Summary

When searching for new drugs, compounds from natural sources displaying a high degree of structural diversity play an important role. By applying High-Throughput-Screening (HTS) technology, a more rapid discovery of biological active components is possible.
In this field EVOTEC OAI enterprise succeeded in developing a revolutionary screening technology with the EVOscreen®/NACONA-System being connected to an HPLC. It allows to detect bioactivity of single compounds directly from an HPLC run. This concept gives rise to a much more selective and efficient search for biological active metabolites since - in contrast to existing approaches - time-consuming isolation processes are only started when activity has been discovered **previously**.
Based on this innovative technology EVOTEC OAI initiated the **"BMBF Leitprojekt Validierte Lead/Target Systeme - eine horizontal integrierte Verbundstruktur zur automatisierten Pharma-Wirkstofffindung ("Drug Discovery Machine")",** a cooperation between industry and research groups of several universities.
In the project part described in this thesis which was titled "**Pharmakologisch wirksame Inhaltsstoffe einheimischer Pflanzen**" about 800 plants and fungi were extracted with solvents of different polarities. Then these extracts were separated and subjected to HTS analysis in total or as fractions, in order to detect new bioactive constituents. In the course of HPLC/HTS analysis two fundamental problems arose:

1. the great number of different extracts, complex and heterogeneous in nature requires a rapid adaption and optimization of the HPLC separation parameters
2. after determination of a bioactive metabolite during HPLC/HTS analysis it is necessary to get structural information about this hit compound as quick as possible. The reason is that it makes no sense to have a screening machine on the one hand being able to perform bioactivity measurements of several 100 HPLC peaks a day and on the other hand waiting several days for any structural information of one single hit compound.

Therefore, the main subject of this thesis was to find solutions for these two problems of HPLC/HTS analysis.

Ad 1.:

The aim to rapidly optimize and adapt HPLC separation conditions when dealing with numerous samples of varying composition seemed difficult to achieve. In our opinion it requires the application of a method developing software like the new designed ChromSword®Auto being capable of automatically optimizing HPLC separation. Thus, efficiency and limits of this software should be explored in this work.
In detail the following investigations were carried out:

- fully automated method development for separating a coumarin mixture:
  The ChromSword®-Software calculated four gradient and two isocratic methods with the chromatograms included within 62.3 hours. It is true that the run time of

these suggestions was rather high but actually the separation process had already been finished after half and two thirds of the time, respectively.
Although the desired parameter values of retention factor and resolution could not be achieved in every case this automatic method development of ChromSword®Auto has turned out to be a good basis for further optimization processes.

- virtual method development for separating a coumarin mixture under consideration of empirical data:
  Under consideration of the experimental data from the fully automated method development ChromSword®Auto was able to calculate the virtual separation of the coumarin mixture using the EVOscreen®NACONA parameters. After a virtual and experimental optimization of compound separation the chromatograms were in good congruence with respect to order and intensity of the peaks despite some limitations.
  Thus, when changing and transferring methods one should execute a virtual method development on basis of empiric data since then it is possible to prejudge the chromatographic results **before** any experiments have been started.
- virtual change of sorbent demonstrated by using a coumarin mixture:
  After a virtual change of the sorbent had been initiated, ChromSword®Auto generated a simulation using the postulated parameters for gradient elution. Again we found that the chromatograms were well corresponding concerning order and intensity of the peaks in spite of some restrictions.
  Method development with using a sorbent database means to avoid a time-consuming replacement of the column and an expensive buy perhaps proves to be not necessary.
- Since the calculated chromatograms of ChromSword®Auto generally exhibit rather long run times this software cannot be applied for on-line-method development on the EVOscreen®NACONA system.
  However, it can help if methods must be adapted in order to rapidly react on new conditions (e.g. addition of acid to the mobile phase, change of solvent and stationary phase). This software also enables pre-optimization and fully automated method development of an extract of unknown composition aiming to separate a maximum number of peaks. This was demonstrated for an extract of the fungi Lenzites betulina showing bioactivity in a diabetes-type-II-assay. After having added three known reference compounds to this extract, ChromSword®Auto offered ten different gradient methods.
  This proves that the capability of this software to optimize the separation of reference compounds among an extract as "impurity" can be applied successfully for automatic method development of a complex mixture of unknowns.

Ad 2:

The great number of data can only be processed and evaluated efficiently, if structural information from the "hit-compounds" detected are available as quick as possible.
Therefore, we investigated members of various compound classes to find out what kind of information could be obtained from different mass spectrometric methods coupled with HPLC.
In this connection also the influence which some instrument parameters exercised on the spectral appearance was studied.
In total, about 70 reference substances deriving from six natural compound classes (coumarins, phenylpropanoids, flavonoids, anthraquinones, bitter substances, cardiac glycosides) were investigated by several mass spectrometric techniques (Particle Beam, ESI, APCI and to some extent also (ESI)MS^n and common EI). The capability of these methods was discussed exemplarily in detail for five selected natural constituents (coumarin, digitoxin, robinin, sennoside B, visnadin).
The result of these mass spectrometric investigations can be summarized as follows:

1) HPLC/(Particle Beam)MS has proven to be a valuable and powerful technique for analyzing plant extracts (see also 3.4.1):
   - Non-glycosidic members and aglycones of many natural compound groups (molecular weight < 1000 Dalton) could be analyzed successfully, even if they bear several polar groups. They show spectra rich in fragments which were in very good accordance with the corresponding EI spectra of great electronic mass spectral databases so that substances could be identified in this way.
     If EI spectra are not available, structural information can be deduced from the fragmentation pattern of these PB spectra by applying common fragmentation rules known from EI spectra.
   - To our surprise, our studies revealed that even some glycosides as esculin, frangulin A and hyperosid being quite different in structure could be measured by particle beam. However, only the spectra of the corresponding aglycones were obtained.
   - In contrast, the spectra of cardiac glycosides additionally exhibited strong ions, deriving from the fragmentation of the sugar-chain. Therefore, no identification was possible by comparing them with the corresponding EI mass spectra of the pure aglycones.
   - In case of flavonoids we found that members of this compound class were analyzed with different sensitivity by HPLC/(PB)MS.
   - Some non-glycosidic substances (e.g. some coumarins) could not be measured by HPLC/(PB)MS too, presumably because of their volatility.
   - In some cases we observed a strong influence on the sensitivity of particle beam signals by changing the temperature of nebulizer and expansion region. However, no regulary trend was recognized, i.e. optimal temperatures have to be determined individually for each compound group and even compound, respectively.

2) Common EI-MS measurements (introduction through direct inlet) are usually performed with pure isolated components, i.e. this technique plays no role in the field of HPLC/On-Line-Screening.
   That is why we only exceptionally recorded spectra of this type in order to compare them with HPLC/PB spectra.
   In general, both spectra were in very good accordance, probably due to the fact that in both cases ionization was done by electron impact.
3) Particularly, if reference spectra for identification are not available, other ionization techniques like ESI and APCI (in connection with HPLC) must be employed. Nearly all of our samples could be analyzed with these mass spectrometric techniques which often provided complementary structural information and covered a broad range of compound types.
   Electrospray ionization proved to be a valuable tool for analyzing natural constituents since it generates the ions $[M + H]^+$, $[M + H_2O]^+$, $[M + Na]^+$, and sometimes $[2 \times M + Na]^+$, partly with high abundances. This allowed a reliable determination of the molecular peak so that sometimes also a more well-founded and extensive interpretation of the particle beam spectra was possible.
   Apart from that, particularly in the ESI spectra of some glycosides specific ions appeared conveying essential structural information: e.g. the spectra of digitoxin and robinin displayed fragments formed by the loss of one, two and three sugar moieties.
   In contrast, the main fragmentation of sennoside B was the fission of the C-10/C-10'-bond forming the two monomeric anthrone glycosides (as Na-adduct ions). Subsequent ejection of glucose yielded the monomeric aglycone. From these ions essential structural information could be gathered both concerning a homo- or heterodianthrone structure and the number of bonded sugars. Moreover, the occurrence of any further substituents could be estimated.
   Pyranocoumarins of the visnadin-type obviously favour the loss of substituents from the dihydropyran ring; at least one of these ions is of high abundance.
   In general we found HPLC/(APCI) spectra to be more "clean" (exhibiting less ions from contaminants) and to contain a lower number of fragments compared with ESI.
   Apart from that a reliable determination of the molecular weight often was not possible (except e.g. for coumarin) since quasi-molecular ions were absent or too weak.
   Nevertheless these APCI spectra often turned out to be helpful, on the one hand confirming results gained from ESI measurements and on the other hand complementing these data by delivering additional structural information.
   E.g., in the case of digitoxin only the APCI spectrum shows a fragment originating from the molecular ion by loss of two sugar moieties and ions indicating a further decomposition of the aglycone (in particular of the lactone ring).
   From the APCI spectrum of the flavonol robinin the loss of the third sugar can be recognized much easier than from the ESI spectrum because under APCI conditions the specific ion represents the base peak.

Finally, in the APCI spectrum of the dianthrone glycoside sennoside B the characteristic fragments representing the monomeric anthrone glycoside and the dimeric aglycone sennidin arise which are of diagnostic value and are deficient in the ESI record.

4) The DLI/(ESI)MS^n experiments have been performed of selected representatives of different natural compound classes to find out, to what degree further structural increments could be established by applying such sophisticated technique.
   Naturally, these measurements could also have been done in coupling with HPLC, but since we have only analyzed single reference components, introduction via DLI ("Direct Liquid Introduction") seemed to be more appropriate.
   The results of these MS^n experiments can be summarized as follows:

- In principle the (ESI)MS^n technique was found to be a very valuable tool for mass spectrometric structure elucidation of natural compounds.
  On the one hand, it confirmed ms information gained from other spectra (PB, ESI, APCI) and allowed a more reliable interpretation since frequently the origin of fragments could be traced back. On the other hand, consecutive CID experiments (up to MS^6) in general imparted more detailed structural information; in some cases further parts of the molecule structure could be deduced.
- For instance, this was true for the flavonol robinin the MS^3 spectrum of which showed a strong fragment $[(Gal + Glu) + Na]^+$ conclusively indicating the existence of a disaccharide chain.
  Just this knowledge led to a correct interpretation concerning the elimination of single sugar moieties in the common ESI spectrum.
- A different situation is encountered for visnadin belonging to a group of secondary metabolites called pyranocoumarins. The main fragments (data from PB, ESI, APCI) could also be well explained by MS/MS experiments but it is not possible to deduce the pyranocoumarin skeleton in case of an unknown component. However, for visnadin data from MS^n measurements indicated a cyclic system with a carbonyl or carboxylic function. In this connection the separation of an acetoxy- and 2-methyl-butyroxy residue observed in the above mentioned spectra can be interpreted as loss of ring substituents.
- The (ESI)MS^n analysis (ESI in positive mode) of the dianthrone sennoside B only confirmed the information already obtained by other ms techniques before and furnished no new results. The two reasons for this behaviour are first that the dominating primary fragmentation process (in MS^2) consisted in a fission of the central C-10/C-10' bond and second that the prominent main fragment in the MS^3 spectrum was formed by the Na-adduct ion of desoxy glucose.
  Since the sennoside B molecule bears a lot of electronegative groups it proved to be promising to alternatively execute ESI measurements in negative mode, i.e. to record the negative ions and subject them to MS/MS experiments.

In this way we could achieve more detailed structural information:
Apart from the confirmation and more reliable interpretation of the ESI and APCI data we both received a sure proof of two carboxyl and hydroxyl functions attached to a homodianthrone structure.

To sum up, our investigations showed that working in the field of HPLC/HTS analysis it is necessary to apply all common and available HPLC/MS-techniques (e.g. PB, ESI, APCI) to rapidly obtain as much structural information as possible about the detected bioactive compounds.
It is true that in this connection MS/MS proved to be a powerful tool for mass spectrometric structure elucidation too, but the limitation of this high sophisticated technique lies in a higher expenditure of time and staff making it unsuitable for routine analysis.
This holds also true for HPLC/NMR coupling an interesting new technique being well established today which provides essential data about the molecular structure quite different from those of ms. Unfortunately, we had no access to this fascinating analytical method which will play an important role in this field of research in future.