LAPPEENRANTA UNIVERSITY OF TECHNOLOGY

Department of Chemical Technology

HIGH THROUGHPUT PURIFICATION OF COMBINATORIAL LIBRARIES BY PREPARATIVE LC/MS

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ABSTRACT

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During the last decade new development in pharmaceutical industry has greatly accelerated the drug discovery process. Combinatorial chemistry has enabled the synthesis of large collections of structurally diverse molecules, so called combinatorial libraries, for biological screening, where the structural activity of the compounds is studied by many different tests and possible hits are identified. Later on leads for new drug candidates are developed. The purity of the synthesised compounds is essential in order to have reliable results from the biological screening. Therefore high throughput purification techniques are required to provide high-quality compounds and reliable biological data. The requirements of ever-increasing throughput have led to the automation and parallelisation of these techniques. Preparative HPLC/MS technology is applied for a fast and effective post synthesis purification of the combinatorial libraries.

Many parameters affect the efficiency of the purification process by prepLC/MS, such as the properties of the separation column and flow gradient. These parameters have to be optimised in order to achieve the best resolution. The performance of alkaline compounds in different flow conditions were studied by preparative LC/MS. The purity analysis by analytical HPLC for a library after the purification by preparative LC/MS was optimised and the purity analysis for some compounds of a crude library also before the purification was implemented.

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Hakusanat: kombinatorinen kemia, komponenttikirjastot, HTP-puhdistus, HPLC, MS, karakterisointi

Viime vuosien nopea kehitys on kiihdyttänyt uusien lääkkeiden kehittämisprosessia. Kombinatorinen kemia on tehnyt mahdolliseksi syntetisoida suuria kokoelmia rakenteeltaan toisistaan poikkeavia molekyylejä, nk. kombinatorisia kirjastoja, biologista seulontaa varten. Siinä molekyylien rakenteeseen liittyvä aktiivisuus tutkitaan useilla erilaisilla biologisilla testeillä mahdollisten “osumien” löytämiseksi, joista osasta saatetaan myöhemmin kehittää uusia lääkeaineita. Jotta biologisten tutkimusten tulokset olisivat luotettavia, on syntetisoitujen komponenttien oltava mahdollisimman puhtaita. Tämän vuoksi tarvitaan HTP-puhdistusta korkealaatuisten komponenttien ja luotettavan biologisen tiedon takaamiseksi. Jatkuvasti kasvavat tuotantovaatimukset ovat johtaneet näiden puhdistustekniikoiden automatisointiin ja rinnakkaistamiseen. Preparatiivinen LC/MS soveltuu kombinatoristen kirjastojen nopeaan ja tehokkaaseen puhdistamiseen.

Monet tekijät, esimerkiksi erotuskolonnin ominaisuudet sekä virtausgradientti, vaikuttavat preparatiivisen LC/MS puhdistusprosessin tehokkuuteen. Nämä parametrit on optimoitava parhaan tuloksen saamiseksi. Tässä työssä tutkittiin emäksisiä komponentteja erilaisissa virtausolosuhteissa. Menetelmä kombinatoristen kirjastojen puhtaustason määrittämiseksi LC/MS-puhdistuksen jälkeen optimoitiin ja määritettiin puhtaus joillekin komponenteille eri kirjastoista ennen puhdistusta.

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LITERATURE SECTION

Symbols and abbreviations

c_{oct}	Concentration of a compound in octanol	, g/l
c_{water}	Concentration of a compound in water	, g/l
P_{ow}	Partition coefficient of a compound between octanol and water	, -
ACD	At Column Dilution	
ACN	Acetonitrile	
DMSO	Dimethysulfoxide	
DMA	Dimethylacetamide	
HPLC	High Performance Liquid Chromatography	
HTP	High Throughput Purification	
HTS	High Throughput Screening	
LC/MS	Liquid Chromatography / Mass Spectrometer	
MS	Mass Spectrometry	
SAR	Structure Activity Relationship	
SLIA	Structure-based Lead Identification and Archiving	
TFA	Trifluoroacetic acid	
THF	Tetrahydrofuran	
UV	Ultraviolet	

1. Introduction

In this thesis the purification of combinatorial compound libraries by preparative LC/MS was studied and the performance of alkaline compounds in different flow conditions was investigated in order to optimise the process conditions. A method to evaluate the compound purity after the preparative LC/MS process was also investigated. Analytical HPLC methods using UV-detection were implemented to determine the purity after the purification process. The purity of some crude libraries was studied also before submitting the compounds to the preparative LC/MS process to find out if the time-consuming purification was worthwhile.

Combinatorial chemistry was introduced in the late eighties as an effective new method for creating large collections of structurally diverse small molecules, so called combinatorial libraries. The size of investments made by most major pharmaceutical companies to implement combinatorial technologies demonstrates its importance today. In recent years the tendency in the pharmaceutical industry has been to develop new, fast and efficient ways to synthesise and biologically screen pharmaceutical compounds in order to generate new drugs. High throughput methods, in other words screening of large numbers of compounds against multiple targets, have been implemented as a means of rapidly identifying hits and developing them into promising lead candidates. Leads are potential candidates for new drugs. The combinatorial chemistry approach has two phases; production of the library and finding the active compound. Combinatorial chemistry has made it possible to accelerate the synthesis of such molecules and, most remarkably, the ability to synthesise large libraries of compounds through automated parallel synthesis and sort-and-combine methodologies [Diggelman et al./1, Kyranos et al./2, Zeng et al./3, Wilson et al./4, Sucholeiki/5].

2. Background

2.1 Combinatorial libraries and the drug discovery process

Figure 2.1 presents schematically the sources and phases of drug discovery.

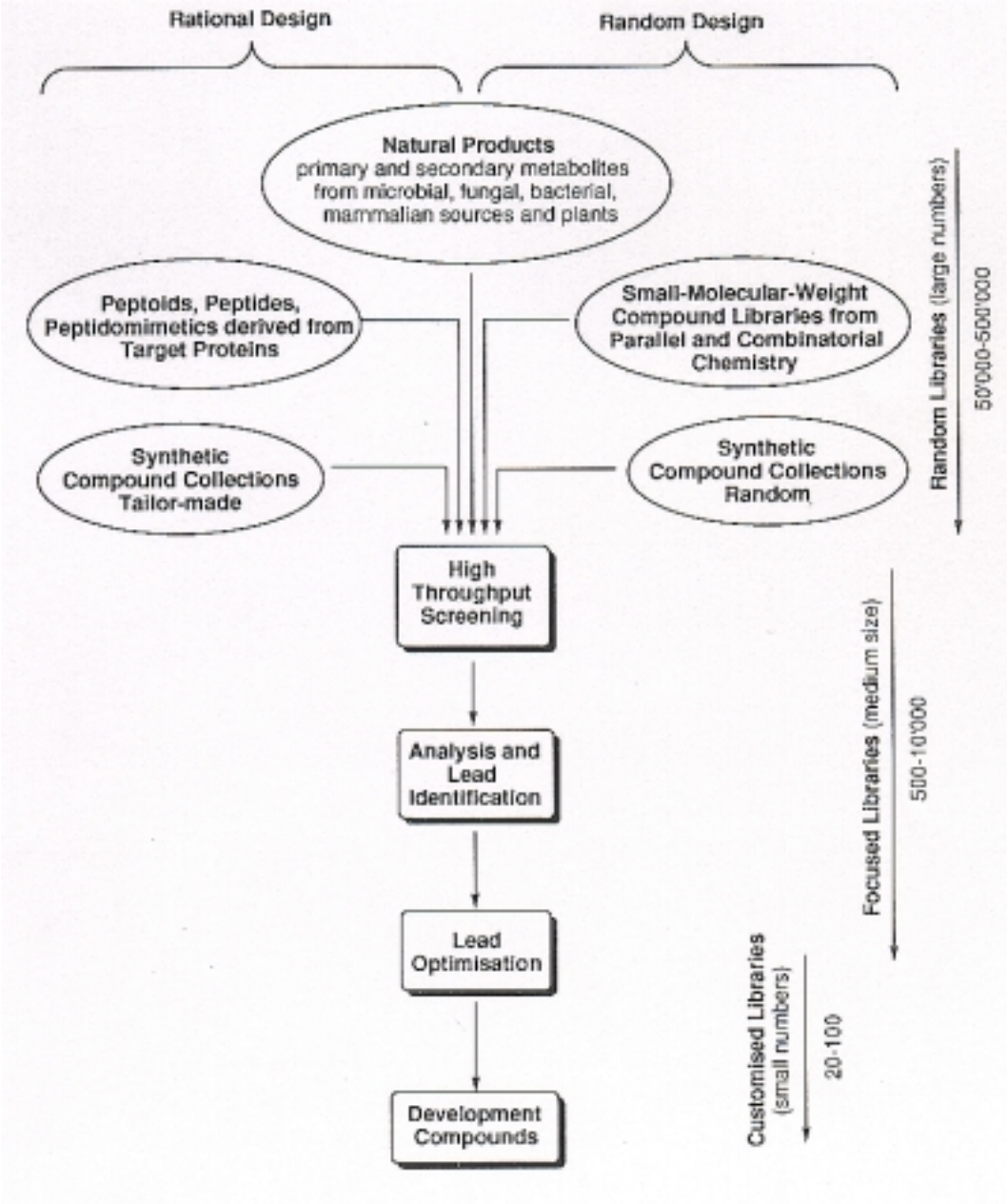


Figure 2.1 The different sources and phases for lead finding.

The traditional source of candidates for new drugs are natural products. They can be isolated from plants, microbes or animals. The lead optimisation of natural compounds is very time-consuming and costs of production of the final drugs are high. However, it is very likely that the importance of the natural products in the lead finding will increase in the future. Another source for new lead candidates are the compound collections that have been accumulated in industry and academic institutions. Their chemical structure is usually more simple compared to the natural products and therefore the leads can be easily optimised and the costs are lower. The peptoids, peptides and peptidomimetics derived from the structural information of the a target protein can also be valuable for lead finding, although their optimisation process is often time-consuming and expensive. An excellent tool for lead finding are the combinatorial small-molecular-weight compound libraries because the optimisation can be made fast and the manufacturing costs are low. A compound library can be defined as a family of compounds that originate from the same assembly strategy and building blocks. All the compounds of a library have the same common structure with different attached substituents. Depending on the strategy of the synthesis, a library can consist of single compounds or mixtures. The only factors limiting the complexity of the compounds are the chemical strategies used, reactive building blocks and the number of available building blocks [Obrecht et al./6].

The purity of these libraries is very important in order to obtain high quality results. If the number of compounds is small, liquid-liquid extraction and solid-phase capture reagents are useful methods in some cases. However, for libraries of a few thousand compounds, preparative HPLC (High Performance Liquid Chromatography) is a powerful alternative. For yet larger numbers of compounds, factors such as solvent consumption and the number of fractions become restrictive [Ripka et al./7, Yurek et al./8].

2.2 The synthesis of compounds

The rapid progress of biological high throughput screening (HTS) methods put a demand for developing high throughput synthesis methods. These methods offer the possibility to screen thousands of molecules a day and therefore a real need for large numbers of small-molecular-weight compounds results. Initially the production of large numbers of compounds was carried out in mixtures and was mostly researched with peptides. This method enabled a more rapid screening. For example, if there was no activity seen in a mixture, all the compounds could be assumed to be inactive. The extensive experience with solid-phase synthesis of peptides was utilised and combined with split-and-mix methods to provide compounds in extensive mixtures. Although being an useful entry into the production of large number of compounds this method had several disadvantages, such as the fact that no purification of products was possible. Other drawbacks were the necessity for deconvolution to identify hits, the small quantities of the compounds prepared, the completeness or actual composition of the library, and large numbers of weakly active compounds, which could lead to many false positive results. The removal towards small-molecular-weight compounds demanded new synthetic strategies. The requirement for ever-increasing throughput has led to the development of ultra-fast methods, parallel high throughput techniques and automation [Wilson et al./4, Obrecht et al./6, Ripka et al./7].

Parallel synthesis is a powerful alternative when libraries of a few thousand to tens of thousands of compounds are produced. It has largely replaced the split-and-mix methods in the generation of compound libraries. In the split-and-mix method the functionalised polymeric resin, which contains many individual beads with reactive sites, is treated with a single monomer. Compared to the polymeric resin a large amount of this monomer is added and a reaction occurs. The unreacted reagent is normally removed by filtration. After this coupling the beads are sorted and treated with a second monomer set, recombined and sorted again. This is repeated until the last set of monomers is added. Parallel synthesis enables the generation of a large collection of individual compounds in much larger quantities of material than split-and-mix methods. In parallel synthesis the number of reaction vessels is equal to the number of final products and it is assisted by robotic devices. Conventional manual methods have allowed a chemist to synthesise from 25 to 50 new compounds a year, whereas parallel synthesis due to its amenability to automation enables a chemist to produce

thousands of new compounds a year [Ripka et al./7, Bannwarth/9, Zeng et al./10, Anonymous/11].

In parallel synthesis the product is one target compound in milligram quantities per well of the synthesis plate. The structure of the target compound is confirmed and the chemical purity defined. New synthetic strategies, polymer supports and polymer-supported reagents and catalysts, novel linker- and split strategies as well as new building blocks are discovered all the time [Ripka et al./7, Obrecht et al./6].

Although a pure compound is targeted, side reactions and unreacted starting materials regularly cause impurities. It has been attempted to optimise the reaction conditions to improve yields and select only the starting materials resulting in good yields in the crude reaction mixtures. Large libraries still usually result in varying purity stages. Besides, to achieve reasonable pure final products, compromises often have to be made when the starting materials are selected. Combinatorial libraries based on solution chemistry, rather than solid-phase chemistry, are even more complicated. Excess reagents can be partially removed by capture reagents, but neutral side products or other contaminants are difficult to remove. Besides, the nature of the capture reagents limits the choice of starting material, because the capture reagents should only interact with the excess reagents and not with the final products. [Ripka et al./7].

In the parallel synthesis individual compounds in single wells of a dispersed array are produced. They have often been mistakenly assumed to be structurally correct and pure compounds, which is true when compared with the situation with the split-and-pool mixtures but not true when compared with the purity state of traditional, indivually synthesised compounds. The target compounds produced as a result of the parallel synthesis do not include the same yields and purity stages across the whole library. Short chemical reaction series can often perform to completion and relatively pure libraries can be produced. In more complicated chemistries on solid-phase the situation is very different. Frequently, the yield of product can be greatly affected if the type of the starting materials is changed. This can be partly avoided by careful selection of only those inputs that result in high yields but this restricts the flexibility in the library design [Ripka et al./7].

In high throughput parallel synthesis the rate limiting step is the purification, isolation and analysis of the target compounds and not the synthesis of the compounds itself. The aim is to create pure compounds for biological screening in order to gain a reliable structure activity relationship (SAR) scan from the testing of these compounds. The time and effort required to characterise these libraries and to isolate and resynthesise active components has greatly increased due to the larger number of compounds [Ripka et al./7, Nemeth et al./12].

3. The need for purification

The results of the biological screening of the impure raw mixtures have led to false positive results and missed active compounds when the yields are low. Time-consuming resyntheses, as well as substantial efforts to confirm positive results become necessary. The idea that a plate of systematically varied molecular structures would lead to SARs has not been realised. To take the full advantage of the potential SAR information it is necessary to know the purity of the compounds, verify the molecular weight and quantify the amounts. The purification of the libraries enables the screening hits to be directly comparable for potency and derivation of SARs as well as the implementation of multiple screens on each compound. Conversely, the purification process consumes plenty of time, since all the few thousand compounds have to be purified and the amount of hits among these is <0.1 % [Ripka et al./7, Yurek et al./8].

The most important properties of the compounds are their potency and selectivity. However, other physical properties, such as solubility and membrane permeability, also have an important role when a new drug candidate is developed. Traditionally, when a compound was selected for further development, the decision was based on a compromise of potency and selectivity and the other physical properties mentioned earlier. Nowadays leads are selected based only on potency and selectivity, optimisation of other properties comes much later. Therefore, in addition to screening for biological properties it could be worthwhile to be able to measure selected physical properties which are important in the lead selection process, including solubility, libophilicity and membrane permeability. The compounds have to be pure in order the reliable results to be available. Then the decision could be based on a compromise of potency, selectivity and important drug-like physical properties [Ripka et al./7].

4. HPLC system for purification

4.1. Preparative HPLC

According to the characterisation of compound libraries the samples are often not pure enough for biological screening. Therefore techniques that can be fully automated, such as preparative HPLC, are used for purification of combinatorial libraries. [Kyranos et al./2].

Traditionally, preparative HPLC systems have been used for the repetitive purification of batches of the same sample with well understood chromatographic properties. In contrast, in high throughput purification there is no prior knowledge of the chromatographic properties of the purified samples. Therefore, generally applicable gradients and peak detection devices are used. Furthermore, collection devices that permit adequate fraction collection are needed. Automated HPLC/MS workstations have been developed to increase the speed of analysis for purifying combinatorial libraries. The HPLC methods used conventionally cannot provide the throughput necessary to handle the size and complexity of combinatorial libraries. Therefore rapid HPLC methods have been developed for the preparative purification of compound libraries. Short columns and fast gradients, typically 7-10 min per sample, have been used to ease analysis and the high-resolution separations these columns can perform have enabled their widespread utilisation in combinatorial chemistry [Kyranos/2, Ripka et al./7, Zeng et al./13].

4.2. Preparative HPLC/MS

Typically, when automated preparative HPLC is used, the fraction collection is triggered upon an observed threshold UV-signal. This leads constantly to the collection of several tubes per each sample. Therefore, a large fraction collector and secondary analysis after the HPLC separation are needed. This consumes plenty of time, when large numbers of compounds are purified. A fully automated and rapid method for preparative scale purification of combinatorial libraries has been developed to ease the purification of compounds. The preparative LC/MS method includes fast chromatography and fraction collection using electrospray ionisation mass spectrometry, where real-time MS ion signals are used to trigger fraction collection. Then only the target mass compound of a compound mixture needs to be identified in order for automated preparative purification to proceed. Thus, per sample only one fraction is collected. This one sample – one fraction paradigm enables batches of compounds to be processed without the need to use large fraction collection beds. Furthermore, there is no need for post-purification screening and pooling in order to identify the purified target fraction. PrepLC/MS can be performed fully unattended and overnight with the capacity to purify multimilligram quantities of single pure compounds from very complex mixtures [Kyranos et al./2, Ripka et al./7].

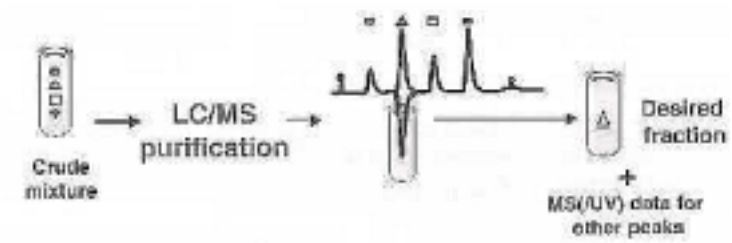


Figure 4.1 Purification based on preparative LC/MS

LC/MS purification system meets many of the requirements of the research and development of drugs [Brailsford/14]:

- scalability for different sample loadings
- speed - more samples by column regeneration
- specifity - only the peaks of interest are collected
- interaction with external systems
- robustness and reproducibility of results
- ease of use

5. The Novartis method for compound purification

5.1 Introduction

Novartis has implemented high throughput purification in their research strategy in combinatorial chemistry. All the compound libraries produced are purified by the preparative LC/MS system before the biological screening in order to obtain reliable results of the possible biological activity of the compounds.

5.2 Instrumentation and process

The preparative purification is performed using the high-pressure Waters 600 gradient pump controlled through Fractionlynx software. The standard flow rate is 20 ml/min. The initial standard flow contains 95 % water and 5 % organic solvent. The linear gradient of organic solvent is from 5 % to 95 % during the gradient run time. The Waters 2700 and Gilson autosamplers are used for injecting the samples. Samples are eluted and separation is made by Waters Symmetry and Waters XTerra prep MS 19×50 mm columns. A splitter divides the flow between the mass spectrometer and the fraction collector. A very small part of the sample (1:1000) is diluted with methanol by the make-up pump and led into a Waters 996 PDA mass detector. The analysis of the molecular weight is performed using a Waters ZMD or ZQ atmospheric pressure electrospray ionisation mass spectrometer. The majority of the sample is carried to the fraction collector. Controlled by software the desired mass is accurately collected by the Gilson 215 fraction collector when the mass spectrometer detects the target molecular mass. Hence the fractionating is controlled with the peak identification and the expected product from each sample present in the input is collected in one fraction and placed at the same position on the output rack. The rest is led into the excess waste. Since products are delivered into individual microtubes, amounts can be obtained by simply weighing the tubes using robotic weighing. The whole system is controlled by Waters MassLynx-software and all the modules except for the make-up pump are directed by the FractionLynx-option of the software. Aside from hardware and software the separation column has a decisive role in a prepLC/MS-system. The detailed information of samples and fractions is saved in a database on a harddisc [Anonymous/11, Burg/15, Chumsae/16, Spatz/17, Burg/18, Burg/19, Godel/20].

Diagram of the equipment arrangement is presented in figure 5.1.

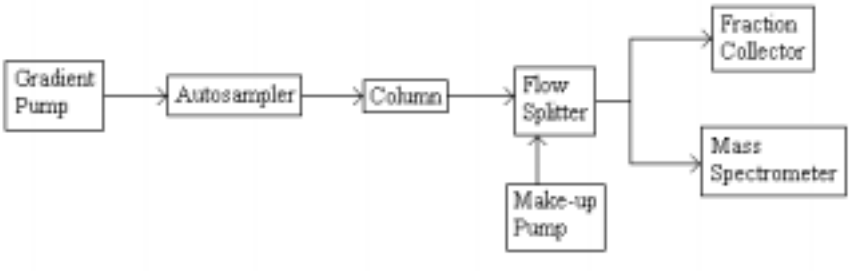


Figure 5.1 FractionLynx system for the routine chromatographic preparation of purified samples from combinatorial libraries.

Each combinatorial library synthesised at Novartis has an identification code name, such as TSA-003. Each library contains a few hundred racks and every rack of a library has its own code number, for example 3456. Each rack contains 96 samples in 12 columns and 8 rows. The vertical columns are identified by numbers and the rows by alphabets. Every sample of a rack can be identified by its position on the rack, for example sample 3456 B7 means the seventh sample of the second row of the rack number 3456 of some library.

6. General process conditions

6.1 Column separation efficiency

There are many parameters that affect the separation efficiency of a column. These include the column length, particle size of the packing material and reverse phase material of the column. Also the pressure capability of the HPLC instrument restricts the column choices. However, these are not the only parameters to be taken into account. Capabilities of the pumping system such as flow rate range or gradient delay volume, detector parameters such as sampling rates and time constants, together with system capabilities such as the overall system band spreading, can affect the result of a separation and should be optimised together with the choice of column length, particle size, and gradient parameters [Neue et al./21].

Optimisation of the LC/MS purification process to achieve shorter and more efficient purification is not very simple. In order to achieve shorter purifying times the effect of the column characteristics on the selectivity and resolution of the separation process needs to be fully understood. The changes in the column and operational parameters such as column length, flow rate and gradient run time, have most influence on the resolution and selectivity of the separation. These operational parameters are used to maximise the desirable aspects of a combinatorial separation which are throughput, resolution and robustness [Carmody et al./22].

6.1.1 Impact of gradient run time, flow and column length

The best resolution can be achieved by the longest gradient time. Long gradient run times require small flow rates. In contrast, the best throughput can be achieved by shortest gradient time, so if only the gradient run time is reduced the resolution is sacrificed. The gradient time must be adjusted to the flow rate in order for the highest throughput to be obtained without sacrificing the resolution. If the gradient is very fast, the resolution obtained with a short column can be very similar to the resolution obtained with a long column. The resolution is higher with a long column only if a slow gradient is used. A shorter column has a lower column back-pressure and is therefore ideal for very fast separations due to the better stability of the column. Therefore, for example a 50 mm column is a good choice for fast separation. However the length of the column has not as much influence on the separation as the number of gradient volumes moving across the

column. By scaling the gradient volume proportionally to the column volume the throughput of a sample can be obtained. The development and optimisation of the gradient methods is usually done empirically. In figure 6.1 the resolution on two different column lengths is presented as a function of time [Neue et al./21, Carmody et al./22].

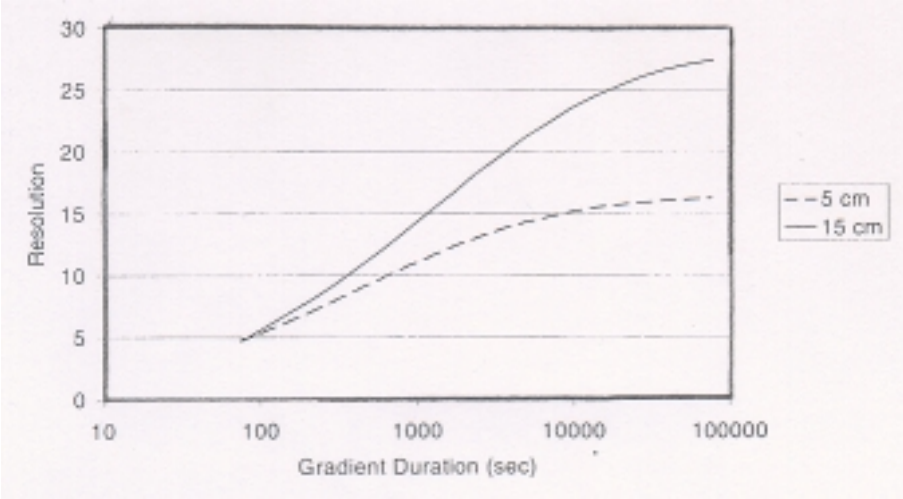


Figure 6.1 A comparison of the performance of two different column lengths.

6.1.2 Effect of particle size

The effect of the particle size on column separation efficiency is very significant. Smaller particle size increases the resolution. By decreasing the particle size and increasing the flow rate an increase in sample throughput can be realised with increasing resolution. Therefore, in order to gain the best resolution and highest throughput for short columns, fast flow rates and small particle size should be applied. When the particle size of a column is optimised, the column back-pressure is the first parameter that should be considered. The back-pressure of a column increases inveresely to the square of the particle size if the length of the column is constant. Thus, column with a large particles reaches the back-pressure limit of the instrumentation at higher linear velocity than a column with small particle size. [Neue et al./21, Carmody et al./22]

6.2 The column material

An important consideration in the column performance is the packing material of the column. Traditionally silica gel is used as the column packing material and it is still most common in the reversed-phase-chromatography. Some other alternatives have been introduced, such as polymer-based materials and also the combinations of both. Generally the polymeric phases have higher sample capacities than monomeric phases as well as wider pH range and better stability. Polymer-based materials can operate at the pH range from 1 to 14, and can easily be cleaned with rather harsh solvents. High-density bonded silica gel-based columns with C18 carbon loadings in the 18-20 % range with monomeric and polymeric bondings should offer more retention and have fewer available silanols to interact with basic compounds [Majors/23, Majors/24].

The most popular carbon chain length is C18 at the range of chain length from C4 to C30. The shorter chain lengths are recommendable for compounds such as proteins and peptides that sometimes interact with the more hydrophobic phases. The longer chains are useful for analysing certain isomers, carotenoids, chlorophyll and flavonoids that display unique selectivities [Majors/23].

Waters Symmetry and XTerra columns with different column lengths, diameters and particle sizes are applied for both analytical and preparative HPLC equipment at Novartis. The Waters Symmetry columns have silicagel as packing material whereas Waters XTerra column contain C_{18}-bonded hybrid organic/inorganic particles. A comparison of the advantages and disadvantages of the inorganic (silica) and organic (polymer) packing material according to Waters is presented in table 6.1 [Anonymous/25].

Table 6.1 A comparison of the advantages and disadvantages of the silica-based and organic column packing material according to Waters.

Packing material	Advantages	Disadvantages
Inorganic C_{18} Silica	Mechanical strength High efficiency Predictable retention	Tailing peaks for bases Limited pH range Chemically unstable
Organic Hybrid Polymer	Wide pH range No tailing for bases Chemically stable	Low efficiency Mechanically weak Unpredictable retention

The traditional silica-based reversed-phase columns are not able to operate for a long time period above pH 8 and therefore restrict the ability to use pH as a selectivity tool. The column material dissolves with an accelerating speed as a greater surface of silanols is exposed with time. XTerra columns have methylsiloxane groups attached throughout the hybrid particle allowing procedures with the pH range from 1 to 12 due to reduced dissolution on the particle surface. The structure of the particles of a Waters XTerra column is presented in figure 6.2 below.

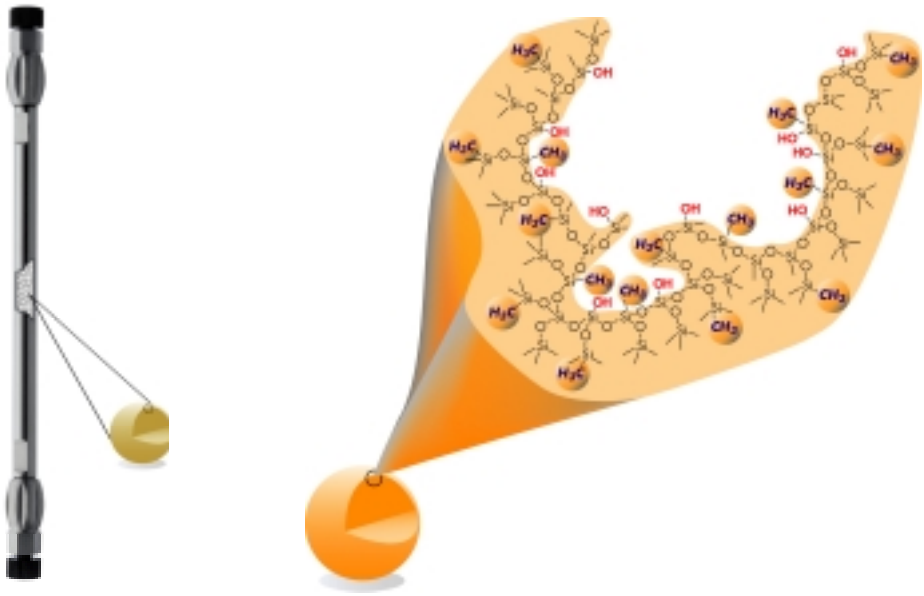


Figure 6.2 Structure of the particles in Waters XTerra columns [Anonymous/25].

The structure and the dissolution of the column packing material at high pH for Waters Symmetry and XTerra columns are presented in figures 6.3 and 6.4 [Anonymous/25].

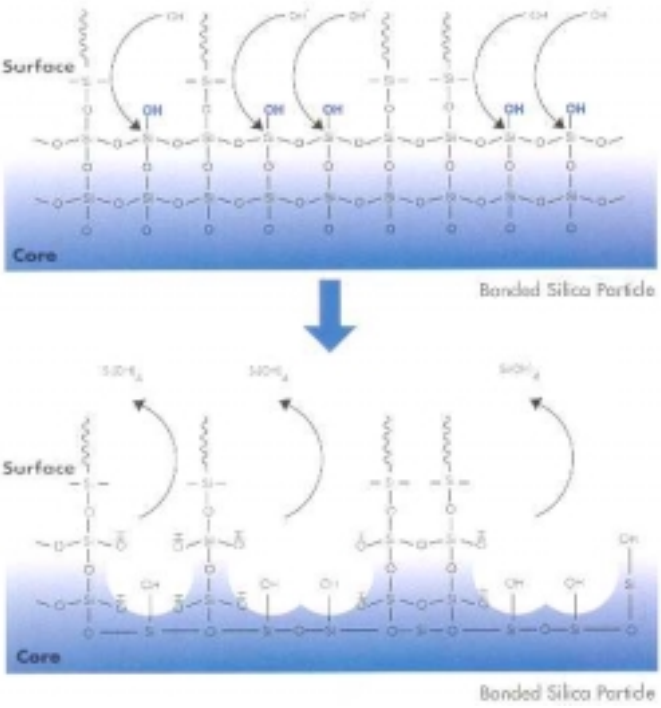


Figure 6.3 The dissolution of a Waters Symmetry column that leads to the eventual bed collapse of the silica-based column material [Anonymous/25].

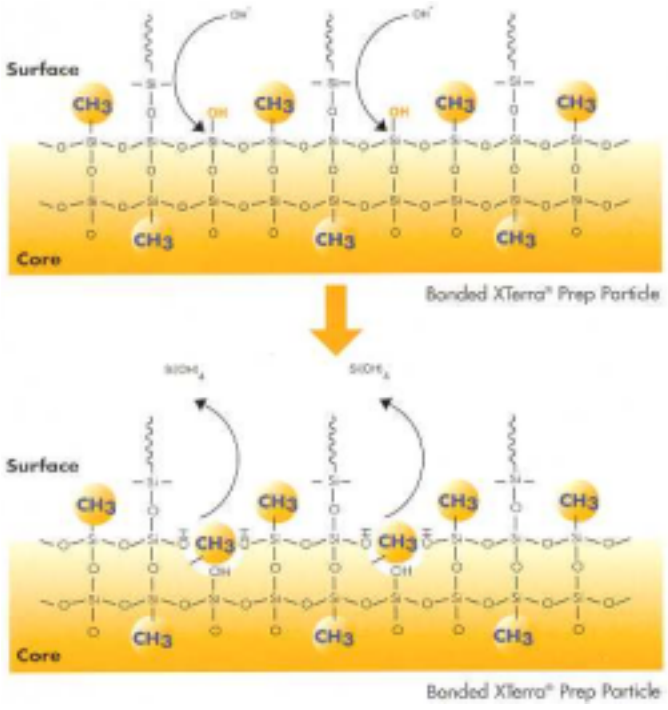


Figure 6.4 The high bonded phase coverage of the Waters XTerra columns minimises the dissolution of the column packing material [Anonymous/25].

6.3 Organic Solvent

Three solvents are generally used as organic modifiers in reversed-phase chromatography: methanol, acetonitrile (ACN) and tetrahydrofuran (THF). The most important factor for fast separations is the viscosity of the mobile phase, since both the pressure drop across the column and the peak width are functions of the viscosity. Methanol and THF have maxima of the dependence of viscosity on their mixtures with water. ACN has only a very small maximum for a mixture of about 10 % ACN in water, as well as a much reduced viscosity compared to mixtures of methanol or THF with water. The reduction in pressure is about 50 % when ACN is used instead of methanol, and the average column plate count is about twice as high with ACN than methanol as organic modifier. Therefore, ACN is the most preferred solvent for fast reversed-phase separations [Neue et al./21].

However, the samples in combinatorial chemistry are usually poorly soluble in an organic solvent such as dimethyl sulfoxide (DMSO) or dimethylacetamide (DMA). The injection of a large amount of organic solvent into a column in 100 % water can result in severe peak distortion or even elution of a large fraction of the sample in the column void volume. If the analytes are dissolved in DMSO, a dilution of the sample with the aqueous buffer (5:1 or even 10:1) together with a larger injection volume gets around this problem. For preparative applications, one would like to dissolve the maximum amount of the sample. Often, DMSO is the best solvent, but an injection of a large volume of DMSO creates peak distortions. The dissolving problem can also be relieved by so called at column dilution (ACD) if the disortions and failures are caused by the strong solvents and large injection volumes rather than over-loading of the column packaging. The sample is diluted by a small solvent flow at injection, and the majority of the flow as well as the actual gradient is mixed with the sample just before the sample enters the column [Neue et al./21, Blom 26].

EXPERIMENTAL SECTION

PART I: ULTRA HIGH THROUGHPUT PURIFICATION OF ALKALINE COMPOUNDS

7. Introduction

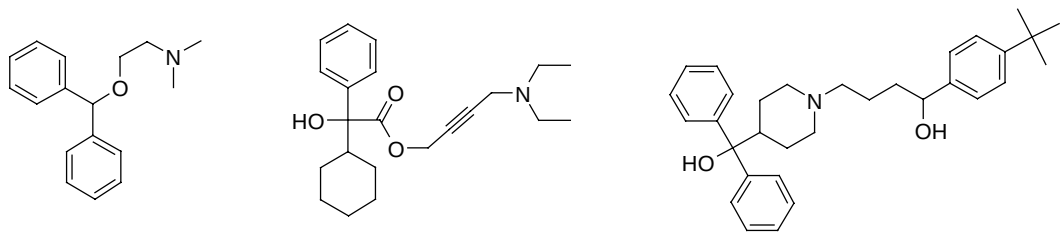
In the first part of the experimental section of this thesis the ultra high throughput purification of compounds was performed by preparative LC/MS. The aim of the research was to examine the performance of alkaline compounds using different buffers, flow gradients, columns and flow rates. The loading capacity of the equipment was also studied.

8. Measurements in different flow conditions

8.1 Measurements in alkaline and acid flow

8.1.1 The Waters results

First, some of the results of fast throughput purification at high pH for alkaline compounds presented by the manufacturer Waters were reproduced. Three compounds were separated on XTerra Prep MS C_{18} columns. These compounds were diphenhydramine, oxybutynin and terfenadine. The structure of the compounds is shown in figure 8.1.



diphenhydramine oxybutynin terfenadine

Figure 8.1 Chemical structure of diphenhydramine, oxybutynin and terfenadine.

In all the experiments done by Waters the mobile phase consisted of 10 mM NH_4HCO_3-buffer with pH 10 and ACN. From 0 to 1.1 min the linear flow gradient of ACN was from 5 % to 40% and from 1.1 min to 7.5 min from 40 % to 90 %. The injection volume was 500 µl in each experiment and the concentration of the analytes was 20 mg/ml DMSO. The flow rates and columns used in the experiments can be seen in table 8.1 and the results in figure 8.2 [Anonymous/27].

Table 8.1 The flow rates and columns used in the fast throughput purification experiments for basic compounds at high pH done by Waters. 10 mM NH_4HCO_3-buffer with pH 10 and ACN were used as the mobile phase. The injection volume was 500 µl and the concentration of the analytes was 20 mg/ml DMSO. The analytes used were diphenhydramine, oxybutynin and terfenadine.

Waters experiments	**Flow rate, [ml/min]**	**Column size, [mm]**
1	20	19×50
2	20	19×30
3	40	19×30

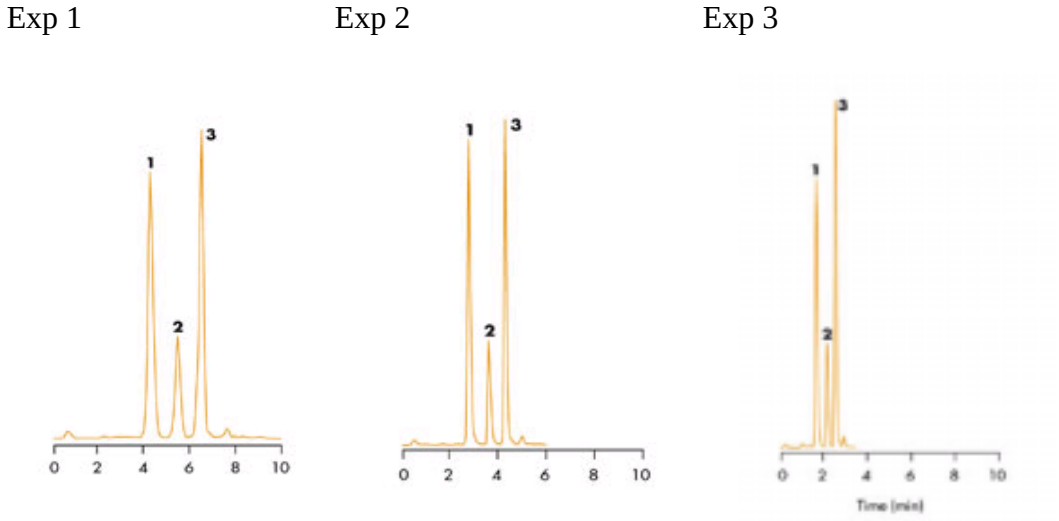


Figure 8.2 The results for fast purification for basic compounds at high pH produced by the column manufacturer Waters. The compounds were diphenhydramine (1), oxybutynin (2) and terfenadine (3). 10 mM NH_4HCO_3-buffer with pH 10 and ACN were used as the mobile phase. The injection volume was 500 µl and the concentration of the analytes was 20 mg/ml DMSO.

8.1.2 Experimental plan for alkaline and acid flow

Some of the experiments presented by Waters were repeated. Two of the compounds used by Waters, diphenhydramine and terfenadine, were used in the experiments. The plan was to test these compounds in both alkaline and acid media, and to find out if there were any residuals of the buffer in the concentrated samples. The buffer, the gradient of ACN and the column used in the experiments are shown in table 8.2.

Table 8.2 The experimental plan for diphenhydramine and terfenadine in alkaline and acid flow.

Experiment number	**Buffer**	**Gradient of ACN**	**Column**
1	NH_4HCO_3	0-1 min:5-40%, 1.1-7.5min:40-90%	XTerra prep MS C_{18} 19×50 mm
2	$(NH_4)_2CO_3$	0-1 min:5-40%, 1.1-7.5min:40-90%	XTerra prep MS C_{18} 19×50 mm
3	0.1 % TFA in H_2O	0-1 min:2-40 %, 1.1-7.5 min:40-90%	XTerra prep MS C_{18} 19×50 mm
4	0.1 % TFA in H_2O	0-1 min:2-40 %, 1.1-7.5 min:40-90%	Grom-Sil 120 ODS-3 CP 50×20 mm

Some experiments were done with a selection of Novartis compounds. Benzimidazoles were used since they represent a typical example of combinatorial library compounds at Novartis. The buffer, the gradient of ACN and the column used are shown in table 8.3.

Table 8.3 The experimental plan for benzimidazoles in basic and acid flow.

Experiment number	**Buffer**	**Gradient of ACN**	**Column**
5	NH_4HCO_3	0-1 min5-40%, 1.1-7.5min:40-90%	XTerra Prep MS C_{18} 19×50 mm
6	0.1 % TFA in H_2O	0-1 min:2-40 %, 1.1-7.5 min:40-90%	Grom-Sil 120 ODS-3 CP 50×20 mm
7	0.1 % TFA in H_2O	0-1 min:5 %, 1.1-7.5 min:5-40%	XTerra Prep MS C_{18} 19×50 mm

8.1.3 Diphenhydramine and terfenadine in basic and acid flow

8.1.3.1 LC/MS Equipment

The experiments were performed using the high-pressure Waters 600 gradient pump controlled through Fractionlynx software. Samples were eluted and separation was made with Waters XTerra Prep MS C_{18} 19×50 mm and Grom-Sil 120 ODS-3 CP 50×20 mm columns in the room temperature. A small part of the sample was diluted by the make-up pump and led into a Waters 996 PDA mass detector. The analysis of the molecular weight was performed using a Waters ZQ atmospheric pressure electrospray ionisation mass spectrometer. No fraction collector was used in these experiments.

8.1.3.2 NH_4HCO_3 as buffer system

A basic 10 mM buffer was made for the experiments by dissolving 2 g NH_4HCO_3 in 2.5 l water and adjusting the pH to 10 with ammonia. The concentration of diphenhydramine and terfenadine was 20 mg/ml DMSO.

First, diphenhydramine was analysed. During each run the amount of alkaline buffer in the flow was constant, 10 %. The gradient of ACN was 5-40% from 0 to 1 min, and 40-90 % from 1.1 to 7.5 min. The rest of the flow was water. The flow rate was 20 ml/min and the XTerra Prep MS C_{18} 19×50 mm column with particle size 5 µm was used for the experiments. The sample injection volume was 100 µl. Terfenadine and a mixture of both compounds were examined at the same conditions. The mixture contained the same amount of each compound in DMSO. Only the gradient time of terfenadine and the mixture was longer because of the longer resolution time of terfenadine. The concentration of ACN was a constant 90 % from 7.5 to 10 min.

8.1.3.3 $(NH_4)_2CO_3$ as buffer system

To study the difference between NH_4HCO_3 and $(NH_4)_2CO_3$ another basic 10 mM buffer was made for the experiments by dissolving 2.4 g $(NH_4)_2CO_3$ into 2.5 l water and adjusting the pH to 10 with ammonia. The gradient of the flow and the flow rate were identical to the experiments made with the NH_4HCO_3 buffer. The same column was used and the injection volume was 100 µl. Diphenhydramine and terfenadine were examined both separately and mixed.

8.1.3.4 TFA buffered eluent

Both components and their mixtures were also examined with TFA buffered eluents. The experiments were carried out with the linear gradient of ACN being 2-40 % during the first minute, 40-90 % from 1.1 to 7.5 min and with the constant concentration of ACN 90 % from 7.5 to 10 min. The rest of the flow was water with 0.1 % TFA. The flow rate was 20 ml/min. Experiments were performed on two columns, XTerra Prep MS C_{18} 19×50 mm and Grom-Sil 120 ODS-3 CP 50×20 mm. Both columns had the particle size 5 µm.

8.1.3.5 Determination of the amount of inorganic salts in the product

Three measurements were made for the flow after column with both buffers to determine if there were remains of NH_4HCO_3 or $(NH_4)_2CO_3$ in the product. Three times 8 ml of the flow was collected, evaporated and weighed.

8.2 Results and discussion for alkaline and acidic flow

8.2.1 Reference compounds in alkaline and acidic flow

Examples of the results for diphenylhydramine and terfenadine in alkaline and acid flow can be seen in figure 8.3.

The chromatograms indicated with numbers 1 and 2 the alkaline buffer system was used at the run, NH_4HCO_3 in the first one and $(NH_4)_2CO_3$ in the second one. All the other conditions were identical for the samples. The chromatograms 3 and 4 show the performance of the compounds on two different columns in acidic elution system.

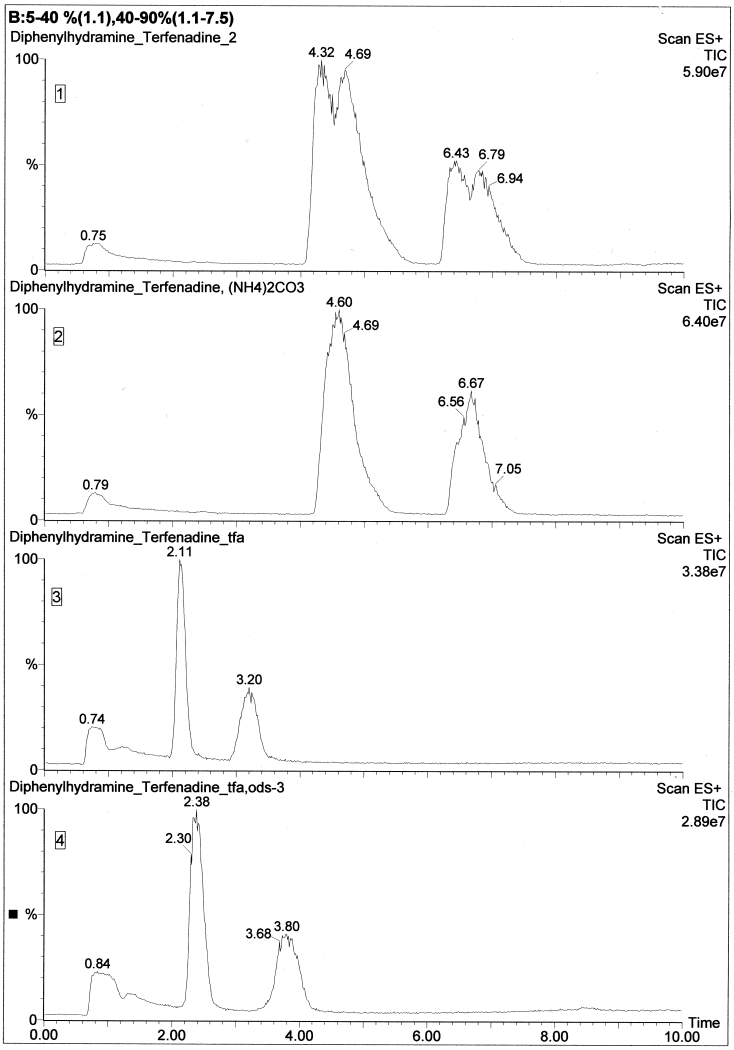


Figure 8.3 The performance of diphenhydramine and terfenadine on preparative LC/MS system when the flow rate was 20 ml/min, injection volume 100 µl, columns Waters XTerra prep MS C_{18} 19×50 mm (1-3) and Grom-Sil 120 ODS-3 CP 50×20 mm (4) and the buffer systems NH_4HCO_3 (1), $(NH_4)_2CO_3$ (2) and 0.1 % TFA in water (3 and 4).

The difference of the performance of the reference compounds on two different alkaline eluent systems can be seen in the chromatograms 1 and 2 in figure 8.3. When NH_4HCO_3 is used as the buffer, the peaks seem to appear earlier and they are wider. However, the more conspicuous factor when the results of NH_4HCO_3 and $(NH_4)_2CO_3$ is compared is the shape of the peaks. With NH_4HCO_3 there is more peak distortion and the peaks of both the reference compounds are divided in two. But when both the chromatograms with alkaline buffer are compared with the appearance of the compounds with acidic eluent system the difference in the shape of the peaks is significant. When acidic TFA buffer is used, the peak shape is much more symmetric and the peaks are not as wide as they are on the alkaline eluent system. On the other hand, the peaks appear earlier and therefore the alkaline eluent system could be applied for polar compounds that resolute in the very beginning of the gradient when acidic buffer system is used. The performance of the Waters XTerra column is better than the performance of the Grom-Sil ODS-3 column when the peak shapes are compared and therefore the Waters XTerra column is recommendable also in the experiments with the acidic buffer system.

Thus, the results accomplished by the column manufacturer Waters could not be repeated. Apparently this is due to the very optimised conditions performed for an advert of the manufacturer in order to improve the sale of the product. Such conditions are very likely impossible to implement in practical purification approach and the information published by the manufacturer does not alone ensure the promised results. However, the Waters experiments have obviously been done by using UV-detector instead of MS and therefore it could be worthwhile to repeat the experiments on basic eluent system by preparative HPLC using UV as the detector.

8.2.2 Inorganic salts in the product

The amount of inorganic salts in the concentrated samples was measured three times for both NH_4HCO_3 and $(NH_4)_2CO_3$. The results are shown in table 8.4. No significant amounts of NH_4HCO_3 or $(NH_4)_2CO_3$ could be detected and therefore both buffer systems are suitable for the designated use. However, NH_4HCO_3 was preferred due to its slightly smaller presence in the samples.

Table 8.4 Amounts of NH_4HCO_3 and $(NH_4)_2CO_3$ in the flow after column. The flow rate was 20 ml/min, collected amount of flow 8 ml and the column XTerra Prep MS C_{18} 19×50 mm.

Measurement	**NH_4HCO_3, [mg]**	**$(NH_4)_2CO_3$, [mg]**
1	0.1	0.4
2	0.0	0.0
3	0.1	0.1

8.3 Experiments with benzimidazoles in alkaline and acidic flow

Three benzimidazoles were chosen to be examined. These were 2-aminomethylbenzimidazole, benzimidazole and PKF 103-397, which is a code name for a compound synthesised at Novartis. The concentration of each compound was 20 mg in 1 ml DMSO.

Each compound was analysed separately and mixed in an acid flow. During the run ACN had a gradient 2-5 % during the first minute, 5-40 % from 1.1 to 7.5 min and constant concentration 90 % from 7.5 to 10 min. The rest of the flow was water with 0.1 % TFA. The flow rate was 20 ml/min, the column used was Grom-Sil 120 ODS-3 CP 50×20 mm and injection volume was 100 µl.

The compounds were examined in a basic flow using NH_4HCO_3-buffer and XTerra Prep MS C_{18} 19×50 mm column. During the run the amount of basic buffer in the flow was constant, 10 %. The linear gradient of ACN was 5-40 % from 0 to 1 min, and 40-90 % from 1.1 to 7.5 min. The flow rate was set to 20 ml/min and the sample injection volume was 100 µl.

Alternative gradient conditions were also studied. The initial hold of ACN was 5 % for 1 min, followed by 5-40 % linear gradient from 1.1 to 7.5 min and constant 40 % from 7.5 to 10 min. The concentration of NH_4HCO_3-buffer was constant 10 %. The flow rate was set to 20 ml/min and injection volume 100 µl. The XTerra Prep MS C_{18} 19×50 mm column was used for the examinations.

8.4 Results and discussion for the benzimidazoles in alkaline and acidic flow

Examples of the results for the three benizimidazoles in alkaline and acid flow can be seen in figure 8.4. In the chromatogram indicated with number 5 the performance of all three compounds on alkaline NH_4HCO_3 buffer system is presented. In chromatogram 6 the performance of the compounds on acidic elution system can be seen.

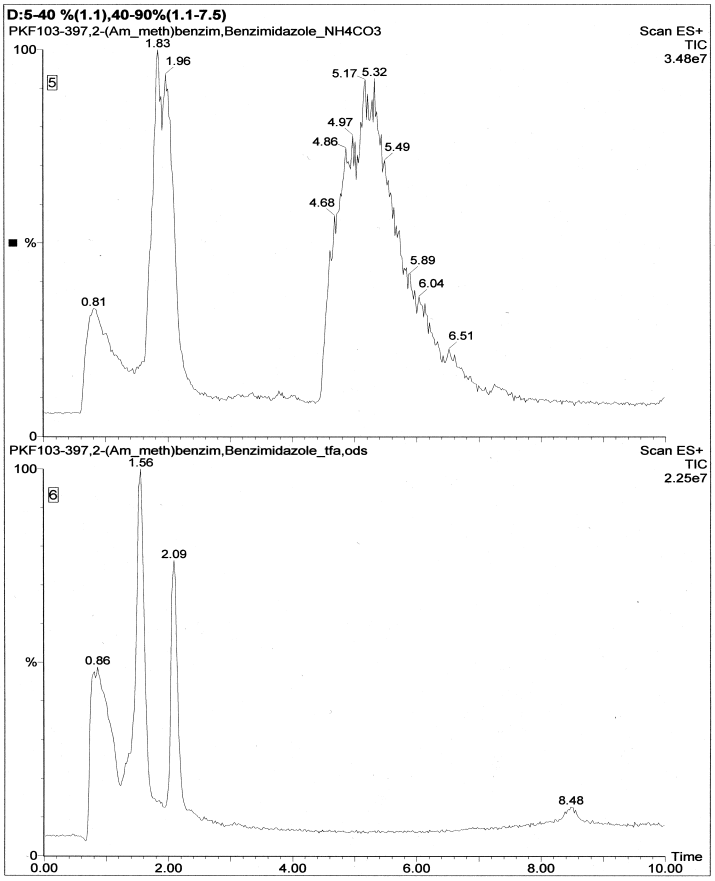


Figure 8.4 The performance of 2-Aminomethylbenzimidazole, benzimidazole and PKF 103-397 on preparative LC/MS system when the flow rate was 20 ml/min, injection volume 100 µl, columns Waters XTerra prep MS C_{18} 19×50 mm (5) and Grom-Sil 120 ODS-3 CP 50×20 mm (6).

The difference in the performance of the reference compounds on basic and acidic eluent systems can be seen in chromatograms 5 and 6 in figure. In the NH_4HCO_3 eluent system in chromatogram 5 the peaks appear later than on TFA system but again the peaks are very wide, especially for the compound that appears last. When acidic TFA buffer is used, the peak width and symmetry are much better although exactly the same amounts and concentrations of the compounds are used. Again the alkaline eluent system could be used to separate polar compounds.

9. The loading capacity

9.1 Experimental plan for loading capacity

Next, the loading capacity of the preparative columns was studied. Diphenylhydramine and terfenadine were used as reference compounds. According to the manufacturer significant amounts of substances could be loaded to preparative LC/MS. The plan was to first use the same concentration of the compounds in DMSO as was used in the experiments with acid and basic flow, 20 mg/ml DMSO. Only a larger injection volume was acquired. Thereafter a larger amount would be analysed. If the analysis with a larger amount of compound gives a good result the amount to be analysed is increased.

9.2 Determination of the loading capacity for diphenylhydramine and terfenadine

In all the measurements with diphenylhydramine and terfenadine in alkaline and acid flow the sample amount was 1 mg in 100 µl DMSO. First, a 10 mg sample of each diphenylhydramine and terfenadine in 500 µl DMSO was analysed. The compounds were analysed both separately and in a mixture. During the run the gradient of ACN was 2-5 % during the first minute, 5-40 % from 1.1 to 7.5 min and had a hold at 90 % from 7.5 to 10 min. 10 mM NH_4HCO_3 with pH 10 was used as buffer and its performance in the flow was 10 %. The flow rate was set to 20 ml/min and Waters XTerra Prep MS C_{18} 19×50 mm column was used.

Since the results at 10 mg were acceptable, the injection volume was increased. A sample of 100 mg was analysed with the injection volume 500 µl. For the injection 50 mg diphenylhydramine was dissolved in DMSO and 50 mg terfenadine in DMA. The gradient of ACN was 2-5 % during the first minute, 5-40 % from 1.1 to 7.5 min and had a hold at 90 % from 7.5 to 12 min. 10 mM NH_4HCO_3 with pH 10 was used as buffer and its performance in the flow was 10 %. The flow rate was 20 ml/min and the XTerra Prep MS C_{18} 19×50 mm column was used.

9.3 The results for the loading capacity of the reference compounds

Examples of the results for the loading capacity experiments are presented in figure 8.5. In all the chromatograms all the other conditions are identical and only the amount of the reference compounds injected was changed. In chromatogram identified with number 7 altogether 1 mg of reference compounds was injected in 100 µl DMSO, in chromatogram 8 10 mg in 500 µl DMSO and in chromatogram 9 100 mg in 500 µl DMSO.

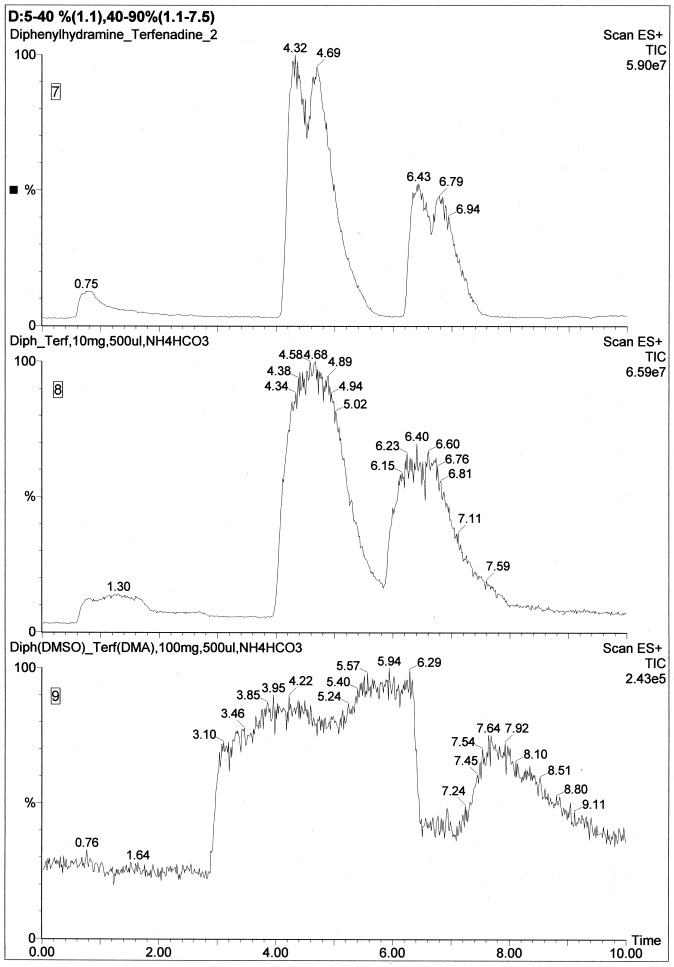


Figure 9.1 The performance of diphenhydramine and terfenadine on preparative LC/MS system when the flow rate was 20 ml/min, column Waters XTerra prep MS C_{18} 19×50 mm and injected amounts 1 mg/100µl DMSO (7), 10 mg/500µl DMSO (8) and 100 mg/500µl DMSO.

As can be seen in the three chromatograms in figure 8.5, the peak distortion increases when the injection volume is increased due to the overloading. Thus, the loading of 100 mg reference compounds in 500µl DMSO is already far too much and a satisfactory resolution cannot be achieved. The peaks become wide and shapeless because such big amounts cannot be separated. Therefore, the loading capacity of the columns is very limited. Due to the poor results the experiments were not continued.

PART II: DETERMINATION OF THE PURITY OF SLIA-COMPOUNDS

10. Introduction

In the second part of the experimental section of this thesis a method to investigate the purity after the purification process by preparative LC/MS for compound libraries produced at Novartis was developed. The aim was to analyse all the purified SLIA-compounds with this method. The abbreviation SLIA means structure-based lead identification and archiving. Some racks of compound libraries were analysed also before the purification in order to see if the synthesis of these compounds had been succesful enough for the time-consuming purification process to be worthwhile.

11. The determination of the purity of a combinatorial library by analytical HPLC

11.1 Experimental design

Some racks of the combinatorial library TSA-003 were analysed by analytical HPLC using different columns and eluent systems. The following columns were used in the experiments:

- Waters XTerra MS C_{18} 4.6×30 mm; 2.5 µm
- Waters Symmetry C_{18} 2.1×50 mm; 3.5 µm
- Waters XTerra MS C_{18} 3.0×30 mm; 2.5 µm
- Waters XTerra MS C_{18} 3.0×30 mm; 3.5 µm
- Waters Symmetry MS C_{18} 3.0×150 mm; 5 µm

The approximate optimal flow rates received from the manufacturer Waters and presented in table 11.1 would be taken into account.

Table 11.1 The approximate optimal flow rates for different column sizes and gradient run times presented by the column manufacturer Waters (* at or outside pressure limit of current instrumentation) [Anonymous/27].

d_p t_g	5 µm 1 min	5 µm 2 min	5 µm 4 min	3.5 µm 1 min	3.5 µm 2 min	3.5 µm 4 min	2.5 µm 1 min	2.5 µm 2 min	2.5 µm 4 min
4.6x50	**7.5**	**5.0**	**2.5**	**10.0 ***	**6.0 ***	**3.5**	**10.0 ***	**6.0 ***	**3.5 ***
4.6x30	**5.0**	**3.0**	**2.0**	**6.5**	**3.5**	**2.5**	**7.0 ***	**4.0**	**3.0**
4.6x20	**4.5**	**2.0**	**1.5**	**4.5**	**2.5**	**2.0**	**5.0**	**3.0**	**2.0**
2.1x50	**1.5**	**1.0**	**0.5**	**2.0 ***	**1.2 ***	**0.7**	**2.0 ***	**1.2 ***	**0.7**
2.1x30	**1.0**	**0.6**	**0.4**	**1.3**	**0.7**	**0.5**	**1.4 ***	**0.8**	**0.6**
2.1x20	**0.9**	**0.4**	**0.3**	**0.9**	**0.5**	**0.4**	**1.0**	**0.6**	**0.4**

The experiments were implemented first by testing the Waters XTerra MS C_{18} 4.6×30 mm column with the particle size 2.5 µm. According to table 11.1 the optimal flow rate for this particular column was 4 ml/min if the gradient run time is 2 min. One or more racks were analysed with this method and some random samples from each on the longer Waters Symmetry MS C_{18} 3.0×150 mm column to compare the results. Next, some racks were analysed on the Waters XTerra MS C_{18} 3.0×30 mm column with particle sizes 2.5 µm and 3.5 µm. Some experiments were carried out on the Waters Symmetry MS C_{18} 2.1×50 mm column to see if the chromatograms were different. Different flow rates and gradient run times were examined.

11.2 Performance of the compounds in different conditions

11.2.1 Preliminary experiments

11.2.1.1 Acidic eluent system

Initially, the library TSA-003 was analysed with analytical Waters Alliance HT HPLC with UV-detector using acid TFA buffer. The wavelength for UV was from 220 to 310. For the first experiments the flow rate was set to 4 ml/min and the gradient run time to 2 min. ACN had a linear gradient from 5 to 95 % from 0 to 1.74 min and a constant concentration 95 % for the rest of the run. The column was Waters XTterra MS C_{18} 4.6×30 mm with particle size 2.5 µm. The temperature was 60 °C in all the experiments. Three racks were analysed with this method. The samples of the racks were prepared by an automatic laboratory robot. For the first rack, the injection volume was set to 50 µl for the first two samples and 25 µl for the rest of the samples on the rack as well as for the second rack. For the third rack the injection volume was set to 10 µl.

Some samples from each rack were analysed on a longer column with smaller flow rate to find out if the results obtained matched with the results on the shorter column. The samples were diluted with ACN before the run if necessary. The column was Waters Symmetry MS C_{18} 3.0×150 mm with the particle size 5 µm. The flow rate was 1.75 ml/min and the gradient run time 7 min. The gradient of ACN was 5-100 % from 0 to 7 min.

The effect of a slower flow and longer gradient run time on the performance of the compounds were studied. The flow was set to 3 ml/min while no other conditions were changed. Some samples of the rack 3310 were analysed with this method and the results were compared to the results from faster flow. For the experiments with longer gradient the run time was set to 3 min instead of 2 min, flow rate was held at 4 ml/min. The linear gradient of ACN was 5-95 % from 0 to 2.7 min and had a hold at 95 % for the rest of the run.

Some samples were diluted with ACN in order to see if better separation would be achieved by diluting the sample. First, 10 µl of automatically prepared sample was diluted with 50 µl ACN. To be certain of the effect of dilution another 10 µl sample was diluted with 80 µl ACN.

11.2.1.2 Results and discussion for the preliminary experiments in acidic eluent system

The chromatograms of the following compounds in table 11.2 are presented as examples of the results.

Table 11.2 Rack numbers and sample positions presented as examples of the results for different kind of experiments.

Experiment type	**Rack number and sample position**
Preliminary experiment	3302:E12
Longer gradient run time and dilution with ACN	3310:D5, C2
Slower flow rate	3310:G5, G7

Examples of the results of library purification analysis can be seen in figures 11.1 and 11.2. By comparing the results on the short Waters XTerra MS C_{18} 4.6×30 mm; 2.5 µm column with the results on the longer Waters Symmetry MS C_{18} 3.0×150 mm; 5 µm column, it is obvious that the method used for analysing the library racks was not optimal. In figures 11.1 and 11.2 there is a chromatogram from rack number 3302 position E12 performed with analytical HPLC on short and long column. In the chromatogram in figure 11.1 only one peak can be seen. The same sample in figure 11.2 shows several peaks. According to the chromatograms the purity of the libraries cannot be determined with this method since the compounds of the sample are not separated on the short column. The same observation could be made with several other samples.

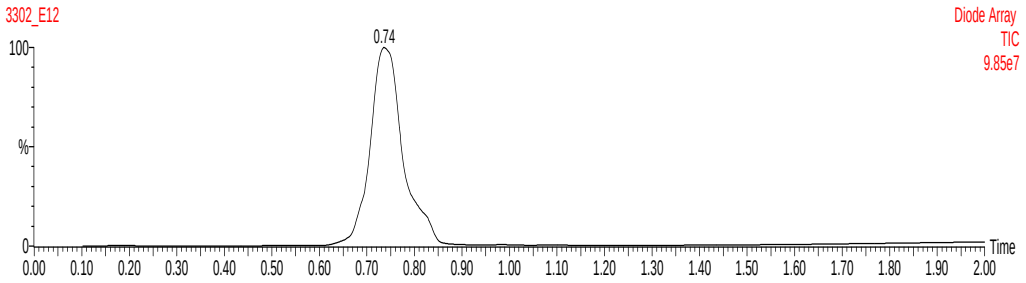


Figure 11.1 Chromatogram of the sample E12 from rack 3302 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm. The flow rate was 4 ml/min, the gradient run time 2 min and the wavelengths from 220 to 310 were measured by the UV detector.

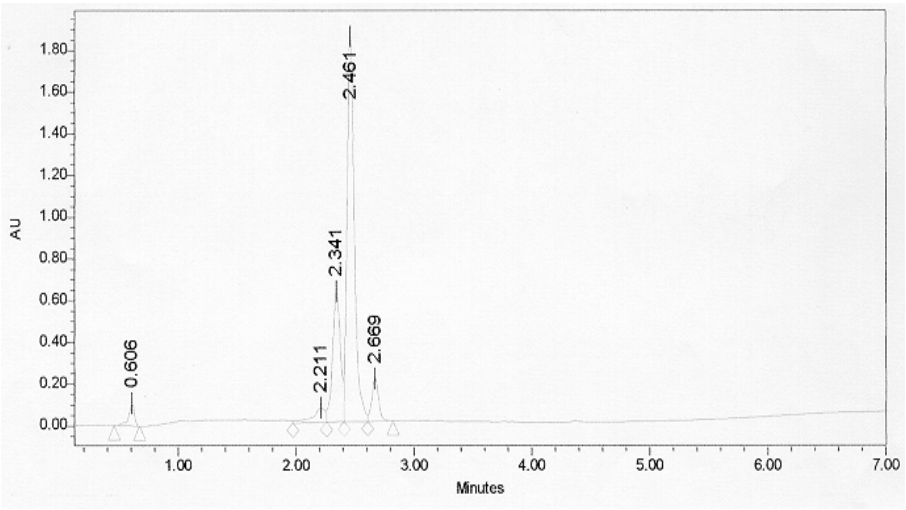


Figure 11.2 Chromatogram of the sample E12 from rack 3302 on Waters Symmetry MS C_{18} 3.0×150 column at the wavelength 214 nm. The flow rate was 1.95 ml/min and the gradient run time 7 min.

For the rack 3310 the injection volume was set to 10 µl instead of 25 µl. Obviously lowering the injection volume alone did not have enough positive effect on the separation. However, for the rest of the samples the injection volume was 10 µl.

The effect of the longer gradient time, higher flow rate and dilution of the sample were examined. The experiments were carried out on the rack 3310. The results with the injection volume 10 µl and longer gradient run time can be compared with the results of shorter gradient run time. It is clear that longer gradient run time only made the the peaks occur later, but had no affect on their separation as can be seen by comparing figures 11.3 and 11.4 where the run time is 2 min for the first and 3 min for the latter. For another sample that included more impurities, like the sample C2 in figures 11.6 and 11.7, the peak separation is already better, but the method should be acceptable for all the samples and therefore longer gradient time does not meet the demands. Furthermore, a shorter gradient rise time is always preferrable. A difference of one minute in the run time means 96 min difference for one rack, but if a library consists of 100 racks, the time needed for analysis is already seven days longer.

Diluting the sample with ACN slightly improved the peak separation, but the peaks were still not separated well enough. The purity of the the product after high throughput purification could still not be determined reliably. In figure 11.5 in the sample D5 from rack 3310 is diluted with ACN at the ratio 1:5 and in figure 11.5. The sample C2 from the same rack is first diluted with the ratio 1:5 in figure 11.8 and in figure 11.9 the amount of ACN is increased with 50 µl. The separation of the peaks does not differ significantly from the experiments done at the normal dilution volume and the baseline is not stable due to an overly diluted sample.

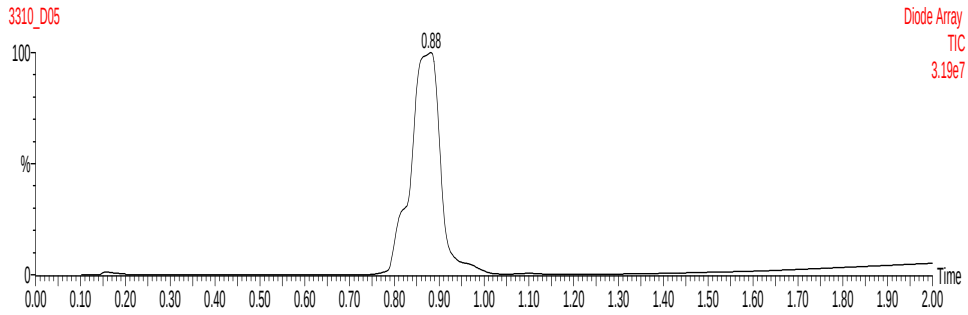


Figure 11.3 Chromatogram of the sample D5 from rack 3310 on Waters XTerra MS C_{18} 4,6×30 mm column with particle size 2.5 µm. The flow rate was 4 ml/min and the gradient run time 2 min.

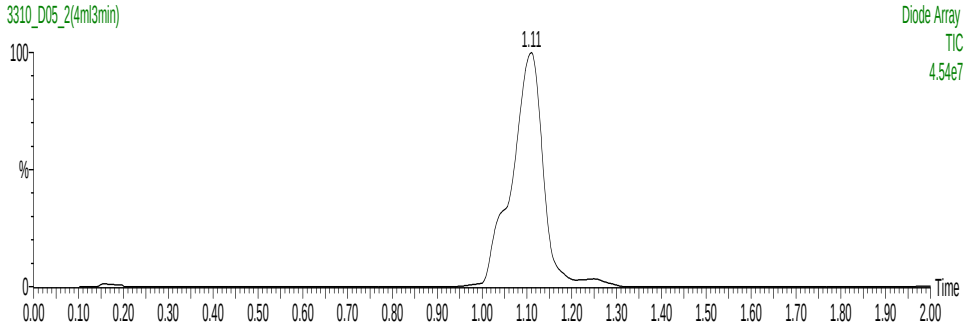


Figure 11.4 Longer gradient run time. Chromatogram of the sample D5 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm. The flow rate was 4 ml/min and the gradient run time 3 min.

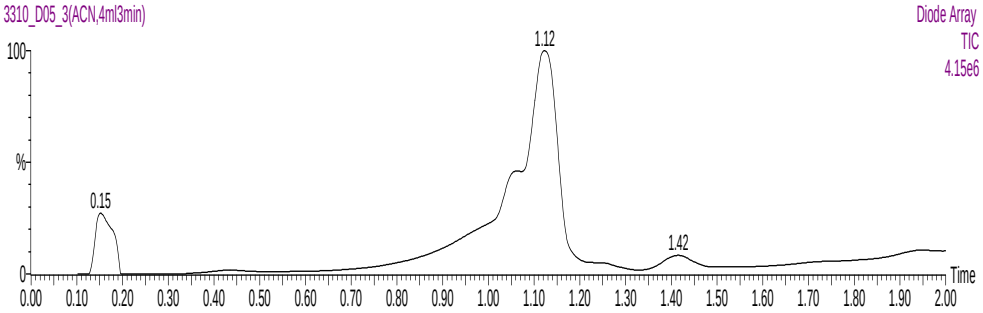


Figure 11.5 Dilution of the sample and longer run time. Chromatogram of the sample D5 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm. The automatically prepared sample was diluted with ACN, the flow rate was 4 ml/min and the gradient run time 3 min.

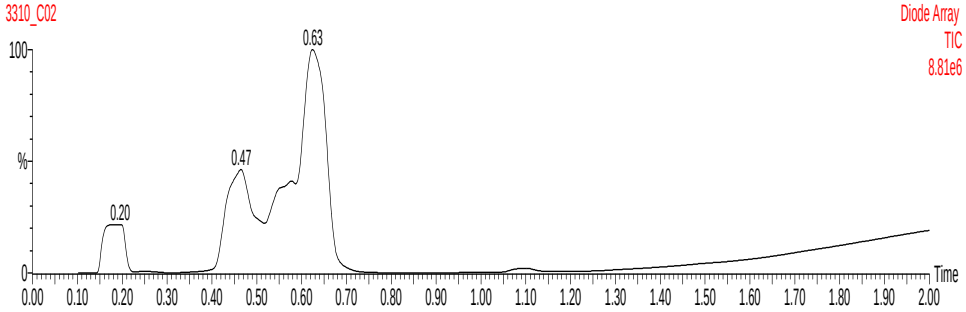


Figure 11.6 Chromatogram of the sample C2 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm. The flow rate was 4 ml/min and the gradient run time 2 min.

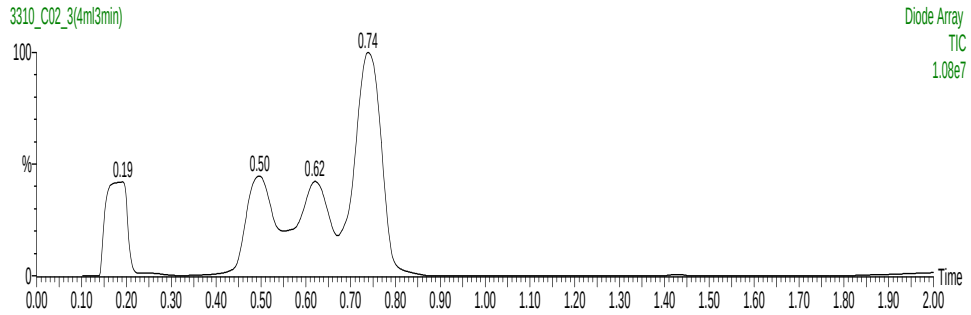


Figure 11.7 Longer gradient run time. Chromatogram of the sample C2 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm. The flow rate was 4 ml/min and the gradient run time 3 min.

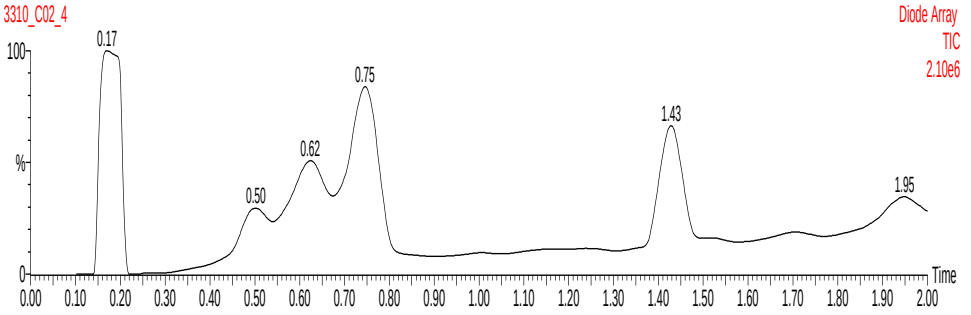


Figure 11.8 Dilution of the sample and longer gradient run time. Chromatogram of the sample C2 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm and ACN addition. The flow rate was 4 ml/min and the gradient run time 3 min..

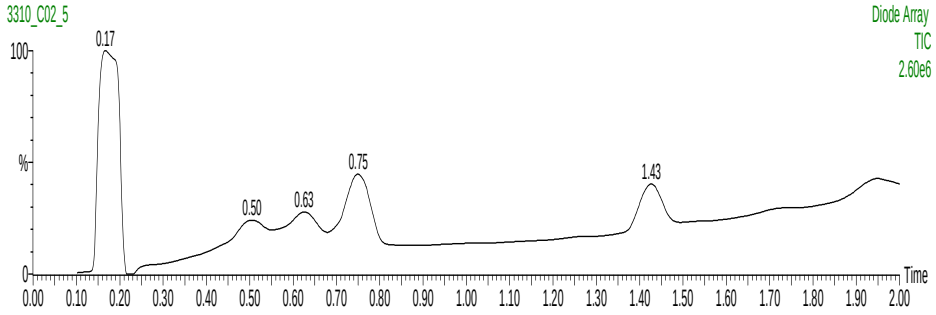


Figure 11.9 Dilution of the sample and longer run time. Chromatogram of the sample C2 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm and ACN addition. The flow rate was 4 ml/min and the gradient run time 3 min.

No better results on the peak performance could be achieved by lowering the flow rate from 4 ml/min to 3 ml/min, which can be seen by comparing figures 11.10 and 11.11. If all the other experimental conditions were the same and only the flow rate was reduced, the peaks appeared later but their shape was almost identical to the results achieved at the higher low rate. This can be seen by comparing figures where samples G5 and G7 from rack 3310 are presented with both the flow rates. Thus, no better separation was achieved with this method.

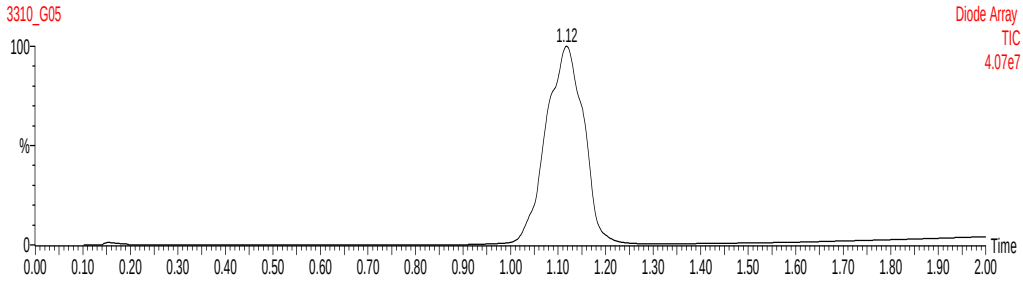


Figure 11.10 Chromatogram of the sample G5 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm. The flow rate was 4 ml/min and the gradient run time 2 min.

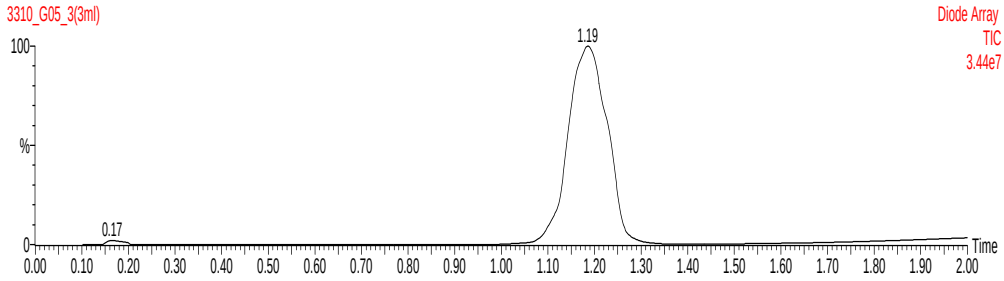


Figure 11.11 Smaller flow rate. Chromatogram of the sample G5 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm in. The flow rate was 3 ml/min and the gradient run time 2 min.

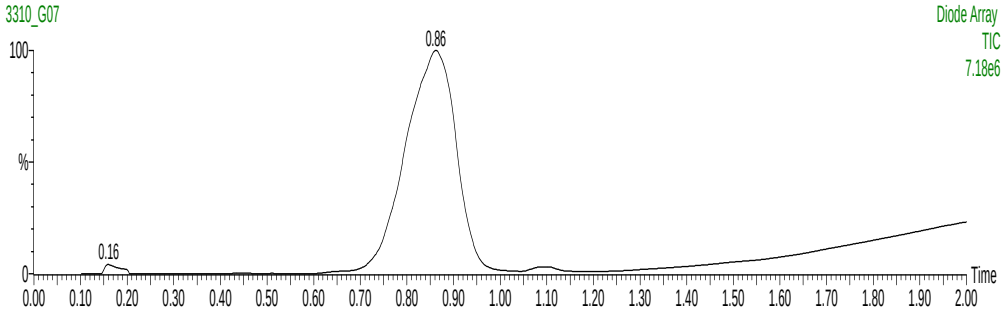


Figure 11.12 Chromatogram of the sample G7 from rack 3310 on Waters XTerra MS C_{18} 4,6×30 mm column with particle size 2,5 µm in. The flow rate was 4 ml/min and the gradient run time 2 min.

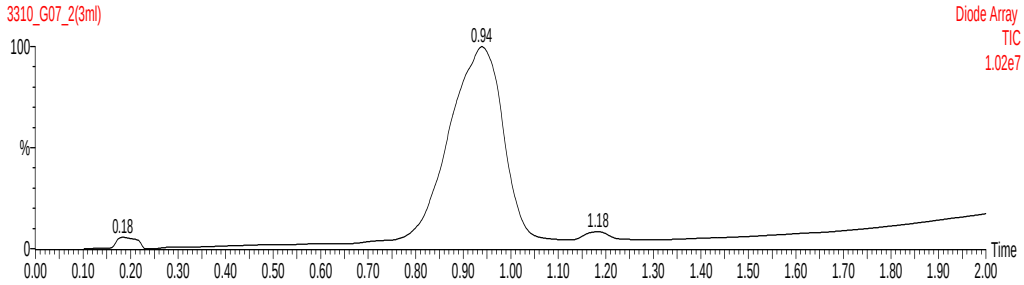


Figure 11.13 Smaller flow rate. Chromatogram of the sample G7 from rack 3310 on Waters XTerra MS C_{18} 4,6×30 mm column with particle size 2,5 µm in. The flow rate was 3 ml/min and the gradient run time 2 min.

A summary of the results is presented in table 11.3.

Table 11.3 The results of the experiments with different flow rates, run times and dilution.

Experiment type	**Comment of the result**	**Figure**
Preliminary experiment	No satisfactory separation	11.1, 11.2, 11.3, 11.6, 11.10
Longer gradient run time	No satisfactory separation, resolution later	11.4, 11.7
Dilution with ACN	No satisfactory separation, unstable baseline with dilution	11.5, 11.8, 11.9
Slower flow rate	No satisfactory separation	11.11, 11.13

11.3 Alkaline eluent system

11.3.1 Analysis on the alkaline eluent system

To optimise the determination of the purity of the library compounds some samples of the rack 3310 were analysed with alkaline eluents. The buffer used was 10 mM $(NH_4)_2CO_3$ with pH 10. The concentration of ACN was 5-95 % from 0 to 1.74 min and 95 % until the run time 2 min was completed. The UV detector scanned the wavelengths from 214 to 310 nm. The column temperature was 60 °C. Five samples from the third analysed rack were examined by this method using the Waters XTerra MS C_{18} 4.6×30 mm with particle size 2.5 µm. The flow rate was set to 4 ml/min and the injection volume 10 µl. The same samples were analysed on the Waters Symmetry MS C_{18} 3.0×150 mm 5 µm column and smaller 1.75 ml/min flow rate at the UV wavelength of 214 nm.

11.3.2 Results for the analysis on the alkaline eluent system

Some samples from rack 3310 were analysed on a alkaline eluent system. The sample G3 from the rack number 3310 is presented as an example of the results in figures 11.14 and 11.15. It is obvious that the use of alkaline eluents resulted in better peak separation, but the shape and size of the peaks was still not acceptable and therefore the method could not be implemented in the determination of the purity. It is relevant that the size of the peaks is correct because the estimation of the stage of the purity is based on the area of a peak.

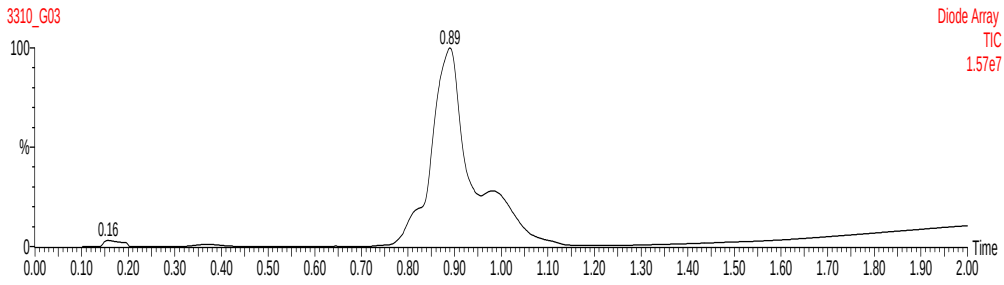


Figure 11.14 Chromatogram of the sample G3 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm in acidic eluent system. The flow rate was 4 ml/min and the gradient run time 2 min.

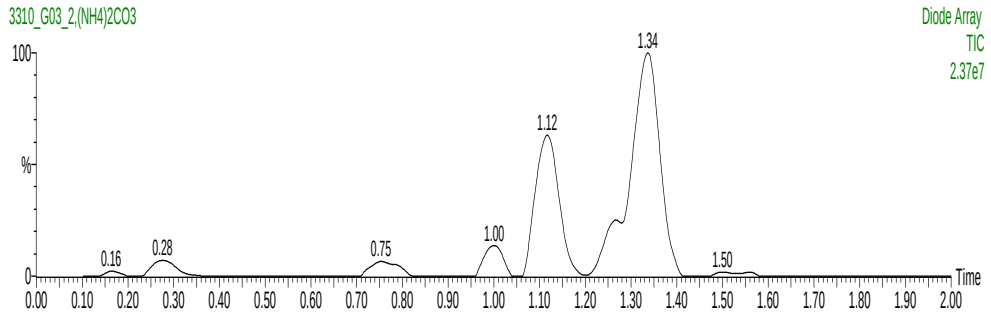


Figure 11.15 Alkaline eluent system. Chromatogram of the sample G3 from rack 3310 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm. The flow rate was 4 ml/min and the gradient run time 2 min.

11.4 Performance of the compounds on different columns

11.4.1 Experiments on different columns

One rack of the compound library was analysed both on the Waters XTerra MS C_{18} 4.6×30 mm column and on the Waters XTerra MS C_{18} 3.0×30 mm, respectively, to see if a smaller column diameter would offer better results. The particle size of both columns was 2.5 µl and the gradient flow conditions were identical. The flow rate was set to 4 ml/min, run time was 3 min and the concentration of ACN had a gradient from 2 to 95 % in 2.7 min and hold at 95 % until 3 min. The flow rate was set to 4 ml/min for the 4.6×30 mm column and the gradient run time was 2 min. For the 3.0×30 mm column the flow rate was set to 2 ml/min while gradient run time was 2 min based on table 11.1. The injection volume was set to 10 µl for every assay. The column temperature 60 °C was applied in all the experiments.

Three racks were analysed on the Waters XTerra MS C_{18} 3.0×30 mm column with the particle size 2.5 µm and on the Waters Symmetry C_{18} 2.1×50 mm column with particle size 3.5 µm. For the XTerra MS C_{18} 3.0×30 mm column the flow rate was set to 4 ml/min. The linear gradient of ACN was 5-95 % during 2 min. In the experiments on the Waters Symmetry C_{18} 2.1×50 mm column the flow rate was 0.7 ml/min. ACN had an initial hold in 5 % for 1 min and the linear gradient of 5-95 % from 1 to 7 min. The UV detector was measuring the wavelengths from 214 nm to 310 nm and the injection volume was 10 µl. Three reference compounds, diphenhydramine, oxybutynin and terfenadine, were also analysed with the Waters XTerra MS C_{18} 4.6×30 mm column and the Waters Symmetry C_{18} 2.1×50 mm column.

Two racks were analysed twice to see the difference between the performance of the particle sizes 2.5 µm and 3.5 µm of the Waters XTerra MS C_{18} 3.0×30 mm columns. For the 2.5 µm column the flow rate was set to 2 ml/min based on table 11.1 and for the 3.5 µm column 1.7 ml/min. The gradient run time 2 min was applied for both columns. The injection volume was set to 5 µl. Two racks were analysed on both the columns.

11.4.2 Results for the performance of the different columns

Some racks were analysed to optimise the method on both Waters XTerra MS C_{18} 4.6×30 mm and 3.0×30 mm with particle sizes 2.5 µm to see if a smaller column diameter would offer better results. The comparison of the chromatograms for sample E4 of rack 3345 in chromatograms 11.16 and 11.17 reveals that the difference in peak performance for the columns is very slight. With the 3.0×30 mm column the resolution time seems to be somewhat shorter and the peak performance slightly better. For the smaller column diameter due to the lower flow rate the solvent consumption is also lower. Therefore the smaller column diameter is recommendable.

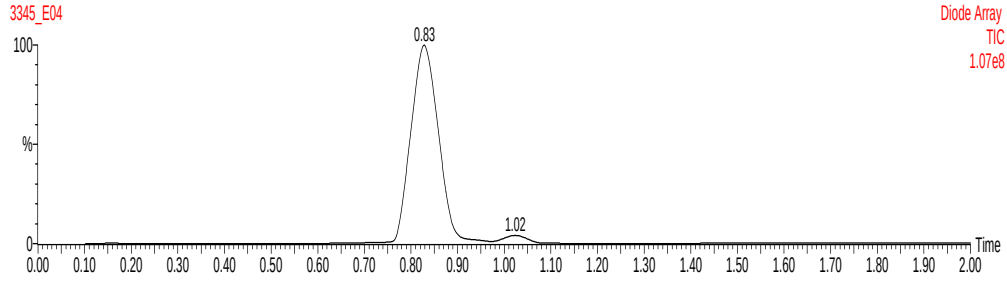


Figure 11.16 Chromatogram of the sample E4 from rack 3345 on Waters XTerra MS C_{18} 4.6×30 mm column with particle size 2.5 µm. The flow rate was 4 ml/min and the gradient run time 2 min.

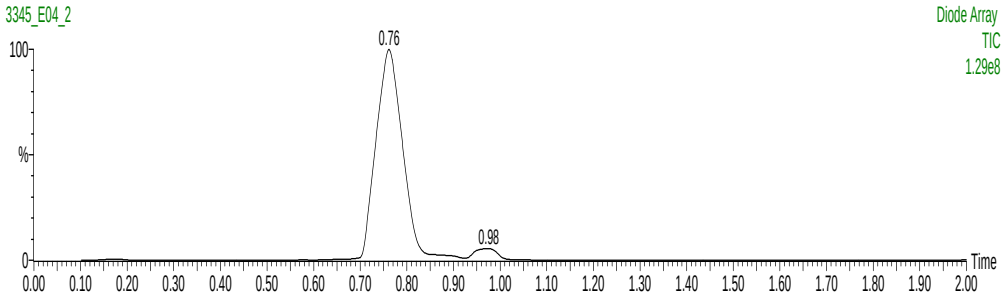


Figure 11.17 Chromatogram of the sample E4 from rack 3345 on Waters XTerra MS C_{18} 3.0×30 mm column with particle size 2.5 µm. The flow rate was 2 ml/min and the gradient run time 2 min.

The Waters XTerra MS C_{18} 4.6×30 mm 2.5 µm column was also compared with a longer column with a smaller diameter. The Waters Symmetry MC_{18} 2.1×50 mm 3.5 µm column was used in the experiments. Some examples of the results are presented in figures 11.18-11.21. There are some contradictions in the results. By comparing figures 11.18 and 11.19 the longer column seems to give a better peak performance and longer resolution time. In the experiments with the reference compounds diphenhydramine, oxybutynin and terfenadine the peak shape is better on the shorter column as can be seen in figures 11.20 and 11.21. Hence, neither of the columns is optimal for this specific purpose.

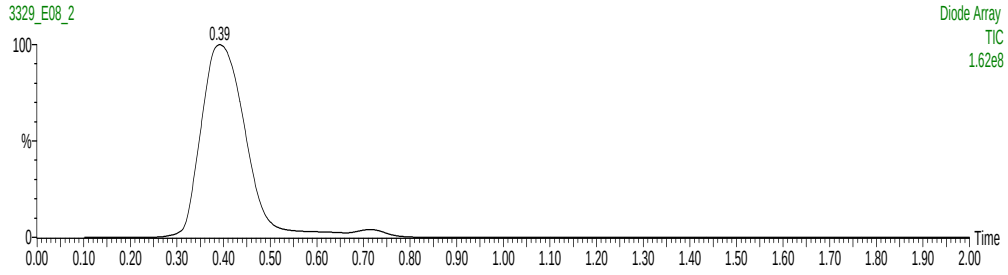


Figure 11.18 Chromatogram of the sample E8 from rack 3329 on Waters XTerra MS C_{18} 4.6×30 mm column at the wavelengths from 214 to 310 nm. The flow rate was 1.95 ml/min and the gradient run time 2 min.

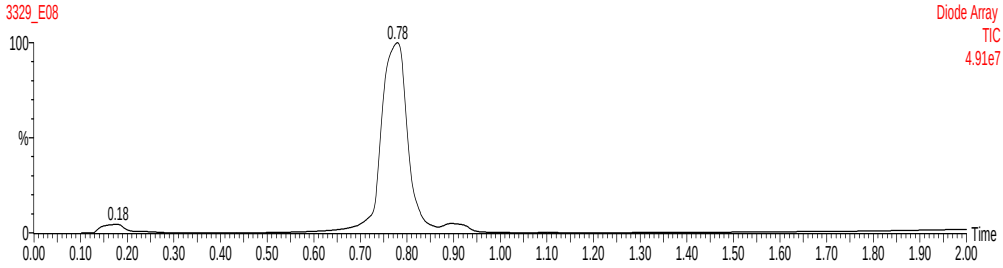


Figure 11.19 Chromatogram of the sample E8 from rack 3329 on Waters Symmetry MS C_{18} 2.1×50 mm column with particle size 3.5 µm at the wavelengths from 214 nm to 310 nm. The flow rate was 1,95 ml/min and the gradient run time 2 min.

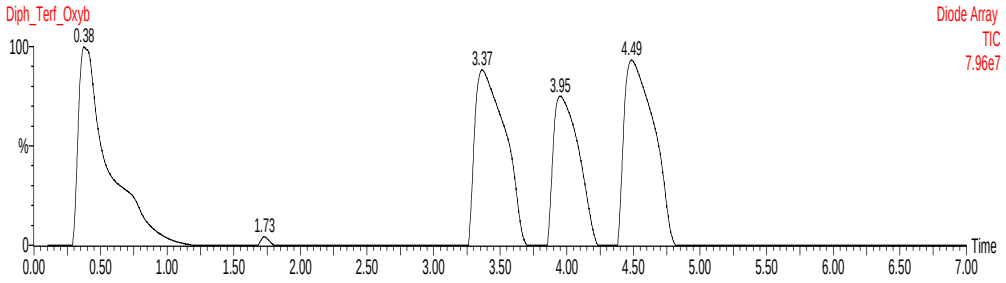


Figure 11.20 Chromatogram of diphenhydramine, oxybutynin and terfenadine on Waters Symmetry MS C_{18} 2.1×50 mm column with the particle size 3.5 µm. The flow rate was 1.95 ml/min and the gradient run time 7 min.

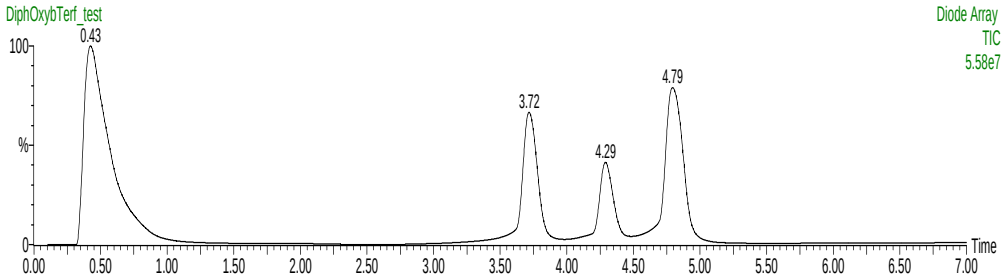


Figure 11.21 Chromatogram of diphenhydramine, oxybutynin and terfenadine on Waters XTerra MS C_{18} 4.6×30 mm column with the particle size 3.5 µm. The flow rate was 1.95 ml/min and the gradient run time 7 min.

Next, the effect of the particle size was studied by analysing compounds by Waters XTerra MS C_{18} 3.0×30 mm with particle sizes 2.5 µm and 3.5 µm. The experiments were carried out in similar conditions, only the flow rate was different and was chosen based on table 11.1. The comparison of figures 11.22 and 11.23 reveals that the particle size 3.5 µm makes the peaks perform later but does not affect the shape and resolution of the different peaks. However, the particle size 3.5 µm is recommendable, because the flow rate and therefore also the solvent consumption is a bit lower.

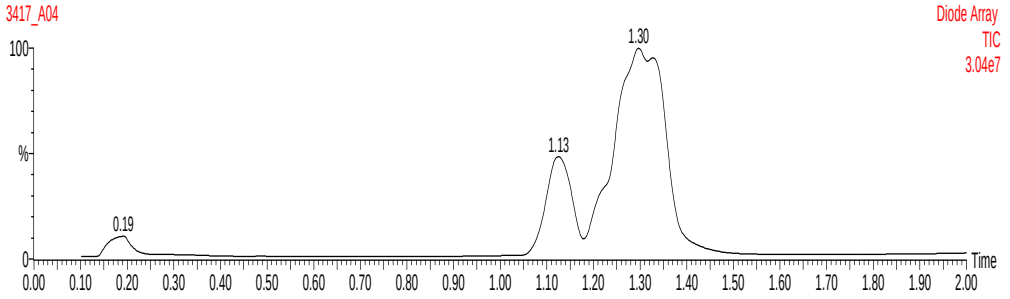


Figure 11.22 Chromatogram of the sample A4 from rack 3417 on Waters XTerra MS C_{18} 3.0×30 mm column with particle size 2.5 µm. The flow rate was 2 ml/min and the gradient run time 2 min.

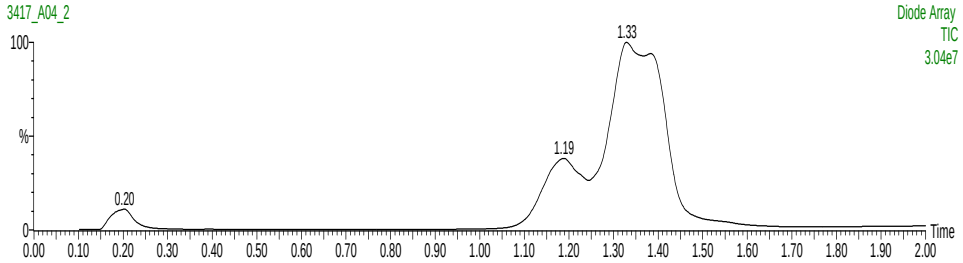


Figure 11.23 Chromatogram of the sample A4 from rack 3417 on Waters XTerra MS C_{18} 3.0×30 mm column with particle size 3.5 µm. The flow rate was 2 ml/min and the gradient run time 1.7 min.

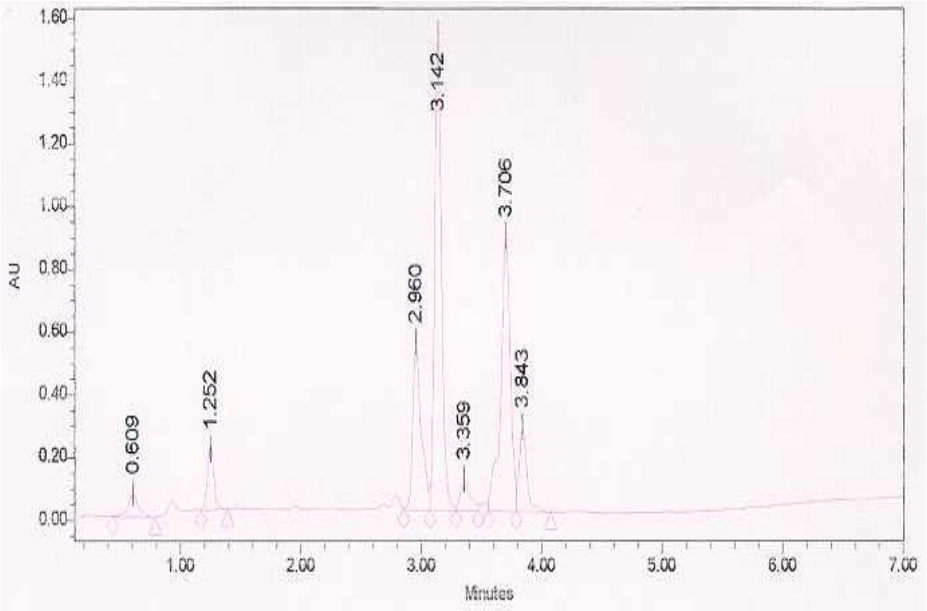


Figure 11.24 Chromatogram of the sample A4 from rack 3417 on Waters Symmetry MS C_{18} 3.0×150 column at the wavelength 214 nm. The flow rate was 1.95 ml/min and the gradient run time 7 min.

In table 11.4 a summary of the results with different columns are presented.

Table 11.4 A comparison of the results achieved on different columns.

Column Type	**Particle Size, µm**	**Comment**	**Figure**
Xterra 4.6×30 mm	2.5	Later resolution compared to smaller diameter, separation not satisfactory, fat peaks	11.16, 11.18, 11.18, 11.21
XTerra 3.0×30 mm	2.5	Separation not satisfactory	11.17, 11.22
Symmetry 2.1×50 mm	3.5	Good peak performance but long resolution time, contradiction in results	11.19, 11.20
XTerra 3.0×30 mm	3.5	Peaks appear a bit later than on smaller particle size, lower solvent consumpition	11.23

The selection of the column type, i.e. column diameter, length and particle size, is not alone enough to solve the problem of optimising the determination of the purity. Apparently Waters XTerra MS C_{18} 3.0×30 mm column with the particle size 3.5 µm gives the best result, but also the other parameters affecting the separation must be considered. Therefore, the effect of different flow gradients were studied.

11.5 The optimisation of the flow gradient based on the clogP value of an organic compound

11.5.1 Theory

11.5.1.1 The partition coefficient

The transport of a drug from the administration site, which can be oral, muscular etc., to the target tissue or organ depends on the structure and the properties of a compound. There is a simple way called the two-phase system for considering and thereby calculating the manner in which an organic compound is distributed in the body, phases being aqueous and organic layers. The proportions of the concentrations at equilibrium of a drug is called the partition coefficient *P*. Such measurements are difficult to accomplish with living organisms, therefore they are frequently determined using n-octanol as the nonpolar lipid phase and water to represent the polar aqueous phase [Thompson/28].

The hydrophobicity (lipophility) of a molecule, which is usually quantified as logP, is an important molecular characteristic in drug discovery. The partition coefficient *P* is defined as the ratio of the equilibrium concentrations c_i of a dissolved substance in a two-phase system consisting of two largely immiscible solvents, usually n-octanol and water

$$P_{ow} = \frac{c_{oct}}{c_{water}} \qquad (1)$$

P_{ow} partition coefficient of a compound between octanol and water

c_{oct} concentration of a compound in octanol

c_{water} concentration of a compound in water

Therefore, the partition coefficient P is a quotient of two concentrations and is usually given in the form of its logarithmic with base 10 (logP). The larger the number is, the more soluble is the compound in lipid. A logP value less than zero indicates that the compound is hydrophilic and would best interact with a polar environment containing hydrogen bond

donors and acceptors. Positive logP value indicates hydrophobicity and the compound would best interact in nonpolar environment. The distance from zero approximates the strength of the potential interaction in the appropriate environment [Anonymous/29, Anonymous/30].

The logP of a compound can be determined experimentally by placing a certain quantity of the substance into a mixture of octanol and water and shaking the phases in a shake-flask. When the octanol and water separate into two layers, the concentration of the substance in question is measured in each layer. Another method to measure logP experimentally is HPLC. Most HPLC procedures used to develop logP values do not use octanol. Thus, they have to be compared to standard curves. If the solutes do not absorb well in the UV, the appearing difficulties in detecting the elution time can eliminate any advantage HPLC might otherwise have over the shake-flask method. Extensive lists of logP values or many groups of compounds are available [Anonymous/30].

11.5.1.2 The calculated logP value

The log P value can also be determined by computational methods based on the structure of a compound. Calculated values of logP, clogP values, can be used for deciding which experimental method is appropriate for selecting appropriate test conditions as a laboratory internal check on possible experimental errors and for providing estimates for logP values if experimental evaluation cannot be accomplished [Anonymous/29].

The computational definition of logP can be divided into two groups: fragment-based and atom-based. In the former the unit is a fragment with organic functional groups. These units are tied together with non-functional carbon atoms which have their own constants. The structural relationships between different fragments are encoded with factors or correction terms for intramolecular activity. The logP is calculated simply as a sum of fragment constants and factors. The atom method divides the organic chemistry into a large number of specific atom types that have specific environmental characteristics and the clogP is the sum of all atom constants. The latter method is easier to program, but is less accurate with complex molecules and therefore not favourable [Kellog et al./31].

Generally, the reliability of calculation methods decreases when complexity of a molecule increases. For simple molecules with low molecular weight and one or two functional groups, a deviation of 0.1 to 0.3 logP units between the results of the calculated and experimental logP can be expected. For more complex molecules the error can be greater. This depends on the reliability and availability of fragment constants, as well as the knowledge of intramolecular actions and the correct use of the correction factors [Anonymous/29, Kellog et al./31].

The clogP value calculated with the ISIS-programme is available for every compound and can be used to choose an optimal flow gradient for each compound, since there ought to be a connection between the clogP value and the behaviour of the compound in the mixture of ACN and water.

Therefore the first task was to find out if the clogP value and the resolution time of a compound correlate. This was done by analysing the resolution times and clogP values of three different combinatorial library racks. If the values correlate, some experiments are done by testing different flow gradients.

11.5.2 The determination of the correlation between clogP and resolution time

The resolution times for the purified compounds of three different racks of one combinatorial library were analysed and plotted versus the clogp value of the compound. The information for the clogP values was available for each library compound with the ISIS-programme and the resolution time could be obtained from the purification results of the compounds. A standard purification protocol for preparative LC/MS was used. The flow rate was 20 ml/min, the gradient of ACN had an initial hold in 5 % for one minute and from 1 to 7 min 5-95 %. The aqueous phase of the flow was water with 0.1 % TFA. The results can be seen in figures 11.25-11.27 and the values of correlation and the equation of the trendline in table 11.5.

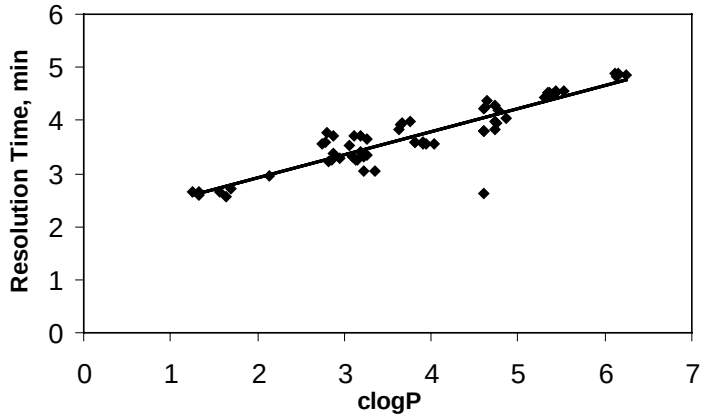


Figure 11.25 The resolution times versus the clogP values of the compounds on rack number 3329. The resolution time was determined at the standard high throughput purification conditions by preparative HPLC.

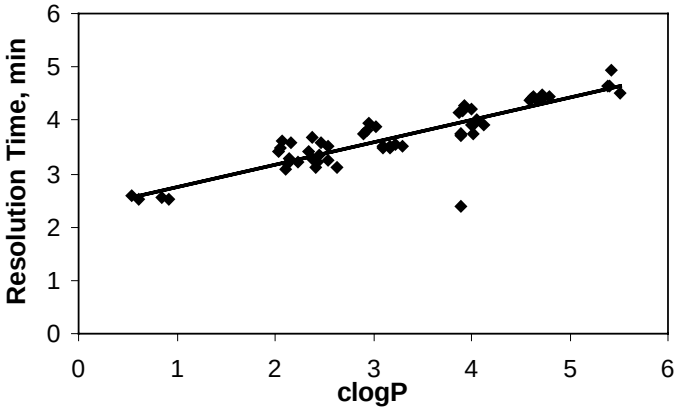


Figure 11.26 The resolution times versus the clogP values of the compounds on rack number 3349. The resolution time was determined at the standard high throughput purification conditions by preparative HPLC.

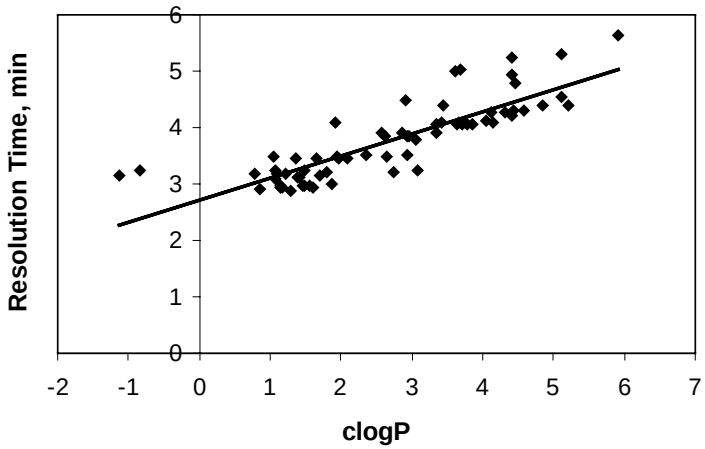


Figure 11.27 The resolution times versus the clogP values of the compounds on rack number 3370. The resolution time was determined at the standard high throughput purification conditions by preparative HPLC.

Table 11.5 The correlation and the equation of the trendline of the resolution time and clogp value for three different racks of the same library.

Rack number	**Correlation r**	**Equation of the trendline**
3329	0.90	0.44x + 2,04
3349	0.88	0.42x + 2,33
3370	0.85	0.39x + 2,72
Average	**0.88**	**0.42 + 2.36**

As can be seen in figures 11.25, 11.26 and 11.27 and in table 11.5, there is an evident correlation between the clogP value and compound resolution times for the analysed racks. Therefore it was worthwhile to study the effect of different flow gradients on resolution.

11.5.3 Experiments on different flow gradients

11.5.3.1 Experimental design for different flow gradients

According to figures 11.25, 11.26 and 11.27 there is an evident correlation between the clogP value of the compound and its resolution time. Therefore the performance of library compounds in different flow gradients was studied. The experiments were accomplished by examining the performance of the substances on different columns and flow gradients. The standard flow gradient is from 5 % to 95 % ACN during the gradient run time. However, when the figures presenting the correlation are studied, it can be seen that for all the compounds the resolution time is between 2 and 5 minutes. Hence, none of the fractions appear in the very beginning of the gradient or in the end of it. Thus, the gradient from 5 % to 95 % is not the best option. Therefore the performance of one or more suitable compounds were studied in the different gradient flow conditions. A flow gradient of 20 % to 80 % was examined, because all the compounds resolute between these concentrations and there are still some resources in both ends of the gradient, if some compounds make an exception. The column temperature was 60 °C in all the experiments.

Some random samples that included more than one peak were analysed by analytical HPLC and UV detector on both the standard 5-95 % flow gradient and also on the 20-80 % gradient. The affect of the delay time in the beginning of the run was also studied. The knowledge obtained from the previous experiments on different columns was exploited. According to that the preferrable column was the Waters XTerra 3,5×30 mm 3,5 µm and the gradient run time 2 min was applied. The optimal flow rate for this column and gradient run time was 1,7 ml/min according to the table produced by Waters.

11.5.3.2 The performance of different flow gradients in purity analysis

The performance of some library compounds was studied in different flow gradients. The results of the correlation between the clogP value and the resolution time were used as a starting-point for the experiments and the composition of the flow was chosen based on that. In the figures 11.28-11.30 the sample number E7 from the rack 3454 of the library TSA-003 is presented as an example of the affect of different flow gradients on the compound separation.

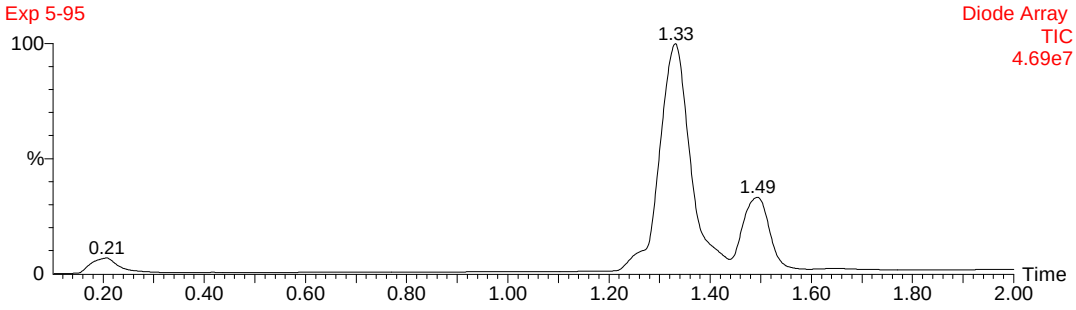


Figure 11.28 Sample E7 from the rack 3454 on the Waters XTerra 3.0×30 mm 3.5 µm column. The flow rate was 1.7 ml/min and the gradient 5-95 % during the 2 min run time. The injection volume was 5 µl.

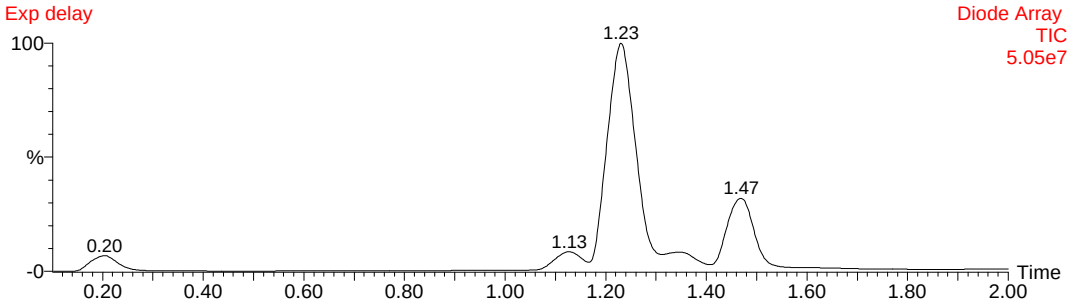


Figure 11.29 Sample E4 from the rack 3454 on the Waters XTerra 3.0×30 mm column with particle size 3.5 µm. The flow rate was 1.7 ml/min and the gradient run time 2 min. The linear gradient of ACN had an initial hold in 5 % for 15 s and was 20–80 % for the rest of the run. The injection volume was 10 µl.

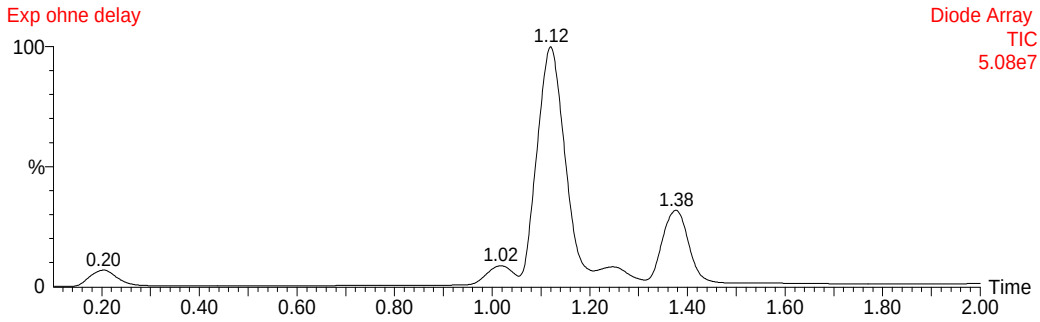


Figure 11.30 Sample E7 from the rack 3454 on the Waters XTerra 3.0×30 mm column with particle size 3.5 µm. The flow rate was 1.7 ml/min and the gradient run time 2 min. The linear gradient of ACN was 20-80% during the run. The injection volume was 10 µl.

In figure 11.28 the standard purification gradient 5-95 % is applied. The performance of the compounds on the flow gradient chosen based on the clogP values are shown in figures 10.29 and 10.30 with and without a delay in the beginning of the run. When the standard flow gradient is applied, the peaks appear later and their separation is not as complete as it is when compared with the performance of the compounds on a gradient based on the clogP values of the compounds. Therefore the latter method is preferrable. The gradient of 20 % to 80 % was analysed with and without the delay time in the beginning of the run. There is no difference in the peak separation whether the delay time is used or not. However, the peaks appear later when the delay is applied in the beginning of the run. For example, the highest peak of the sample E7 form rack 3454 resolutes at 1.23 min with the delay and at 1.12 min without the delay. Thus, the method without the delay was preferred due to the short gradient run time. Some unpolar compounds might resolute quite late and might not be seen by the UV-detector during the 2 min run.

Table 11.6 The optimal conditions for analysing the purity of the compounds after preparative LC/MS .

Column	Waters XTerra 3.0×30 mm, 3.5 µm
Eluent system	ACN with 0.1 % TFA, H_2O with 0.1 % TFA
Flow rate	1.7 ml/min
Run time	2 min
Gradient	20-80 % ACN
Injection volume	5 µl

There are four examples of the results obtained with the analysing system where the optimised conditons are applied. The samples were first analysed with the optimised method on the short Waters XTerra 3.0×30 mm 3,5 µm and longer Waters Symmetry 3.0×150 mm 5 µm columns. The following samples are presented as examples of the results: rack 3317: D9, 3362:E1, 3382: E8, 3379: H3.

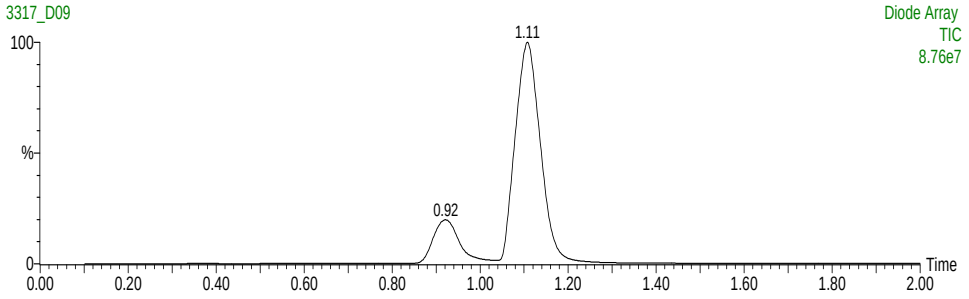


Figure 11.31 Sample D9 from the rack 3317 on the Waters XTerra 3.0×30 mm column with particle size 3.5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 10 µl.

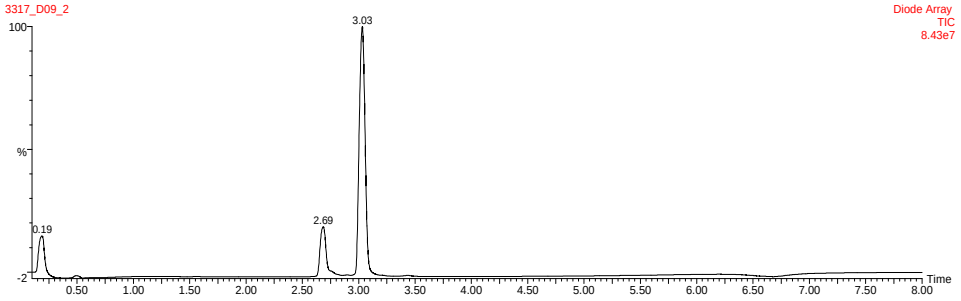


Figure 11.32 Sample D9 from the rack 3317 on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 10 µl.

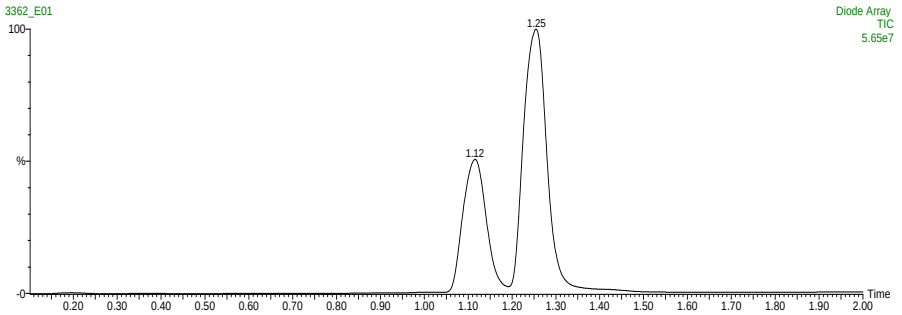


Figure 11.33 Sample E1 from the rack 3362 on the Waters XTerra 3.0×30 mm column with particle size 3.5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 10 µl.

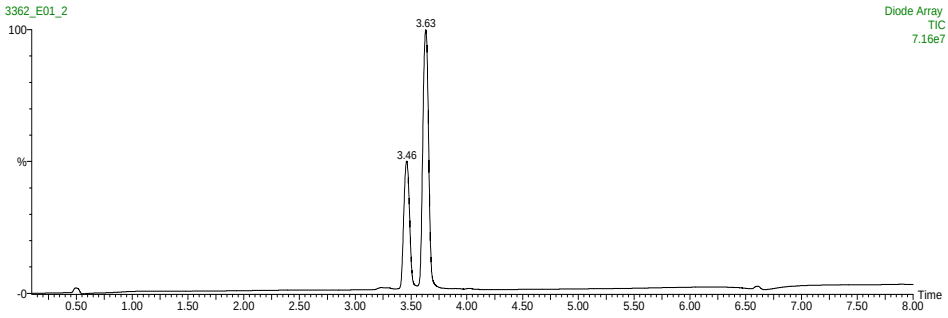


Figure 11.34 Sample E1 from the rack 3362 on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 10 µl.

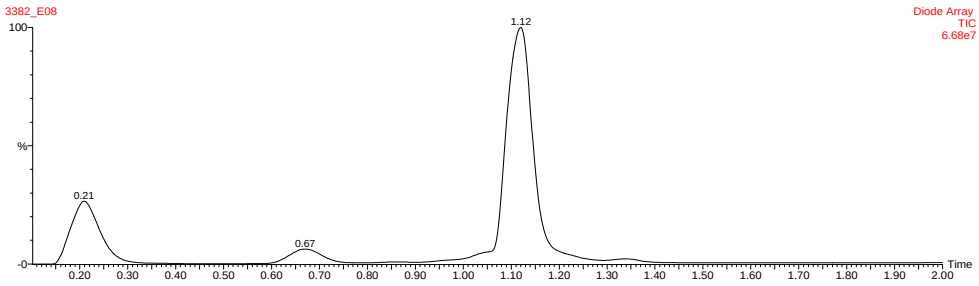


Figure 11.35 Sample E8 from the rack 3382 on the Waters XTerra 3.0×30 mm column with particle size 3.5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 10 µl.

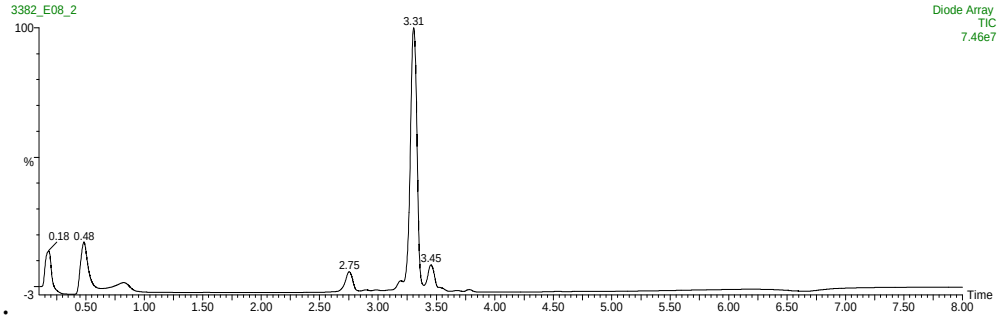


Figure 11.36 Sample E8 from the rack 3382 on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 10 µl.

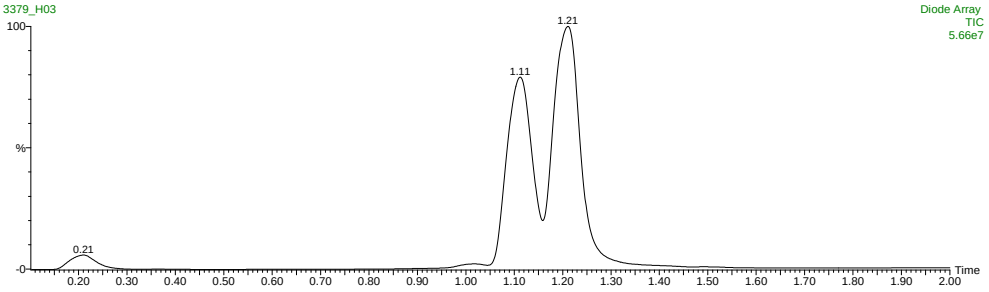


Figure 11.37 Sample H3 from the rack 3379 on the Waters XTerra 3.0×30 mm column with particle size 3.5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 10 µl

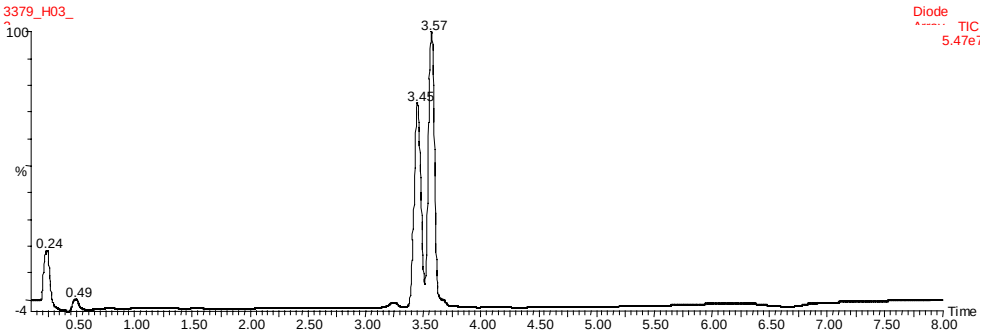


Figure 11.38 Sample H3 from the rack 3379 on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 10 µl.

The whole library TSA-003 was analysed with the optimised method and the results were processed by the Openlynx application of the Masslynx-software to obtain the information of the proportional area of the diverse compounds in each sample. The area of the peaks offers the information of the purity of the sample and can be exploited when the decision whether the sample is pure enough for a reliable biological screening is made.

11.6 Optimisation of the injection volume

11.6.1 Experimental Design

Three reference compounds, diphenhydramine, oxybutynin and terfenadine were analysed to optimise the injection volume and to see which injection volume was too small for the equipment. Six different injection volumes were examined. These were 10, 5, 4, 3, 2 and 1 µl. The experiments were carried out using the Waters Symmetry 5.0×150 mm 5 µm column. The flow gradient was from 5 % to 100 % ACN for 7 minutes and constant 100 % for the last minute of the run. The flow rate was 1.75 ml/min. The compounds were dissolved in 20 mg/ml DMSO.

11.6.2 The results and discussion for optimisation of the injection volume.

In figures 11.39-11.43 the performance of different injection volumes at the same conditions can be seen.

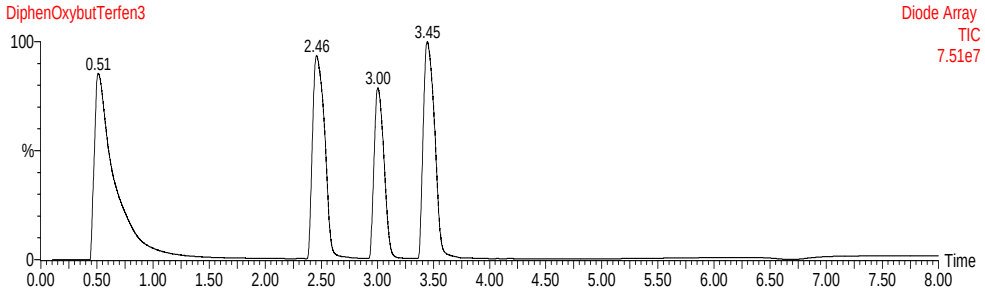


Figure 11.39 Three reference compounds diphenhydramine, oxybutynin and terfenadine on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 10 µl.

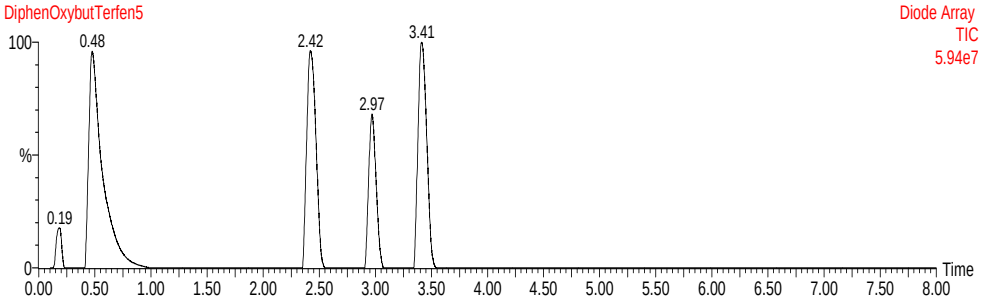


Figure 11.40 Three reference compounds diphenhydramine, oxybutynin and terfenadine on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 5 µl.

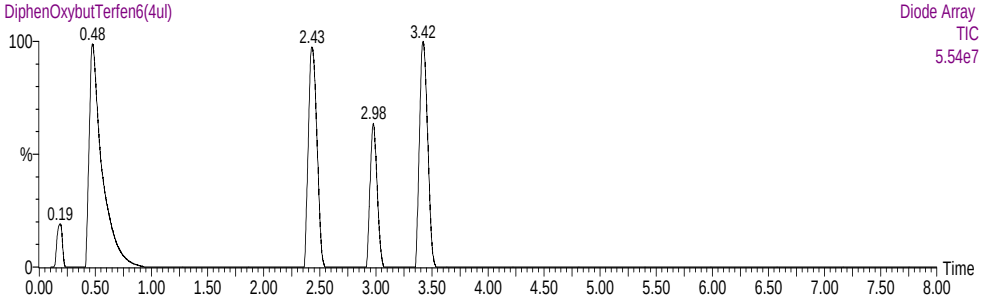


Figure 11.41 Three reference compounds diphenhydramine, oxybutynin and terfenadine on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 4 µl.

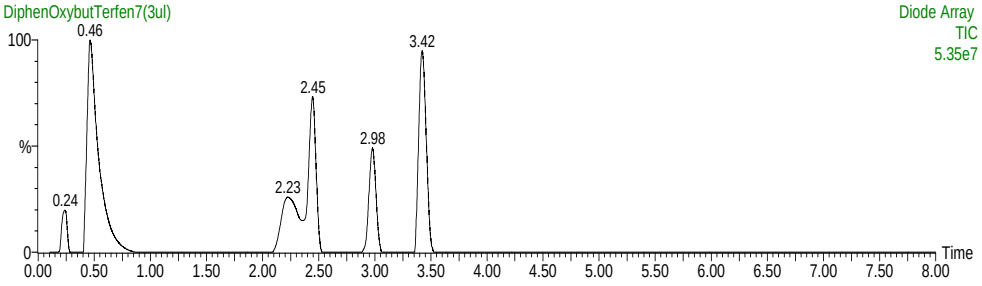


Figure 11.42 Three reference compounds diphenhydramine, oxybutynin and terfenadine on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 3 µl.

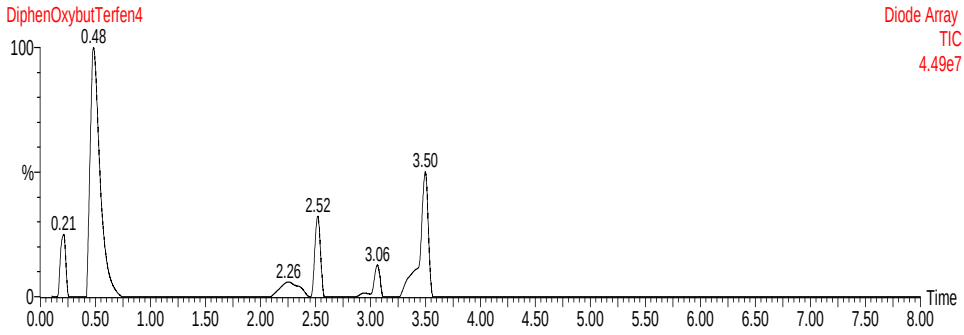


Figure 11.43 Three reference compounds diphenhydramine, oxybutynin and terfenadine on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min and the gradient run time 8 min. The injection volume was 1 µl.

In figure 11.39 the separation of the peaks is complete but the shape and the symmetry are not optimal. The performance of the smaller injection volumes can be seen in the next four figures. When the injection volume is 4 and 5 µl, the peaks are well separated and also their shape is acceptable compared to the larger injection volumes. For smaller volumes, a preliminary peak appears, which can be seen in figures 11.42 and 11.43 with the injection volumes of 3 and 1 µl as examples of the results. Therefore, the smallest practicable injection volume is 4 µl. However, the injection volume 5 µl was preferred for the experiments.

12. Optimisation of the purity analysis for a library before purification by LC/MS

12.1 Experimantal plan for the analysis before the LC/MS purification

The purification was also optimised for another compound library which seemed to be unsuccesfully synthesised according to the previous analysis data. The aim was to examine whether the library contained enough target compounds for the purification by preparative LC/MS to be advocating. Some racks of the library were analysed by using different columns and buffer systems by the analytical Waters Alliance HT HPLC equipment. The plan was to analyse one example rack on two different columns, using both acid and basic eluent systems. One rack had been relatively succesfully synthesised, one not so well and one with moderate success. The flow conditions were optimised for each column and buffer system individually.

The two columns used for the analytical experiments were Waters Symmetry 2.1×50 mm and Waters XTerra 3,0×50 mm columns with the particle size 3.5 µm. The sample A1 of the rack number 3245 from the combinatorial library HEL-001 was examined on different flow rates and gradient run times to optimise the flow conditions. The gradient of ACN was from 5 to 95 % during the run in all the experiments accomplished on acidic eluent system. The sample was diluted with 200 µl mixture of ACN and water (9:1). The injection volume was set to 5 µl and the wavelengths from 214 to 310 nm were detected. The column temperature was 60 °C to enable the applying of higher flow rates.

12.2 The Measurements for the analysis before the purification

12.2.1 Analysis on the Waters Symmetry column

In the experiments on Waters Symmetry 2.1×50 mm, 3.5 µm particle size column the used eluent system consisted of water and ACN with 0,1 % TFA added in both. Initially, the flow rate was set to 0.7 ml/min and the gradient run time was 6 min. The sample was also examined on the longer Waters Symmetry 3.0×150 mm, 5.0 µm particle size column since a reliable reference result was required. For the longer column the gradient of ACN was from 5 to 100 % in 7 min and the flow rate 1.75 ml/min. The results on the shorter columns were compared with the performance of the sample on the longer column.

For the further experiments with the sample A1 of the rack 3245 the flow gradient was increased to 1 ml/min and the run time was first held at 6 min and then reduced to 5 min. The flow rate was further increased to 1.2 ml/min on the 5 min run time and the 4 min run time was also examined.

12.2.2 Analysis of the Waters XTerra column

The experiments on Waters XTerra 3.0×50 mm, 3.5 µm particle size column were implemented by using an eluent system consisting of water and ACN both including 0.1 % TFA. The conditions accomplished on the experiments on Waters Symmetry 2.1×50 mm, particle size 3.5 µm column were also utilised for the experiments on the Waters XTerra 3.0×50 mm, 3.5 µm particle size column. Therefore, the flow rate was set to 1.2 ml/min and the run time to 4 min. Rack 4325 from library HEL-001 was analysed with this method.

12.3 The results for the analysis before the purification

12.3.1 Waters Symmetry column

The flow rates and run times used in the experiments in acidic buffer systems are presented in table 12.1 and performance of sample A1 from rack 4325 in different conditions in figures below.

Table 12.1 The used columns, flow rates and gradient run times for the experiments performed on sample A1 from the rack 3245 of combinatorial library HEL-001.

Figure	Column	Flow rate, ml/min	Run time, min
1	Waters Symmetry 3.0×150 mm	1.75	8
2	Waters Symmetry 2.1×50 mm	0.70	6
3	Waters Symmetry 2.1×50 mm	1.00	6
4	Waters Symmetry 2.1×50 mm	1.00	5
5	Waters Symmetry 2.1×50 mm	1.20	5
6	Waters Symmetry 2.1×50 mm	1.20	4

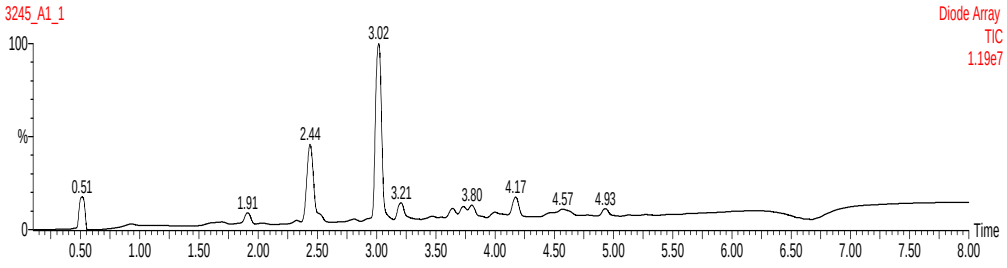


Figure 12.1 Sample A1 from the rack 3245 of the library HEL-001 on the Waters Symmetry 5.0×150 mm column with particle size 5 µm. The flow rate was 1.7 ml/min, the gradient run time 8 min. The injection volume was 5 µl.

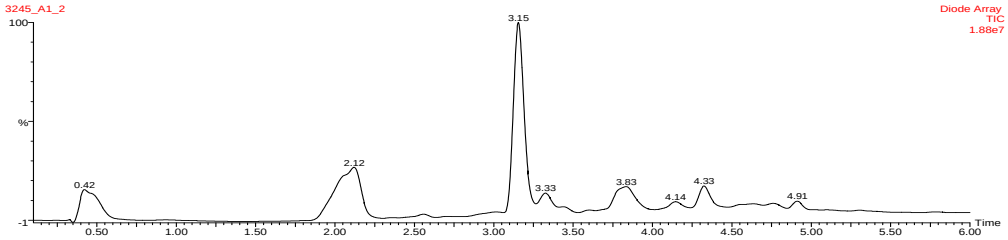


Figure 12.2 Sample A1 from the rack 3245 of the library HEL-001 on the Waters Symmetry 2.1×50 mm column with particle size 3.5 µm. The flow rate was 0.7 ml/min, the gradient run time 6 min. The injection volume was 5 µl.

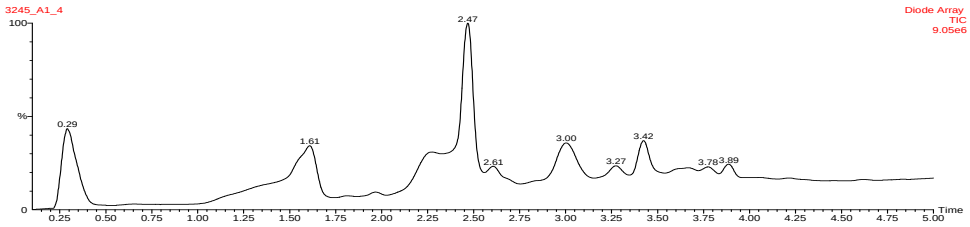


Figure 12.3 Sample A1 from the rack 3245 of the library HEL-001 on the Waters Symmetry 2.1×50 mm column with particle size 3.5 µm. The flow rate was 1.0 ml/min, the gradient run time 5 min. The injection volume was 5 µl.

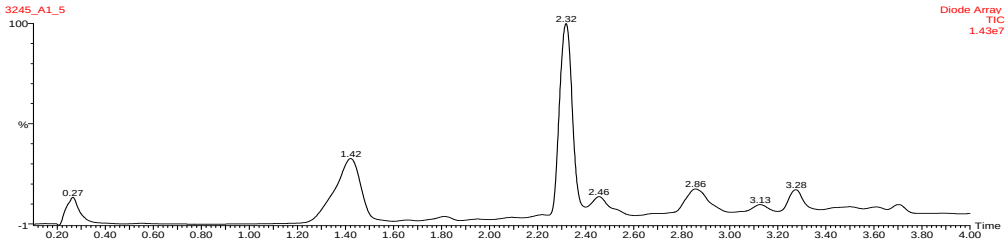


Figure 12.4 Sample A1 from the rack 3245 of the library HEL-001 on the Waters Symmetry 2.1×50 mm column with particle size 3.5 µm. The flow rate was 1.2 ml/min, the gradient run time 5 min. The injection volume was 5 µl.

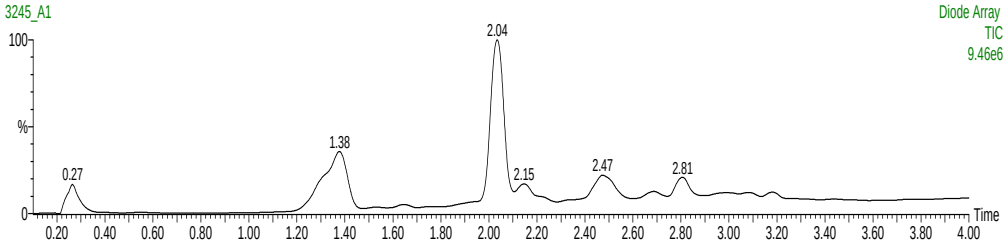


Figure 12.5 Sample A1 from the rack 3245 of the library HEL-001 on the Waters Symmetry 2.1×50 mm column with particle size 3.5 µm. The flow rate was 1.2 ml/min, the gradient run time 4 min. The injection volume was 5 µl.

By comparing the resolution on different flow rates and gradient run times with the reference result on the long 3.0×150 mm column it can be seen that the sample A1 of rack 3245 of the library HEL-001 achieved an adequate resolution on the flow rate 1.2 ml/min with gradient run time 4 min. Longer gradient run times or slower flow rates do not offer any significant benefits and therefore the shorter gradient is preferrable.

12.3.2 Comparison of the analytical Waters Symmetry and XTerra columns

The conditions applied to the Waters Symmetry 2,1×50 mm, 3,5 µm particle size column were adequate and therefore utilised also for the Waters XTerra 3,0×50 mm, 3,5 µm particle size column. The sample A1 is presented in figure 12.6 as a demonstration of the feasibility of the method.

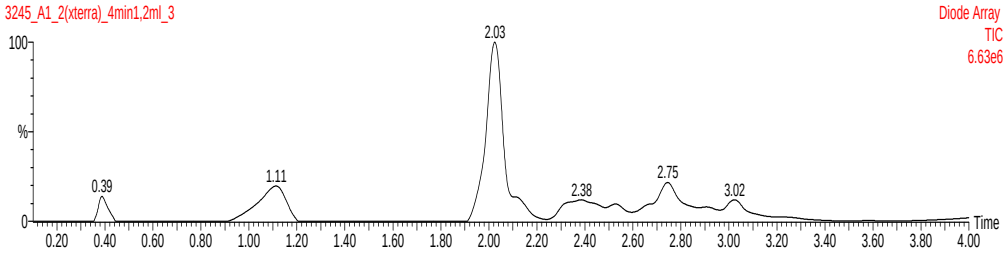


Figure 12.6 Sample A1 from the rack 3245 of the library HEL-001 on the Waters XTerra 3.0×50 mm column with particle size 3.5 µm. The flow rate was 1.2 ml/min, the gradient run time 4 min. The injection volume was 5 µl

In figure12.7-12.10 below the samples A8 and B8 of the rack 3245 of the combinatorial library HEL-001 are presented both on the Waters Symmetry and Waters XTerra columns as examples of the difference between the two columns.

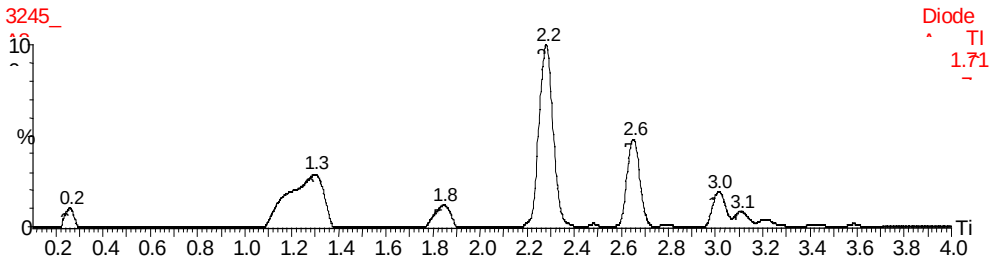


Figure 12.7 Sample A8 from the rack 3245 of the library HEL-001 on the Waters Symmetry 2.1×50 mm column with particle size 3.5 µm. The flow rate was 1.2 ml/min, the gradient run time 4 min. The injection volume was 5 µl.

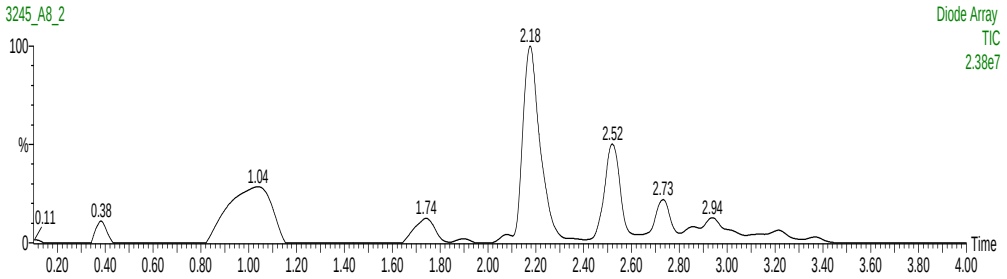


Figure 12.8 Sample A8 from the rack 3245 of the library HEL-001 on the XTerra 3.0×50 mm column with particle size 3.5 µm. The flow rate was 1.2 ml/min, the gradient run time 4 min. The injection volume was 5 µl.

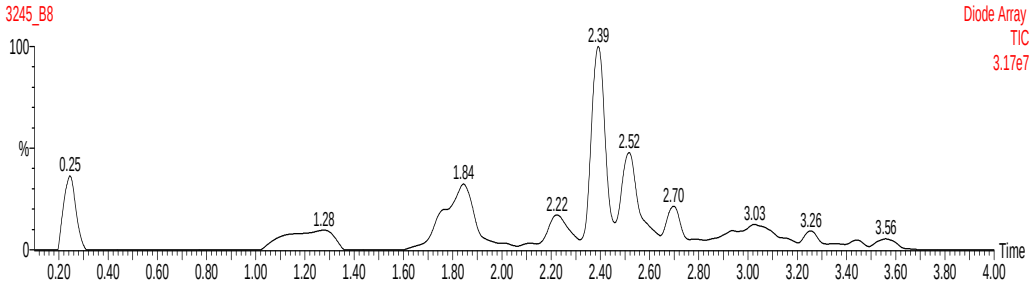


Figure 12.9 Sample B8 from the rack 3245 of the library HEL-001 on the Waters Symmetry 2.1×50 mm column with particle size 3.5 µm. The flow rate was 1.2 ml/min, the gradient run time 4 min. The injection volume was 5 µl.

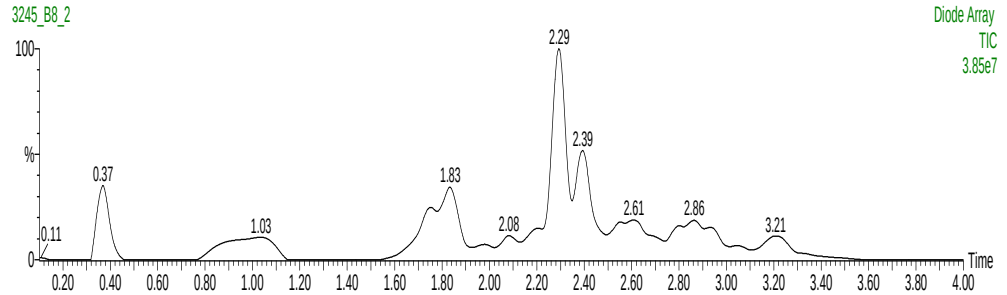


Figure 12.10 Sample B8 from the rack 3245 of the library HEL-001 on the XTerra 3.0×50 mm column with particle size 3.5 µm. The flow rate was 1.2 ml/min, the gradient run time 4 min. The injection volume was 5 µl.

The performance of the two compounds on two columns packed with diverse column material demonstrates, that the difference is not so significant whenever all the other conditions are identical. Only the column diameter is different. On the Waters XTerra column the peaks appear earlier, but even there the difference is not major. The highest peak of sample B8 resolutes at 2.39 min on the Symmetry column and at 2.29 min on the XTerra column. The peak separation is almost the same, maybe slightly better on the Symmetry column, apparently due to the smaller column diameter. However, Waters XTerra column could be recommendable because of its wider pH range and feasibility for alkaline buffer systems.

13. Summary

The tendency of pharmaceutical industry to find new drug candidates in an accelerating speed has put a demand for faster synthesis and lead, i.e. a promising compound for a new drug candidate, development. Many manufacturers have introduced new technologies and devices to meet the requirements of ever-increasing sample throughput. Efforts to enable a faster purification of samples by preparative LC/MS have been made. New process conditions were introduced by the column manufacturer Waters to perform a better purification for alkaline compounds. Alkaline buffer systems and alternative flow gradients were applied in these experiments. The same conditions were implemented at Novartis in order to test if a better resolution and thereby purification could be achieved using the same test compounds and following the instructions published by Waters. However, the introduced results could not be repeated due to the very optimised process conditions that could not be accomplished in practical approach. It could be worthwhile to repeat the experiments on basic eluent system by preparative HPLC using UV as the detector.

It is necessary to know the compound purity after the purification by preparative LC/MS equipment in order to obtain reliable biological screening results. Therefore a fast method for analysing the samples after the purification by analytical HPLC had to be implemented. Different flow conditions and columns were tested to optimise the method. For the applied analysis method Waters XTerra 3.0×30 mm 3.5 µm column was used and the flow rate was set to 1.7 ml/min. The buffersystem was ACN with 0.1 % TFA and H_2O with 0.1 % TFA. The gradient of ACN was from 20 % to 80 % during the gradient run time 2 min.

It is also necessary to know how succesful the synthesis has been before the LC/MS purification is implemented. Valuable time can be saved if it is known that samples are not worth purifying. Unlike after the purification, all the samples of the library need not be analysed and only some examples were investigated instead. Therefore the method to analyse the samples would not have to be as fast as for the post-purification analysis. The optimised method could be implemented on both the Waters Symmetry 2.1×50 mm and XTerra 3.0×50 mm columns with particle size 3.5 µm. The flow rate was 1.2 ml/min, the gradient run time 4 min and the standard flow gradient 5-95 % ACN during the gradient run time was applied.

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