and the product in biotechnological synthesis of drugs is also important in the process development. However, the existing analytical thinking has not yet been routinely adapted to this area. For example there still exist no detailed guidelines for quantification and validation in this field. In the present thesis the detailed recommendations and rules given by the FDA for bioanalytical methods have been applied and modified to the field for analysis of biotechnological synthesis. In **paper II** an interesting and new approach to choose internal standards was demonstrated and compared to ordinary bioanalytical methods. The **paper II** also contains guidelines for method development and validation demonstrating proof-of-principle with a great potential to achieve excellent accuracy and precision of the quantification results.

HPLC coupled to DAD or to MS, for identification and/or quantification is today an established method in the pharmaceutical industry. Additionally in the fermentation field this technique has great potential for the same issues. In this thesis I have developed HPLC-DAD and MS methods that can be used in analyzing fermentation liquids, so that hopefully, more and more laboratories will begin to use these methods and validation guidelines.

Preparative chromatography is widely used for the purification of different compounds, but this procedure needs to be optimized to achieve the minimum production costs. This can be done by computer-assisted modeling. However, this approach requires a priori determination of accurate competitive adsorption isotherm parameters. The methods to determine this competitive information are poorly developed and hence often a time limiting step or even the reason why the computer-assisted optimization is still seldom used. In this thesis in **papers IV-VI**, a new injection method was developed that makes it possible to determine these competitive adsorption isotherm parameters more easily and faster than before. The use of this new

method in p**aper VI,** allowed the determination of quaternary isotherm parameters for the first time. With this method available, computer assisted optimization for preparative chromatography has hopefully reached one step closer to being used more routinely in the future.

I hope that my thesis can be used as a contribution in the future for analysis and validation in biotechnology, and also for the rapid determination of competitive adsorption isotherm parameters so that computer-assisted simulations may be used more extensively, in scaling-up and optimization of large scale chromatography.

6 Acknowledgements

I have a lot of people to acknowledge for their support during these past five years working on my thesis, and I would like to express my sincere gratitude to:

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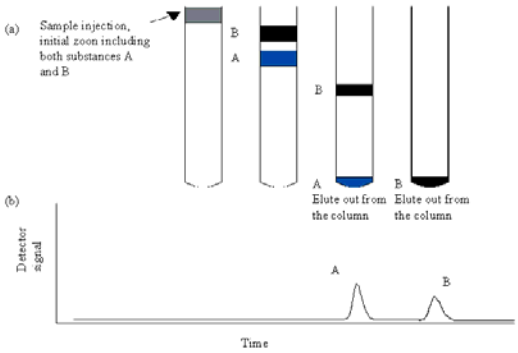
7 Swedish Summary

Utveckling och validering av analytiska och preparativa vätskekromatografiska metoder

7.1 Vätskekromatografi

Kromatografi är en kemisk separationsteknik som utnyttjar förhållandet att de ämnen som skall separeras fördelar sig på olika sätt mellan en stillastående (stationär) och en rörlig (mobil) fas. Vid vätskekromatografi är den mobila fasen en vätska. En kolonn packas med små fina partiklar till vilka ett selektivt adsorptionsmedium bundits fast. Adsorptionsmediet kan exempelvis vara kolkedjor som selektivt binder till vissa hydrofoba delar på molekylerna som skall separeras. Ett prov med olika komponenter injiceras på kolonnen samtidigt som den mobila fasen pumpas genom kolonnen. Den komponent som adsorberar bäst till kolkedjorna stannar kvar längst tid i kolonnen. En dator registrerar via en detektor hur molekylerna flödar ut genom kolonnen. Kan man registrera två toppar väl skilda från varandra, är separationen av två komponenter lyckad. Figur 1 visar schematiskt hur två komponenter separeras.



Figur 1. (a) Schematisk skiss av en separation av de två komponenterna *A* och *B* vid vätskekromatografi i den packade kolonnen. Komponent *B* kvarhålls mera än komponent *A* i kolonnen varvid ämnena separeras. **(b)** Kromatogram från separationen.

Vätskekromatografi är idag den dominerande separationstekniken inom analytisk kemi för separation av läkemedelsmolekyler och andra liknande ämnen.

7.2 Analys av bioteknologiska synteser

Analytiska kemister arbetar med metoder för att kvalitativt identifiera och kvantitativt bestämma enskilda komponenter i komplexa provblandningar. Exempel på detta är prover från bioteknologiska synteser, blod- eller urinprover vid dopingkontroll. För att kunna genomföra dessa uppgifter behövs metoder som är både känsliga och selektiva.

7.2.1 Identifiering

Vid bioteknologisk syntes är det av ekonomiska skäl viktigt att snabbt kunna avgöra att rätt produkt bildas under processens gång för att omedelbart kunna avbryta produktionen om så inte är fallet. I denna avhandling demonstreras hur snabba analysmetoder baserat på separation med vätskekromatografi kopplat till avancerade detektorer, en multivåglängdsdetektor (DAD) eller till en masspektrometer (MS), kan användas för snabb identifiering vid bioteknologisk syntes.

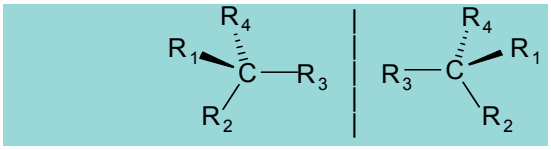
7.2.2 Kvantifiering och validering

Vid optimering av bioteknologisk syntes är det viktigt att snabbt kunna mäta (kvantifiera) koncentrationen av produkten som bildas, samt att säkerställa (validera) att mätmetoderna ger tillförlitliga resultat. Inom det här området saknas det fortfarande detaljerade riktlinjer för kvantifiering och validering, trots att det närbesläktade området bioanalys (analys av biologiska prover som t.ex. urin) inom läkemedelsindustrin är utrustat med välutvecklade riktlinjer. I denna avhandling har därför de metoder och riktlinjer som finns för bioanalys inom läkemedelsindustrin modifierats för att även kunna användas vid analys av små molekyler från bioteknologiska synteser.

7.3 Storskalig separation (preparativ kromatografi)

Vid bioteknologisk produktion krävs att produkten separeras och renas från exempelvis utgångssubstanser och biprodukter. Ett särskilt exempel utgörs av kiral syntes, varvid det bildas två spegelbildsmolekyler (se Figur 2) vilka kan ha helt olika effekter i kroppen och därför ofta måste separeras innan den ena formen kan användas som läkemedel. Ett skrämmande exempel på våndan av att inte avlägsna en oönskad enantiomer utgörs av läkemedlet

Neureosedyn som i slutet av 1950-talet orsakade svåra fostermissbildningar hos gravida användare av det lugnande medlet. I själva verket innehöll medlet två olika varianter av samma ämne, vilka var "spegelbilder" av varandra. Av dessa två hade den ena formen hade den eftersträvande effekten medan den andra orsakade fostermissbildningarna. Det är därför viktigt att kunna separera molekylerna. Vid produktionstillverkning är det viktigt att kunna skilja de två molekylerna åt, så att endast den med medicinskt gynnsam verkan når kroppen i form av ett läkemedel. Vid industriell tillverkning krävs att man kan separera molekylerna i stor skala. Storskalig vätskekromatografi är en ofta använd separationsmetod inom läkemedels- och kemiteknisk industriell produktion, men den är både kostsam och komplicerad att optimera genom ”trial-and-error”.



Figur 2. De båda molekylerna som är spegelbilder av varandra kallas för ”spegelbildsmolekyler” (enantiomerer) och är svårseparerade p.g.a. sin kemiska snarlikhet, trots att de kan ha mycket olika effekt i kroppen. Den ena formen kan vara ett bra läkemedel medan den andra formen kan vara giftig.

7.3.1 Optimering med datorberäkningar

För att de storskaliga separationerna skall bli så effektiva som möjligt och därigenom kunna utföras till kraftigt sänkta produktionskostnader så bör separationerna optimeras, vilket med fördel utförs med datorberäkningar. Datorprogrammen behöver dock ”indata” i form av adsorptionsparametrar som förutsäger (predikterar) hur olika molekylslag konkurrerar med varandra om separationsmediet. De praktiska metoder som finns utvecklade idag för att ta fram dessa parametrar är tidskrävande och kräver dessutom redan ren substans för att kunna genomföras, material som ofta inte finns tillgänglig under processens utvecklingsfas. Vidare så ger de flesta befintliga metoder inte konkurrerande parametrar. Ett välkänt problem vid datasimuleringar är att icke-konkurrerande parametrar ibland resulterar i felaktiga prediktioner beträffande hur substanserna elueras från kolonnen. I dagsläget används inte datorsimuleringar i den utsträckning som skulle kunna vara möjlig om de konkurrerande parametrarna vore enklare att bestämma.

I denna avhandling har nya metoder utvecklats med vilka de konkurrerande parametrarna kan bestämmas snabbare och enklare än vad som tidigare varit möjligt. Med dessa metoder kan parametrarna bestämmas direkt från blandningen av exempelvis två spegelbildsisomerer. En ytterligare fördel är

att bestämningarna kan genomföras med mycket små mängder av de rena substanserna.

7.3.2 Bestämning av adsorptionsparametrar med störningstoppsmetoden

Metoden bygger på att en konstant ström av en molekylblandning pumpas genom den kromatografiska kolonnen tills jämvikt uppnåtts. Därefter störs det jämviktade systemet, vilket svarar genom att framkalla s.k. störningstoppar. Dessa toppar kan efter databearbetning ge den informationen som behövs för att utföra korrekta och informativa simuleringar av separationsförloppet. Emellertid ger befintliga teknikerna ofta otillfredsställande resultat och vid höga platåkoncentrationer registreras exempelvis bara en topp. I denna avhandling har därför ekvationer tagits fram som beskriver hur det injicerade provet skall vara sammansatt för att framkalla båda topparna i en två-komponentblandning utan tillgång till stora mängder ren substans.

I den senare delen av avhandlingen visas hur konkurrerande parametrar från fyra olika komponenter kan tas fram från en blandning på liknande sätt som för två komponenter. Det är första gången någonsin som parametrar för fyra komponenter tas fram samtidigt. Parametrar framtagna med de nya metoderna valideras genom att datorprogrammen predikterar elueringsprofiler, vilka jämförs med experimentella profiler. Överrensstämmelsen mellan experimentella separationer och de av datorn predikterade separationerna är mycket goda. Detta visar att de utvecklade metoderna fungerar väl och har möjligheter att bli både tids och kostnadsbesparande vid industriell processutveckling.

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