On-line multidimensional LC-techniques.

Determination of known and unknown compounds in limited and complex samples.

Dissertation for the degree Philosophiae Doctor

by

Steven Ray Haakon Wilson

Department of Chemistry

Faculty of Mathematics and Natural Sciences

University of Oslo

Contents

Preface

The work presented in this thesis was carried out at the Department of Chemistry, University of Oslo in the period 2003-2007 under the supervision of Prof. Elsa Lundanes, Prof. Tyge Greibrokk and Prof. Frode Rise. I am so grateful for having been under their wings.

I would also like to thank Anders Holm and Pål Molander, who were my supervisors (alongside Prof. Lundanes and Prof. Greibrokk) during my Masters degree. They have also been of great help during these years I have been a PhD student.

Thanks also to all of my wonderful collegues, especially Helle Malerød, Mikolai Jankowski, Milaim Pepaj, Hege Lynne, Hanne Rødberg Larsen, Dirk Petersen, Nebojsa Simic, Albena Mihailova. Also, I would like to thank Roger Trones and Thomas Andersen at G&T Septech.

Special thanks to my students; were would I have been without you?

A big round of applause for my band "Superfamily"; my face is wrinkled with channels of joyful tears, from all the fun we've had.

Thanks to all my friends. I love you all very much.

Thanks to my brother Todd, and his wife and son; Cecilie and Anker. Todd, you have always been my hero, and you always will be.

I would literally not have been here if it wasn't for Mom and Dad. Thanks for all the love, Star Wars toys, laughs, encouragement, tuna fish sandwiches and moments we have had.

Finally, I would like to thank My Girl, Taran, whom this thesis is dedicated to. I love you like the Sun loves the dawn.

Steven Wilson
Oslo, June 2007-06-04

List of papers

This thesis is based on the following papers, referred to Roman numerals in the text:

I. **Determination of Bradykinin and Arg-bradykinin in Rat Muscle Tissue by Microdialysis and Capillary Column-switching Liquid Chromatography with Mass Spectrometric Detection.** Steven Ray Wilson*, Fernando Boix, Anders Holm, Paal Molander, Elsa Lundanes, Tyge Greibrokk
Journal of Separation Science (2005), 28(14), 1751-1758.

II. **Controlling LC-SPE-NMR Systems.**
Steven Ray Wilson*; Helle Malerød, Dirk Petersen, Nebojsa Simic, Maria-Magdalena Bobu, Frode Rise, Elsa Lundanes, Tyge Greibrokk
Journal of Separation Science (2006), 29(4), 582-589.

III. **An Alternative LC-SPE-NMR Design.**
Steven Ray Wilson*, Helle Malerød, Dirk Pedersen, Frode Rise, Elsa Lundanes, Tyge Greibrokk
Journal of Separation Science (2007), 30(3), 322-328.

IV. **Identification of the major metal complexing compounds in *Blepharis Aspera.***
Edward Eddie Mmatli, Helle Malerød*, Steven Ray Wilson, Berhanu Abegaz, Tyge Greibrokk, Elsa Lundanes, Karl E. Malterud, Dirk Petersen, Frode Rise
Analytica Chemica Acta (2007), 597 (1), 24-31.

V. **2D LC Separation and Quantitative Determination of Bradykinin in Rat Muscle Tissue Dialysate with On-line SPE-HILIC-SPE-RP-MS.**
Steven Ray Wilson*, Mikolai Jankowski, Milaim Pepaj, Albena Mihailova, Fernando Boix, Gabriel Vivó Truyols, Elsa Lundanes, Tyge Greibrokk
Chromatographia (accepted 15th june, 2007).

In addition, seven supplementary papers related to the thesis were published or accepted for publication in the same time frame:

SI. **Determination of Perfluorooctane Sulfonate and Perfluorooctanoic Acid in Human Plasma by Large Volume Injection Capillary Column Switching Liquid Chromatography Coupled to Electrospray Ionization Mass Spectrometry.**
Anders Holm*, Steven Ray Wilson, Paal Molander, Elsa Lundanes, Tyge Greibrokk
Journal of Separation Science (2004), 27(13), 1071-1079.

SII. **Determination of perfluorooctane sulfonate and perfluorooctanoic acid in river water by nano-liquid chromatography coupled to nano spray ionization mass spectrometry**
Steven Ray Wilson*, Helle Malerød, Anders Holm, Paal Molander, Elsa Lundanes, Tyge Greibrokk
Journal of Chromatographic Science (2007), 45(3), 146-152.

SIII. **Comparison of Advanced Oxidation Processes and Identification of Monuron Photodegradation Products in Aqueous Solution.**
Maria Bobu*, Steven Ray Wilson, Tyge Greibrokk, Elsa Lundanes, Ilie Siminiceanu
Chemosphere (2006), 63(10), 1718-1727.

SIV. **Comparison of Fenton and Sono. Fenton Bisphenol A Degradation**.
Iordache Ioan*, Steven Ray Wilson, Elsa Lundanes, A. Neculai
Journal of Hazardous Materials, available on-line August 12. 2006.

SV. **Two-dimensional Capillary Liquid Chromatography: pH Gradient Ion Exchange and Reversed Phase Chromatography for Rapid Separation of Proteins.**
Milaim Pepaj*, Steven Ray Wilson Katerina Novotna, Elsa Lundanes, Tyge Greibrokk
Journal of Chromatography, A (2006), 1120(1-2), 132-141.

SVI. **Determination of oxomemazine in human plasma by capillary LC-ESI-MS**.
A. L. Saber, S. R. Wilson*, M. A. F. Elmosallamy , Elsa Lundanes, Tyge Greibrokk
Journal of Liquid Chromatography and Related Techniques, (2007), 30, 393-403

SVII. **Improving the resolution of neuropeptides in rat brain with on-line HILIC-RP compared to on-line SCX-RP**

Albena Mihailova[1], Helle Malerød[1*], Steven Ray Wilson[1], Bartosz Karaszewski[2], Roman Hauser [3], Elsa Lundanes[1], Tyge Greibrokk[1]

Journal of Separation Science (accepted September 5th, 2007).

Abstract

The work presented in this thesis deals with instrumental-and method development of various multidimensional on-line liquid chromatography-solid phase extraction-spectroscopy combinations and applications performed utilizing such instrumentation.

Reasons for employing on-line instrumentations are, amongst others, the possibility of automation with a lowered risk of human error and greater lab throughput, and the increased ability to handle small sample sizes and low analyte concentrations.

Liquid Chromatography (LC)-based on-line systems (i.e. on-line LC-NMR or on-line two dimensional (2D LC)) are available without Solid Phase Extraction (SPE) columns incorporated in the system. However, employing SPE columns have many benefits, such as enabling large volume injections on capillary LC (cap LC) columns, aiding the separation of very large amounts of compounds with conventional HPLC columns and eliminating inter-dimension solvent incompatibility in 2D LC systems.

In this thesis it is argued that on-line LC-SPE combinations are well suited tools for solving several key problematic situations encountered in chromatography; when the target compound(s) is often present at very low concentrations in a small sample, and is hence not easy to detect, when the compound(s) of interest must often be separated from the other compounds present, and when the identity of target compound(s) in a mixture is unknown.

Paper I describes the method development of an **on-line SPE-cap LC** system designed for identification of kinins in rat muscle tissue. The kinins were extracted from muscle by microdialysis, and since the target compounds were expected to be low in concentration, cap LC was employed instead of conventional LC to improve the sensitivity of the method. An on-line SPE system was employed to allow samples of 450 µL to be injected without compromising the chromatographic performance of the cap LC column. Also, the on-line system elegantly traps and transfers the entire amount of target compounds without compound loss to the LC column, which would have been difficult/ impossible with such limited samples if off-line techniques had been employed instead.

A second on-line SPE-LC based multidimensional technique, **LC-SPE-NMR**, is based on separating compounds on an LC column, and trapping the individual compounds on separate

SPE columns. This step can be repeated, trapping bands of the same compound on the same SPE. When a sufficient amount of compound is trapped on an SPE for NMR analysis, it is transferred to the NMR for structural elucidation. This process can enable the isolation of large amounts of the compound, and can be used to separate the compound from NMR-noisy, non-deuterated solvents. However, this process is prone to difficulties, typically due to problems with trapping a compound on the SPE or transfer of the compound from the SPE to the NMR.

Paper II describes approaches that will increase the chance of performing successful LC-SPE-NMR, by varying the separation column diameter, SPE stationary phases and employing a novel and robust SPE-NMR transfer technique.

Paper III continues to describe ways of improving the chance of successful LC-SPE-NMR. In this paper, an alternative approach to SPE trapping is described, which is vastly simpler and much more flexible than SPE trapping units used in commercial LC-SPE-NMR systems. In addition, it was shown that a common LC-SPE-NMR step (SPE drying) can be omitted, as it appeared to have no real function. The paper also includes a discussion of which NMR probes are logical to employ together with the alternative/modified LC-SPE system.

In **Paper IV**, the technical developments and understandings from papers **I-III** were applied for LC-SPE-NMR of unknown compounds in the plant *Blepharis Aspera*. *B. Aspera* has the ability to survive in soils with high levels of metals, which are usually poisonous to plants. It was speculated that the plant contained compounds in high amounts that could complex with the accumulated metals, disabling their toxicity.

Compounds which were high in concentration and found to be metal complexing were identified as the phenylpropanoids verbascoside and isoverbascoside using LC-NMR.

In **Paper V**, the instrumentation and understandings developed in the previous papers were combined for the development of a novel on-line, multidimensional LC system. Hydrophilic Interaction Chromatography (HILIC) and Reversed Phase (RP) chromatography were used for 2D LC separation (**SPE-HILIC-SPE-RP**) of peptides arising from tryptic digestion of proteins, as well as selective isolation and quantification of kinins, again in rat muscle dialysate samples.

Abbreviations

Arg-bradykinin	Arginine bradykinin
ACN	Acetonitrile
capLC	Capillary Liquid Chromatography
cLOD	Concentration Limit Of Detection
ESI	Electrospray Ionization
ELSD	Evaporative Light Scattering Detector
EIC	Extracted Ion Chromatogram
FA	Formic Acid
HILIC	Hydrophilic Interaction Chromatography
ID	Inner Diameter
IT-MS	Ion Trap-Mass Spectrometry
LC	Liquid Chromatography
MeOH	Methanol
mLOD	Mass Limit Of Detection
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PFHA	Perfluoroheptanoic Acid
PFOA	Perfluorooctanoic Acid
PFOS	Perfluorooctane Sulfonate
PGC	Porous Graphite Carbon
RP	Reversed Phase
RSD	Relative Standard Deviation
SCX	Strong Cation Exchange
S/N	Signal-to-Noise
SPE	Solid Phase Extraction
TBA	Tetrabutylammonia

TCA	Trichloroacetic Acid
TIC	Total Ion Chromatogram
TOF	Time-Of-Flight
UV	Ultraviolet
ZIC	Zwitter ionic

Definitions

Classification of LC according to internal diameter (ID) of columns (Y. Saito, K. Jinno, T. Greibrokk, J. Sep. Sci. 2004, 27, 1379-1390):

.

Column designation	Typical ID [mm]
Conventional HPLC	3-5
Narrow-bore HPLC	2
Micro LC	0.5-1
Capillary LC	0.1-0.5
Nano LC	0.01-0.1
Open tubular LC	0.005-0.05

Dimension: *Any measurable extent, as length, width, depth etc. (Webster's Dictionary)*
Thus, the Author considers e.g. *m/z* values (MS) and chemical shifts (NMR) to be dimensions, and therefore e.g. an LC-MS-NMR system is in this text defined as a multidimensional technique.

INTRODUCTION

Whenever a scientist is going to determine which compounds are in a sample, and how much of them are in the sample, there is a strong chance that the analyst will use chromatographic techniques. If the analytes are possibly thermolabile, High Performance Liquid Chromatography (HPLC) is often the technique of choice, most often coupled with a UV detector or a MS mass spectrometer.
The first articles describing HPLC were published in the late 1960's [1-4], and since then HPLC has become an established technique, used in thousands of laboratories all over the world, each performing up to several hundreds of HPLC analyses per day.
To achieve a successful HPLC analysis, there are often several potential challenges that must be overcome. Some of these are:

1) The compound(s) is often present at very low concentrations in a small sample, and is hence not easy to detect.
2) The sample is complex.
3) The identity of compound(s) in the sample is unknown.

Regarding **1)**, there are several ways of overcoming this challenge. For example, the analyst can employ a **miniaturized HPLC column**.
Miniaturized HPLC is based on using LC columns which have reduced inner diameters (e.g. capillary LC [capLC] columns have I.Ds of 0.1-0.5 mm [5]). Miniaturized HPLC was introduced by Horváth and coworkers already in 1967 where stainless steel columns with an I.D of 0.5-1 mm packed with pellicular particles were used for the separation of ribonucleotides [6]. However, it was as late as the 1990's that miniaturized HPLC was considered to be an attractive alternative to conventional HPLC [7].
With miniaturized HPLC, the chromatographic peaks become higher (but not narrower in time) than with a regular HPLC column, due to reduced radial dilution over the column, and hence higher concentration of the analytes [8].
Therefore, miniaturized LC systems are more sensitive when employing a concentration sensitive detector, as is common in HPLC. However, a cap LC column requires reduced

injection volumes relative to conventional HPLC to maintain chromatographic efficiency [7]. Thus, cap LC is often a suitable choice of instrumentation for the detection of compounds present at low concentrations or present in small, limited samples.

2) Sometimes a sample is so complex that a one dimensional (1D) separation is not adequate. Although the employment of a MS detector allows for monitoring analytes that co-elute with other compounds, it is often the case that the co-eluting compounds cause a distortion in the quantification, even when employing an internal standard [9], or can even erase the signal of the analytes(s) due to suppression effects [10]. To overcome this problem, the analyst will often prepare the sample so that interfering compounds are removed (e.g. protein removal by precipitation and centrifugation [11]), or isolate the target analyte prior to analysis (e.g. a selective sample preparation based on the chemical characteristics of the target analyte, e.g. pI value [12]).
However, sometimes the analyst considers virtually *all* the compounds in a complex sample to be interesting, and would therefore not wish to perform extensive compound-removing/isolating steps. Since a single HPLC column (that can produce 100,000 plates/meter) is not expected to be able to separate more than ~40 separate compounds [13], one approach to satisfy this goal is to use **comprehensive two-dimensional (2D) LC** (LC × LC) which was pioneered by Erni and Frei [14] and Buhey and Jorgenson [15].
In 2003, a set of conditions (based on definitions formulated by Giddings [16]) were proposed [17] to define what a comprehensive, two-dimensional separation is:

-Every part of the sample is subjected to two different separations.
-Equal percentages (either 100% or lower) of all sample components pass through both columns and eventually reach the detector.
-The separation (resolution) obtained in the first dimension is essentially maintained.

The performance of a 2D LC system depends on the *peak capacity* [16, 18] and the *orthogonality* [19].

Peak capacity can be defined as the maximum number of component peaks that can be packed side-by-side in the available separation space with just enough resolution to satisfy analytical goals of the separation modes/chromatographic dimensions [16]. The peak capacity of 2D separation can be defined as a *linear combination* of peak capacities in both separation dimensions [16].

The term orthogonality (which means "at right angles") is often used to describe the extent of non-correlation between separation mechanisms [20].

For instance, if the two LC techniques employed in a 2D separation have peak capacities of 50 and 100 respectively, the resulting peak capacity should (in theory) be the linear combination: LC × LC = 50 × 100 = 5000 peaks. However, this number can be drastically lowered if the two separation modes are not "orthogonal" as shown in **Figure 1.**

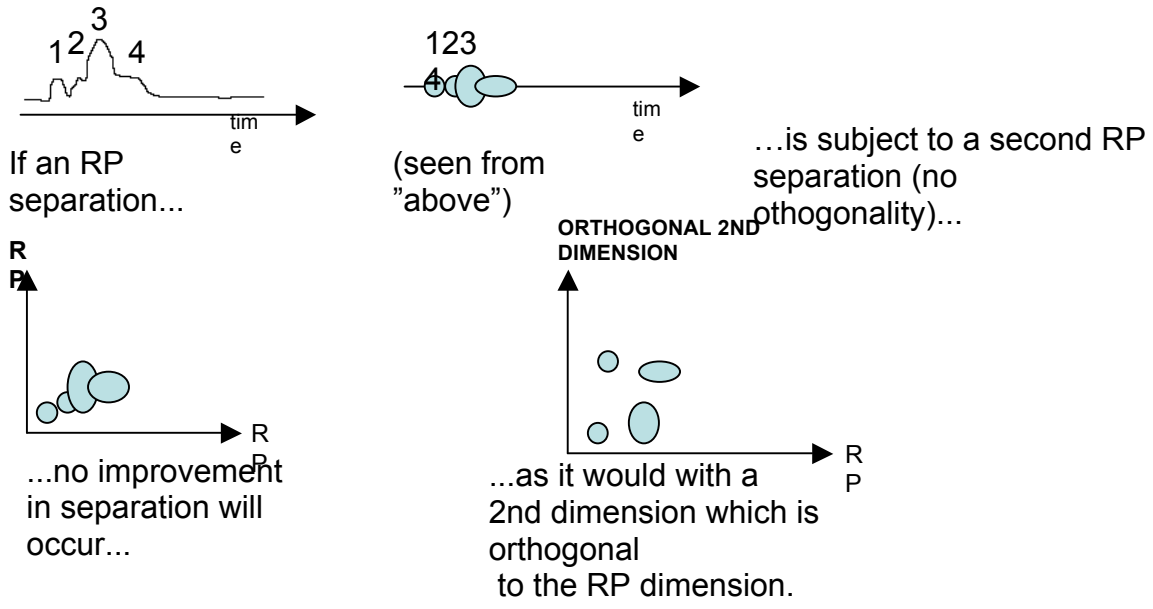


Figure 1: orthogonality in chromatography.

3) In most cases, the identity of the target compounds in a sample is established prior to analysis, and the analysis consists of confirming the eventual presence of a compound, and perhaps the amount of the compound in the sample. However, if the target compound is an

unknown, it can be very difficult to identify the compound with LC-MS/UV instrumentation alone [21]. One of the most used techniques for determining the structure of unknown organic compounds is **Nuclear Magnetic Resonance (NMR) spectroscopy**.
Traditionally, an unknown is manually isolated from the matrix in several *off*-line steps (e.g. liquid extraction, freeze-drying, preparative HPLC). However, in the last couple of decades LC has been successfully *on*-line coupled with NMR instruments for compound isolation followed by e.g. analysis [22-26] or screening purposes [27-29].

OFF-LINE VERSUS ON-LINE METHODOLOGY

In this thesis, to employ an off-line method means that the analyst must manually participate in the preparation of the sample prior to the final analytical separation/identification/quantification. An example of an off-line methodology is if the operator collects the eluent from a preparative HPLC column and places the eluent in the NMR *by hand*. The on-line version of this scenario would be that the eluent is transported directly from the LC and in to the NMR by a tubing connecting the two instruments. The advantages of on-line methods are that they are **automatable**, are more capable of handling **small samples** than off-line methods and are **less prone to contamination.** (In addition, on-line methodology gives the flexibility of being able to inject smaller or larger volumes than normal, directly on to an HPLC system. This more subtle point will be discussed in detail later).
The following is a brief introduction of on-line instrumentations based on the techniques described above, namely capLC, 2D LC and LC-NMR, and issues that need to be addressed when developing these systems or methods based on these systems.

On-line SPE-cap LC

SPE is usually used to clean up a sample before chromatographic analysis of e.g. plasma, urine and water etc. Also, SPE is used to enrich the analyte on the column prior to separation, for more sensitive methodology. However, off-line SPE steps can be laborious, can be prone to contamination and can be difficult to use with small, limited samples. An alternative is to perform the SPE and LC steps on-line, and this technique is often referred to as *column switching* [30-39],(**Figure 2**) which was first reported by Huber et al [33].

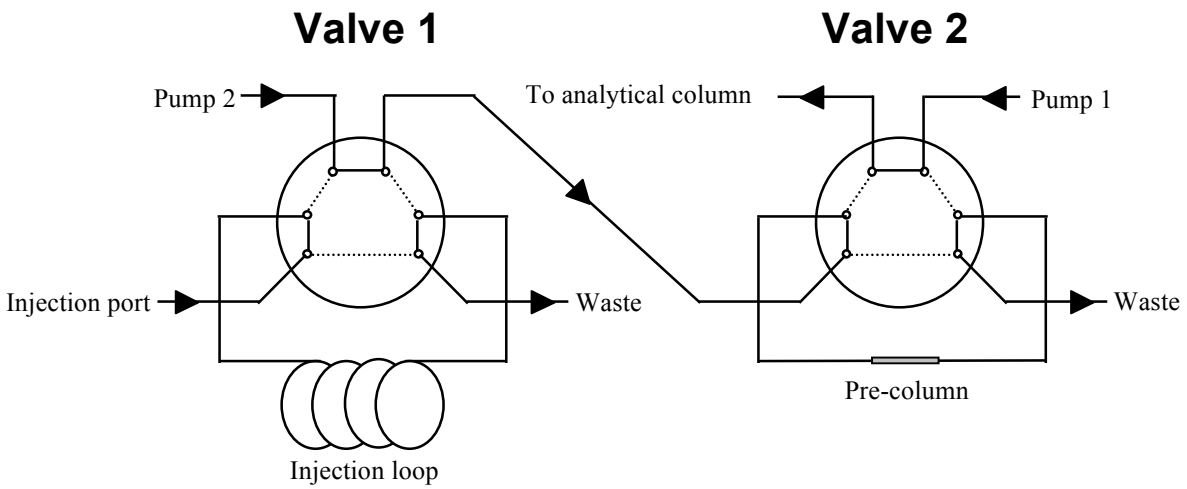


Figure 2: Systematic description of a column switching system, used in **Paper I**.

The principle of column switching is established and quite well known, but will be described here, as it serves as a base for more intricate schemes that will follow in this thesis:
A weak mobile phase is used for transfer of the sample to an SPE enrichment column. The mobile phase must be weak enough so that the analyte(s) are retained on the enrichment column. When a reversed phase system is used, solvent, salts and less hydrophobic compounds which are not retained will pass through the enrichment column and to waste. When the entire sample has been loaded on to the SPE enrichment column, the easily automated plumbing of the system is set so that a second mobile phase (from a second pump) passes through the enrichment column in the backflush mode [39], and is strong enough to elute the analyte(s) from the enrichment column and on to an analytical reversed phased column for chromatographic separation.
The switching system effectively allows the injection of larger sample volumes, e.g. 10 to 1000 μL [40, 41], than what is normal for a cap LC column (~50 to 1 μL) or even a conventional HPLC column (~5-40 μL).
Due to the sensitivity and sample utilization of column switching combined with cap LC, it is a technique that is capable of solving issue **1)**.

Although the principle of column switching is well known to be successful, several issues must be addressed when developing a method based on this technique:

-Can the analyte(s) be focused on the SPE column?
-How large sample volume can be injected?
-How fast can the sample volume be introduced to the SPE column?
-How does the matrix affect these issues?

These issues will be discussed in detail later in this thesis.

On-line 2D LC

LC × LC is a powerful approach for resolving complex samples and is thus an approach to solving **2)**. LC × LC is very often performed on-line [42-44], due to many of the same incentives associated with on-line SPE-LC, namely the ability to automate the system and handle small samples/1st dimension fractions. However, there are three issues which must be addressed when designing an on-line LC × LC system:
i) Which separation modes are to be used, and are they orthogonal regarding the separation of compounds in the sample to be analyzed?
ii) Will the on-line combination of the two separation modes be restricted due to lack of inter-dimension eluent compatibility?
iii) Which restrictions appear regarding time, the choice of flow rates and column diameters?

i) Examples of implementations of LC × LC are reversed phase LC in combination with normal phase LC (RPLC × NPLC) (used for e.g. the separation of oil extracts) [45], RPLC in combination with size exclusion chromatography (RPLC × SEC) (used for e.g. separation of complex polymers [46]) and strong cation exchange LC in combination with RPLC (IEC × RPLC) [47], with the latter being the most common combination for the separation of peptides. However, it was recently reported that this combination was not as orthogonal as expected since tryptic peptides are mostly doubly charged, resulting in a somewhat poor distribution of peaks in the 1st dimension, and therefore alternative combinations of separation modes are under investigation [19].
ii) When an analyte is transferred from one dimension to the next, an aliquite of the 1st dimension solvent will also be transferred. This can be problematic, e.g. if a compound elutes

from the 1st dimension with hexane, this solvent can create severe chromatographic problems for an RP 2nd dimension [48]. Another problem can occur if e.g. the 1st dimension and 2nd dimension are packed in the same column and the mobile phases of both dimensions enter the MS detector [49]; if an IEC mode is employed, the ESI-disturbing salty mobile phases can cause poor analyte response.

iii) Due to the third criterion to 2D separations of maintaining the separation in both dimensions (see above), it is generally agreed upon that the second dimension in on-line 2D LC systems must be capable of completing separations very rapidly compared to the first dimension [50].

Schoenmakers et al has described a protocol for designing LC × LC systems [51], and one of the main points was to employ a much narrower 1st dimension column (i.e. a 1 mm I.D) compared to the 2nd dimension column (i.e. a 4.6 mm I.D). Such an approach can reduce the effects of incompatible mobile phases, but is a difficult system to use for the analysis of compounds present at low concentrations.

Many of these issues can be overcome by refocusing the analytes with a **SPE unit** placed between the two dimensions [52]. Hence, the approach of using an **LC-SPE-LC** system will be prone to the same challenges that must be addressed when developing on-line SPE-LC systems, which are described above.

On-line LC-NMR

As mentioned, LC and NMR are most often combined in an off-line manner: following a collection of target compound-containing eluent, the eluent must be evaporated to remove the non-deuterated solvents, which almost always produce very strong NMR signals, which will e.g. suppress the signal of the compound, disabling structural elucidation. This process can potentially alter or remove the compound due to e.g. interactions with the drying equipment or low boiling points of the compound. Also, this process can be impractical if the target analyte is preset at very low levels. Since such an approach can be tedious, unreliable and is a poor approach for the handling of small amounts of target compound, **on-line LC-NMR** would seem to be an attractive alternative. On-line NMR was first pioneered by Watanabe and Niki [53] in the late 70's. Since that time, the technique has been employed in fields such as

biomedical and pharmaceutical applications [54-56] metabolism studies [23, 57], natural product analysis [58-60] and environmental analysis [61-64]. LC-NMR has also been a subject to technological variations, such as the combination of cap LC and NMR [65] and superheated-water LC-NMR [66]. Despite these technological explorations, the coupling with NMR has ultimately proven to be difficult, even with several technological advances in recent years, such as the development of solvent suppression methods [67] and improvements on probe design [68-70]. The main problem of the on-line coupling is that the compound amounts needed for NMR analysis are usually much larger than the amounts that can be injected on to a HPLC column without the occurrence of a column overload. This problem has previously been addressed by Albert et al, where an overloaded C_{30} HPLC column was used to separate 4 out of 5 tocopherol isomers without loss of chromatographic resolution compared to a not-overloaded C_{18} column [25]. However, this approach requires long run times at low flow rates, and will likely not be attractive to users who deal with more complex samples. Using a preparative sized column instead will not improve the on-line approach either, because the volume in which the peak elutes in will be too big for an NMR probe.

To overcome these mismatches, LC-NMR systems often include a SPE trapping unit, placed between analytical scale HPLC instrumentation and the NMR in an on-line system (**LC-SPE-NMR** [71-79]). This allows for performing multiple trapping of compounds on individual SPE cartridges. Hence, regular HPLC can be performed without overloading effects, and the compound of interest can be trapped on the same SPE many times, and finally the compound can be eluted to the probe in an appropriate elution volume.

For economic reasons, the chromatography and trapping is performed with non-deuterated solvents, and compound elution is performed with deuterated solvents. LC-SPE-NMR is now used with commercially available instrumentation. However, several groups have expressed dissatisfaction with this approach, and have either reverted to manual procedures or designed alternative instrumentation [80-82]. One root of dissatisfaction is that the target compound is not retained on a SPE cartridge during the multiple-trapping stage, echoing the issues needed to be addressed when developing column switching methodology.

AIM OF STUDY

Research laboratories are frequently met with challenging samples that are available in small amounts, with target compounds in minute concentrations and which structure is unknown. To meet these challenges, it is often an advantage to use **on-line systems**, as they can maximize the potential of the individual parts of the system, and minimize the effects of off-line handling (e.g. contamination and cost). However, there are often difficulties in combining instruments in an on-line coupling, but many of these can be overcome by using SPE trapping units between the "mismatching" instrumentations or techniques, resulting in various **LC-SPE combinations**. However, due to the increased complexity of on-line LC-SPE systems versus off-line procedures, careful method development, and sometimes technological improvements are needed. This thesis describes technological advances, method developments and applications of the three on-line techniques described above; SPE-CapLC, LC-SPE-NMR and 2D LC.

ON-LINE SPE-CAPLC

Limited sample volumes, low concentrations: Kinins in muscle tissue, extracted by microdialysis

Often, the samples that are to be analyzed by HPLC are available in ample amounts, such as wastewaters, urine, pharmaceutical products, polymers, etc. Therefore, if the sample contains a target compound present at low levels, the operator can simply utilize more of the sample during the preparation. However, if the sample is limited, as is the case with e.g. *microdialysis* samples, the operator must often use techniques associated with elevated sensitivity, for instance miniaturized LC.

Microdialysis

The microdialysis technique [83] allows for the continuous sampling of substances from the interstitial space by inserting in an animal or human tissue a probe with a dialysis membrane, which is constantly perfused with a solution. The recovery of a compound is dependent on e.g. the concentration, flow velocity, and diffusion coefficient of the compound through the membrane [83].

The total volume of a microdialysis sample is often small (~10-500 µL), and the time of sampling can be many hours. The samples are precious in that the sampling is somewhat difficult, and qualified health personnel must perform the sampling. Also, the procedure can be uncomfortable for the subject.

Kinins in muscle tissue, sampled with microdialysis

Kinin peptides (bradykinin and kallidin) can be released by muscle contractions in various animals, and have been coupled to the appearance of muscle pain [84]. However, in humans, the delivery of kinins is due to the increase in kallikrein enzymes, which liberate bradykinin and lys-bradykinin from high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK), respectively [85]. It has been assumed that rats can only release bradykinin [86], but an immunoassay study employing a kallidin antibody revealed the

presence of a kallidin-like peptide [87], most likely arginine-bradykinin. In **Paper I**, capLC-MS was used to test this hypothesis.

The samples that were to be analyzed were ~500 µL in volume, and the level of kinins was expected to be in the pg/mL range. Since these low concentrations were close to the mLOD and mLOQ of the instrument, it was desirable that entire samples would be subject to analysis. The crudest way of transferring the entire sample would be to inject the sample directly on to the capillary column, but this approach would not be feasible since the injection alone would have taken one hour per sample. Also, the matrix was high in salt content (the perfusion solvent was physiological Ringer-acetate solution, with a molality of 300 mOsm, which would likely contaminate the MS interface.

An off-line approach to solving these issues would e.g. be to perform an extraction with an SPE cartridge, eventually followed by a drying step to remove an excessive amount of organic modifier, prior to HPLC analysis. This approach would most likely result in a loss of sample, and therefore an on-line column switching system was employed.

As mentioned in the introduction, there are several issues that need to be addressed when developing a switching system for a specific application, and most of these concern **the trapping of analyte on the SPE column**.

Analyte (kinin) focusing on the SPE column

One simple way to assess whether an analyte can be trapped on an SPE (C_{18}) column is to inject a standard of the analyte on to the SPE column which is directly coupled to the detector. However, the compound may elute in such a broad band that it is impossible to determine if it has been eluted or has been fully retained. A more practical approach is to inject the compound and elute it from the SPE with relatively strong mobile phases (e.g. 30, 40 and 50 % organic modifier), and plot the logarithm of the retention time versus the percentage of organic modifier in the mobile phase. With C_{18} columns, this relationship will often be linear [88], and by means of regression analysis, the maximum amount of organic modifier that can be used without a *breakthrough* [89] occurring for a specific time interval can be estimated. If this value has a negative sign, the implication is that the SPE material cannot be used no matter the amount of organic modifier. Kinins were found to be retained on a C_{18} SPE column

with loading solvent compositions with ~5 % ACN (with 0.1 % FA added to the mobile phase) (**Paper I**).

Effect of sample volume injected and speed of sample transfer to SPE column

Although it was observed that C_{18} SPE columns could fully retain kinins, it was possible that the volume injected and the loading flow rate could affect the trapping process, resulting in an analyte breakthrough. However, it was found that the kinins could be injected in aqueous volumes up to 800 µL at a flow rate of 250 µL/min without effecting on the peak area (**Paper I**).

Matrix effects

It was speculated that the large concentration of salt in the sample could affect the ion pairing between the formate ions in the loading mobile phase and the analytes, and cause a plug elution of the analytes. To assess this, the experiments described above were repeated, but with the kinins injected in Ringers acetate instead of water. No difference in peak areas could be observed compared to the not-salty, aqueous standards.

Transfer to the LC column

When an SPE trapped compound is back-flushed on to an LC column, it is dissolved and transferred to the column in a cap LC-compatible amount of eluting mobile phase (~1-2 µL), which would be impossible to achieve with an off-line approach. In addition, a refocusing effect will also occur on the analytical column, due to the employment of stronger retaining column material (5 µm particles with C_{18} stationary phase) on the LC column compared to the SPE column (10 µm particles with C_{18} stationary phase). Therefore, no extra band broadening effects were observed when employing a switching system compared to a regular system.

Analysis of rat muscle tissue

The switching-system method for determination of kinins in microdialysate samples finally consisted of injecting samples of 500 µL (450 µL sample + 50 µL internal standard solution) on to a SPE at a flow rate of 250 µL/min, and back-flush the contents to the LC column at a flow rate of 10 µL/min. The kinins were separated with a C_{18} capillary column, and arginine-

bradykinin was identified in the sample (**Figure 3),** confirming the hypothesis made in the previous immunoassay study [87].

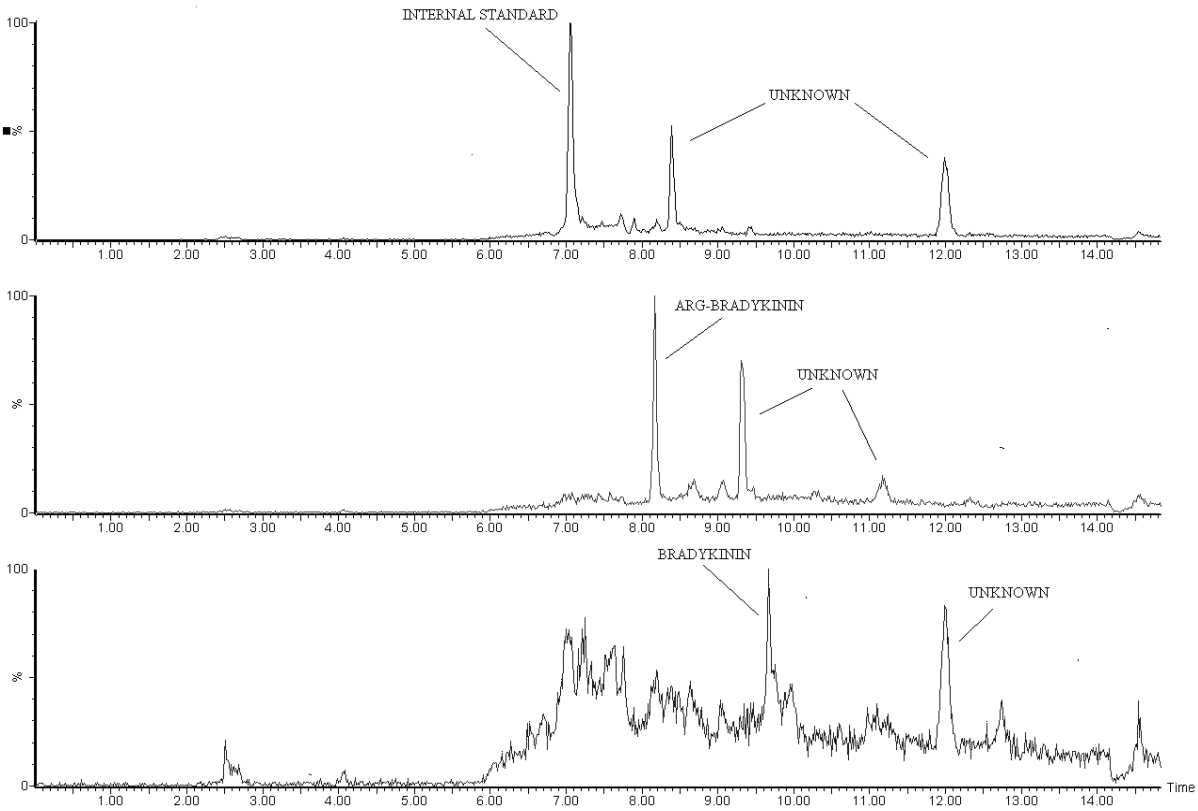


Figure 3: Analysis of dialysate collected from rat muscle tissue using the developed column switching system. The extracted SIC for (tyr^8) bradykinin, arg-bradykinin and bradykinin are displayed (**Paper I**).

On-line SPE-capLC: some conclusions

On-line SPE-cap LC is an approach which can transfer virtually all of a dilute target compound in a limited sample to the separation column and is not prone to human error during the sample preparation. This approach was successfully used for target compound determination of small, precious microdialysis samples. The instrumentation, although more complicated than regular HPLC instrumentation, is very robust as long as steps are made to ensure an effective trapping of the compounds on the SPE.

ON-LINE LC-SPE-NMR

Technological developments

In **Paper I**, a column switching system was employed to enable the injection of a 500 µL sample volume on to a cap LC system, where a regular cap LC system would only be able to handle 2-3 µL of sample volume.

With LC-SPE-NMR, the SPE component allows an analytical LC system to resolve large *amounts of compounds* in mixture (5-50 µg or more per compound), since multiple trappings can be performed. A regular HPLC system is often only able to resolve ~0.5 µg per compound. The reason for wanting to resolve large amounts of compound is to **compensate for the poor sensitivity of the NMR spectrometer.**

In commercial LC-SPE-NMR systems, an HPLC separated compound is detected by a UV (and/or MS) detector and this detection triggers an SPE trapping system, which directs the flow to an SPE cartridge, which is placed in front of the outgoing flow by a robotic arm **(Figure 4**).

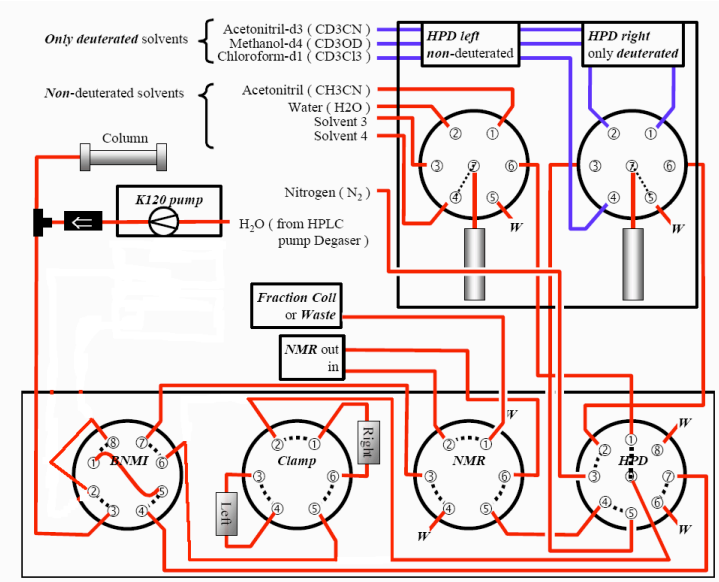


Figure 4: Scheme of Bruker LC-SPE-NMR system.

After an eventual multi-trapping step (e.g. 500 ng of a resolved compound × 10 injections = 5 µg trapped on the same SPE), the SPE column is flushed with nitrogen with the intent of

removing non-deuterated solvents in the SPE cartridge. The SPE cartridge is subsequently washed with deuterated water, and finally a fixed volume of deuterated ACN flushes the trapped compound in to the NMR probe.
Unfortunately, it was experienced with this system that after a compound was detected, it was difficult to tell exactly where the compound was in the system, since the NMR spectra were often blank. There could be several reasons for this: *The compounds were not trapped on the SPE column; an SPE-trapped compound was not accurately transferred to the NMR probe; the amount of transferred compound was not sufficient for NMR detection*.

The next pages describe how these potential problems were assessed and resolved.

SPE trapping
To assess if compounds were being trapped on the SPE columns, a UV detector was connected to the outlet of the LC-SPE system and experiments with standard compounds showed that SPE breakthroughs would easily occur. There could be several reasons for this, and are familiar from the previous chapter: The elution strength of the eluent was too strong; the flow rate was too high over the SPE column; the SPE stationary phase was not appropriate for the application.
To weaken the eluent strength, the eluent must be mixed with a diluting solvent (using a second pump) prior to SPE trapping. An adequate mixing ratio needed for trapping a specific compound (for a specific time) can be estimated by running a number of experiments consisting of injecting the compound on to the SPE column and varying the mobile phase composition, as described in the on-line SPE-cap LC chapter above. For many compounds, the amount of organic modifier must be less than 10 % when using a C_{18} based SPE column (for multiple trapping, the amount must often be less). Subsequently, if a e.g. 50 %ACN separation mobile phase flows through the HPLC column at 1 mL/min, the mobile phase must be post-column diluted 5 times with an aqueous solvent to obtain a composition of 10 % ACN. Unfortunately, this approach can be very impractical. Not only will a flow rate of e.g. 6 mL/min create a pressure problem within the complicated system, a high flow rate can cause a breakthrough over an SPE column [90, 91]. Ways to avoid this is to lower the HPLC flow rate or to use a weaker separation solvent. Both of these approaches will make the separation times

substantially longer, and possibly compromise the chromatographic separation due to e.g. longitudal diffusion. However, employing a 2.1 mm I.D. column instead of a 4.6 mm column allows for a flow rate reduction by a factor of ~5, without compromising the column efficiency (**Paper II**). Although the system pressures were lowered using a 2.1 mm I.D. column, it was difficult to monitor breakthroughs with a UV detector placed after the SPE unit. Valve switching effects during the trapping procedure often resulted in baseline shifts in the SPE-UV chromatogram, causing the operator to be unsure of whether a breakthrough had occurred or not (**Figure 5**). Therefore, to ensure an unambiguous breakthrough monitoring, an MS detector was placed after the SPE unit (**Paper II)**.

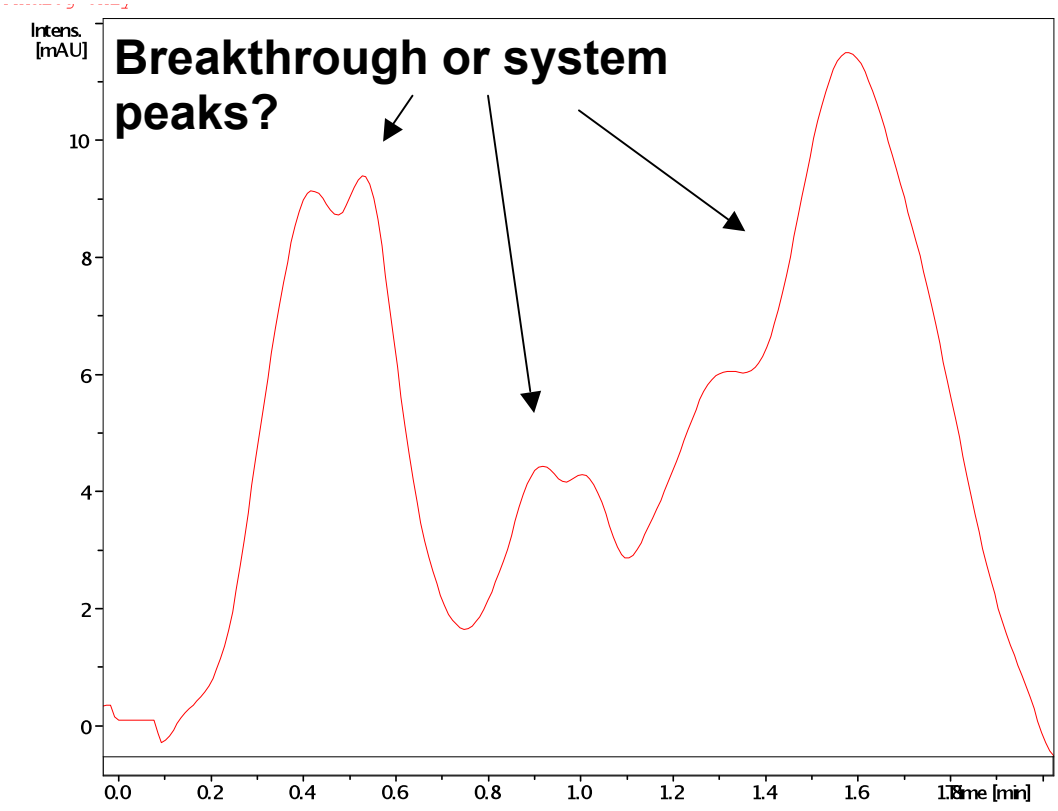


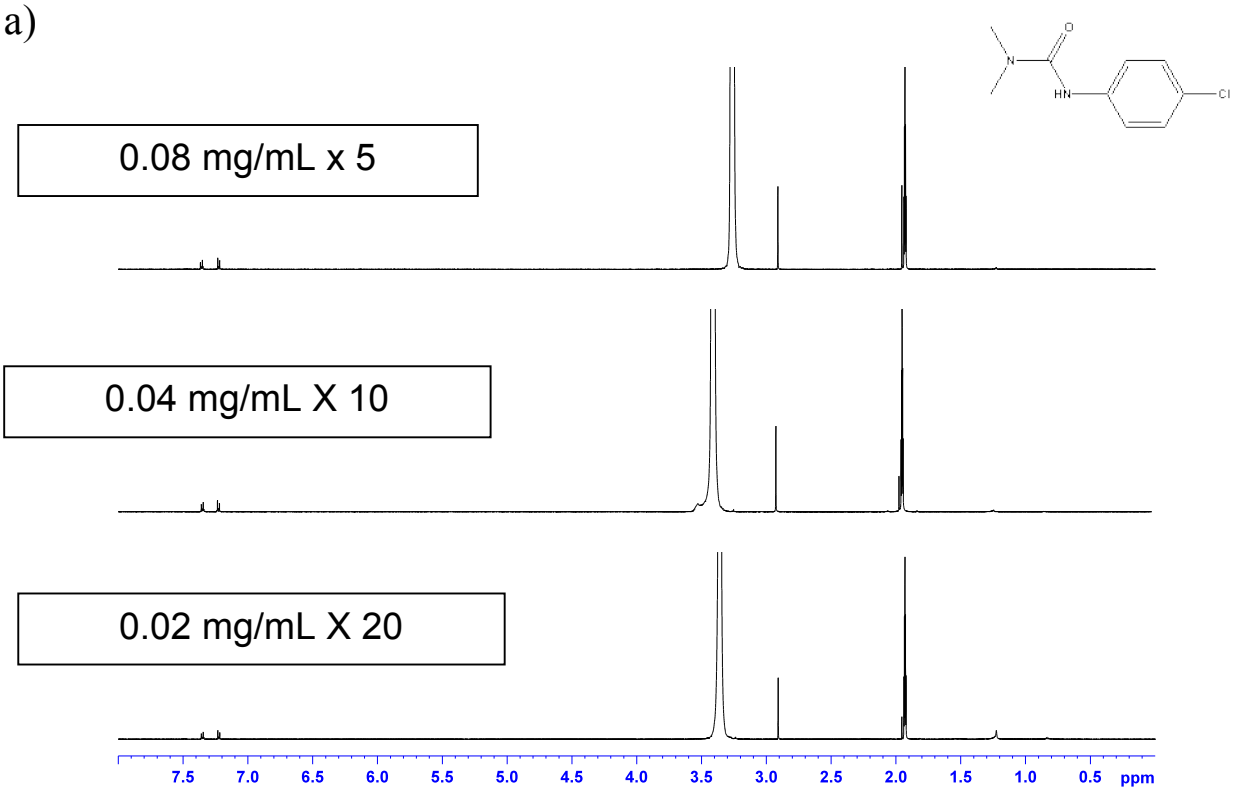
Figure 5: Monitoring of an eventual breakthrough during the trapping stage of an LC-SPE experiment. When employing a UV detector for this purpose, it can be difficult to interpret whether a peak is a system peak caused by e.g. pressure differences, or a breakthrough of the target analyte.

Due to the improvement in robustness of the system, because of virtually no pressure problems and unambiguous breakthrough monitoring, it was noticed that many compounds would not be (multi) trapped with C_{18} columns, even with ~0 % organic modifier in the mobile phase. Due to the lack of success of using C_{18} SPE columns for LC-SPE multi-trapping, alternative materials were explored. Some SPEs with hydrophilic interaction phases (HILIC) were examined, but were found to have insufficient capacity. Ion exchange SPEs were not considered since eluting IEC mobile phases must contain salts that are often not compatible with NMR instruments.

Instead, SPEs packed with porous carbon (Hypercarb) were examined. Hypercarb consists of fully porous spherical carbon particles comprised of flat sheets of hexagonally arranged carbon atoms. This material has been reported to have significantly stronger hydrophobic interactions than C_{18} materials [92].

SPE cartridges (1 mm I.D, 5 mm length) packed with Hypercarb material were found to be satisfactory for multiple trappings of compounds unable to be multi-trapped with C_{18} SPEs: When trapping monuron standard solutions of 80, 40 and 20 μg/mL, 5, 10 and 20 times respectively (total mass of 40 μg), the UV detector peak areas were very similar (RSD $\leq$ 6 %) when the compound was eluted from the SPE. Also, the NMR signals were also nearly identical in height (**Figure 6**) (**Paper II**).

a)


0.08 mg/mL x 5
0.04 mg/mL X 10
0.02 mg/mL X 20
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

b)

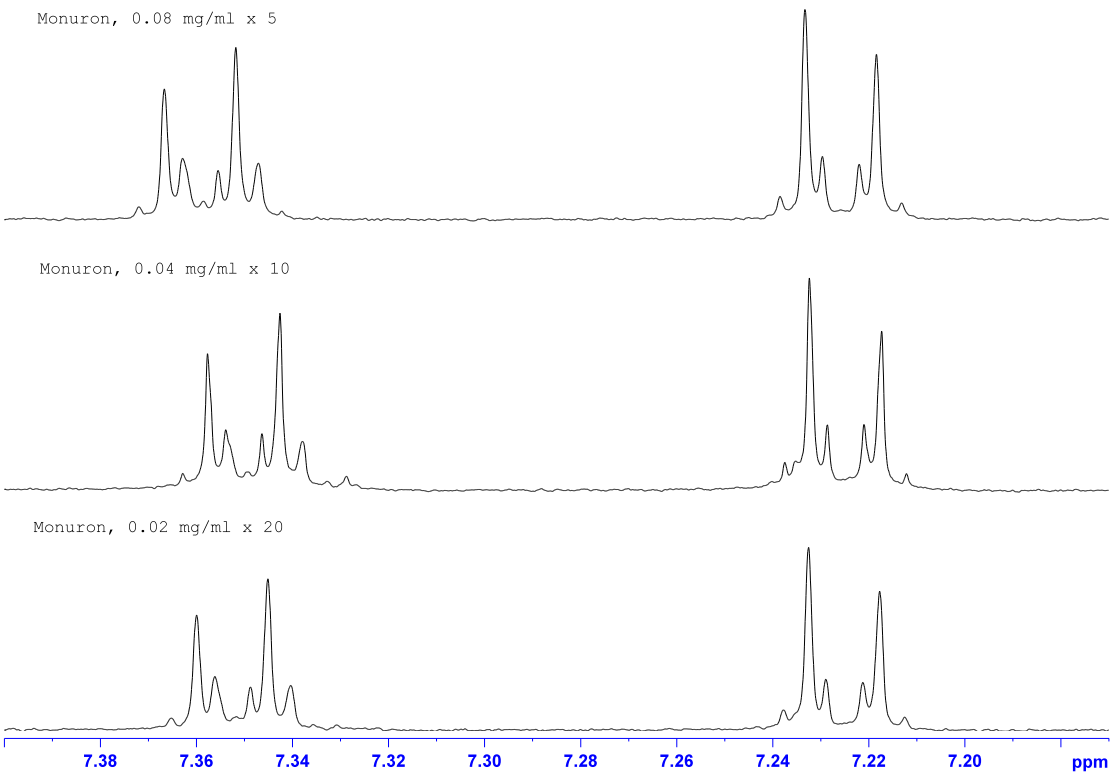

Monuron, 0.08 mg/ml x 5
Monuron, 0.04 mg/ml x 10
Monuron, 0.02 mg/ml x 20
7.38 7.36 7.34 7.32 7.30 7.28 7.26 7.24 7.22 7.20 ppm

Figure 6 (previous page): a) NMR spectra of monuron (structure shown in top right corner, 0.08, 0.04, 0.02 mg/mL) trapped 5, 10 and 20 times respectively. b) Close-up of the aromatic signals of monuron.

Breakthrough could occur on Hypercarb columns with single injections containing 10 μg or more of monuron, probably due to plug-elution. When injected amounts were less than 10 μg, multiple trapping could be used to trap 50 μg monuron before a breakthrough occured (**Paper II**).

SPE system

To summarize the last section, SPE trapping was found to be most successful when using a HPLC column with a reduced I.D. and SPEs with an alternative stationary phase (i.e. Hypercarb material) providing higher retention. However, the selection of SPEs packed with alternative stationary phases was limited with the commercial system employed; the SPE trapping unit employed SPE with non-standard fittings, and the price of custom packing SPEs with alternative phases was unacceptably high. In addition, the SPE unit was difficult to handle due to complicated and unstable software, and the hardware was prone to malfunctions that were impossible to repair without sending the unit abroad.

Due to these difficulties, an alternative SPE unit was employed (**Paper III**) (**Figure 7**) which was based on employing a high pressure column selector, which could direct HPLC-separated compounds to one of nine possible SPEs. The column selector is essentially the same unit as the switching system employed in **Paper I**, in that it is a flow directing-valve unit rather than a robot-arm unit. Since this unit had such a simple design, it was vastly easier to operate and troubleshoot, and required only a fraction of the maintenance needed for the commercial SPE "robot" unit. The columns selector unit can be also be controlled by a wide variety of instrument control systems. Using the 10-column selector is also a more flexible unit to operate, since any SPE column/cartridge with standard steel fittings can be employed.

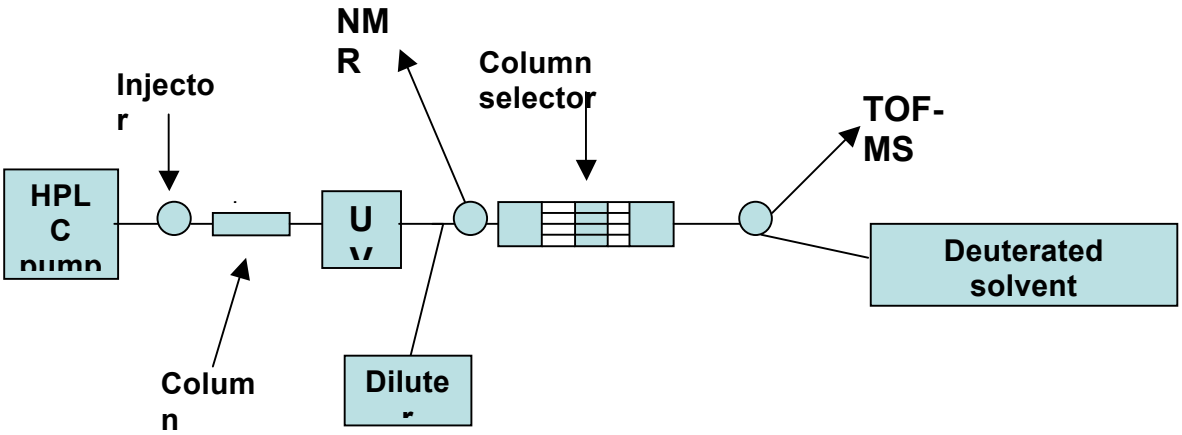


Figure 7: Scheme of the in-house designed LC-SPE-NMR system. Compounds are separated by HPLC, and when monitored by UV detection (and diluted with an additional pump), the target compound-containing eluent(s) are led to a distinct SPE(s) on the column selector. SPE trapping is monitored by MS. After trapping, an analyte-containing SPE is flushed with D_2O, and back flushed to the NMR probe with deuterated ACN.

Column selector performance

The column selector was evaluated regarding response time and dead volume, and the performance was satisfactory. Since it is essential that all compounds trapped are subject to identical conditions, the repeatability and reproducibility of the Hypercarb SPE columns (retention time, peak half width, pressure) were examined and was also found to be satisfactory (**Paper III**).

So far this chapter has described approaches that improve the chance of executing a successful trapping of separated compounds on individual SPE columns (e.g. using a separation column with a reduced diameter, SPE columns with alternative stationary phases, breakthrough monitoring with MS and employing an alternative SPE trapping unit). However, when the compound has been trapped, it must be transported the NMR. Two main obstacles are associated with this process: removing remains of NMR-noisy undeuterated solvents on the SPE column, and accurately transferring the compound to the NMR probe.

SPE-NMR transfer

In **Paper I**, the SPE-LC transfer of SPE-trapped analytes did not need to be optimalized, since the compounds would elute off of the SPE with the HPLC gradient solvent, and subsequently

be refocused on the LC column, resulting in a perfect compound transfer to the LC column. However, no such convenient refocusing effects are present regarding the SPE-NMR transfer: the band eluted from the SPE must be pumped through a tube volume of ~15-30 µL and be placed in the active volume of the NMR probe (2-40 µL, depending on the probe design). With the commercial "robot" SPE unit, this is executed by sending a fixed volume of deuterated ACN through the SPE cartridge, which is intended to both dissolve the compound from the SPE and transfer it in the active volume of the probe. However, there are several problems with this approach. Although ACN is a strongly eluting solvent for many/most compounds, there are exceptions, especially when employing a strong hydrophobic stationary phase such as Hypercarb. Also, the pumps assigned for this task were syringe-based dispenser pumps, which were found to be unreliable regarding flow rate repeatability.
Instead, an alternative procedure was developed (**Paper II**). The dispenser pumps were replaced with a "cap LC quality" gradient pump, which continuously sent flow through the probe. When an analyte was to be transferred from an SPE to the probe, the analyte was back-flushed off a column selector-attached SPE and through a UV detector placed between the SPE and the NMR probe. The employment of the post-SPE UV detector allowed for monitoring the compounds' departure off the SPE. In addition, since the exact volume between the post-SPE UV detector and the probe was known, and the flow rate could be accurately set and monitored, the placement of the band in to the probe was highly repeatable (the relative standard deviation of the NMR signal-to-noise ratio was <5 %.)

Evaluation of the necessity of N_2 flushing of SPE columns

In commercial LC-SPE-NMR instruments, it is common to flush the SPE columns with nitrogen gas to remove traces of undeuterated solvents. However, such a step requires additional valves and tubing, making the instrument even more complicated, and thus less attractive. Also, a N_2 flushing step takes ~20-30 minutes per cartridge, prolonging the LC-NMR procedure. In order to reduce the time of analysis and (perhaps more importantly) simplify the instrumentation, the necessity of this step was examined when employing Hypercarb packed SPE columns (**Paper III**).
25 µg amounts of monuron (**Figure 6, upper right corner**) were trapped on Hypercarb SPE columns, which were/were not dried with nitrogen for 30 minutes, washed with D_2O for 10

minutes, and eluted with deuterated ACN. The non-deuterated solvent peaks in the NMR spectrum of monuron eluted from not-dried columns were acceptably low. Drying the cartridges for 30 minutes did not significantly decrease the non-deuterated ACN signal compared to the always-present deuterated ACN signal (**Figure 8**). Thus, an SPE column-drying step was considered unnecessary.

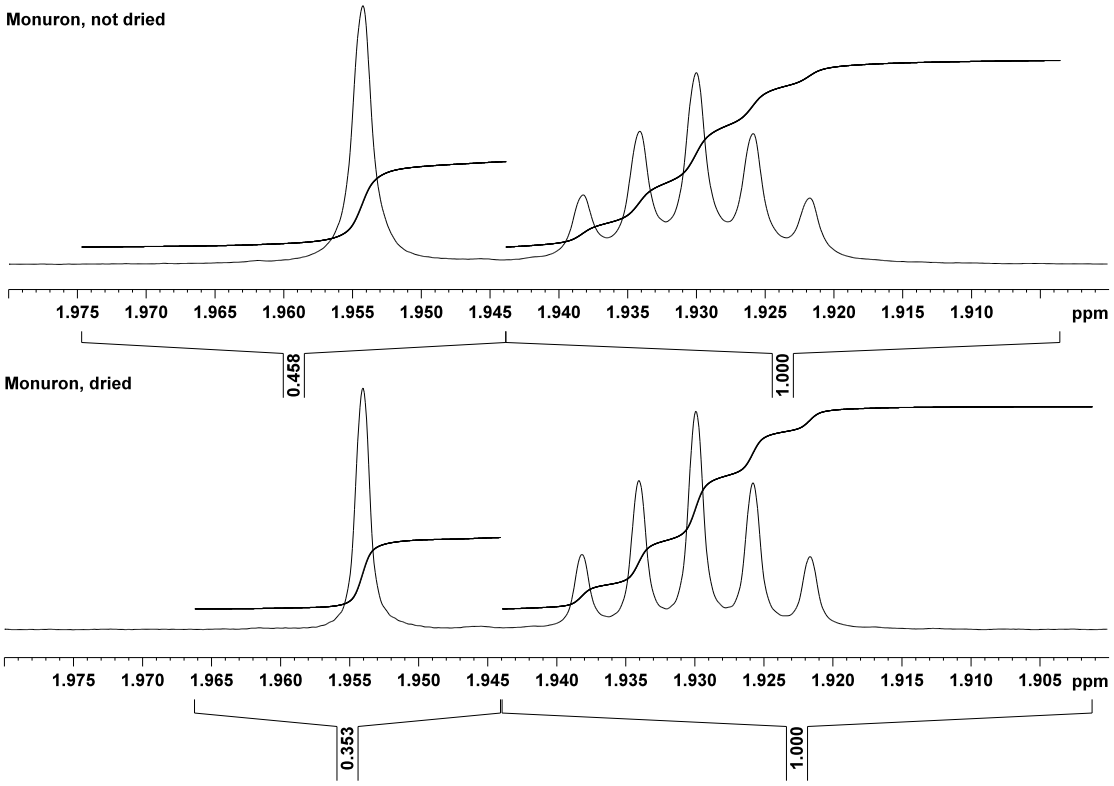


Figure 8: Close-up of the ACN and ACN-d_3 NMR signals eluted from a not-dried SPE cartridge (top) and a dried cartridge (bottom).

Optimizing elution conditions and compatibility with NMR probes

Since the active volume of many sensitive NMR probes or tubes are fixed and small (1.5 µL, 30 µL and ~40 µL for micro-flow probes, conventional HPLC-NMR flow probes, and 1 mm

NMR tubes, respectively [65, 70, 71, 93] it is desirable to transfer the trapped compound from the SPE unit to the probe in a controlled and small volume. This is also a main reason for not employing SPEs with larger I.D. than 1 mm; the chromatographic band would be too dilute before reaching the probe.

Using monuron as a test substance (1-10 µg), the efficiency (N) of 1.0 mm Hypercarb SPE columns was assessed as function of flow rate. The minimum peak elution volume (~35 µL) was achieved with the lowest repeatable flow rate (20 µL/minute). Hence, the elution volumes of 1 mm I.D SPE cartridges match well with conventional HPLC-NMR flow probes and 1 mm I.D. NMR tubes, since the amount of analyte necessary for ^{1}H NMR (5-20 µg when <1000 Dalton) elute in volumes of ~40 µL.

However, this elution volume does not match with the more sensitive micro-flow probe (1.5 µL), and the sensitivity of the probe (approximately a 4 time increase in S/N) does not compensate for the fact that only a small portion of the chromatographic band is placed in the active volume. In addition, attempts to downscale the SPE I.D. resulted in poor capacity and breakthroughs. Thus, LC-SPE-microcoil NMR was therefore not further considered in this thesis.

Curiously, at approximately the same time as these results were published, several other papers reported coupling LC-SPE systems with microcoil NMR [94, 95].

In paper [94], the authors used an on-line combination of SPE I.D.s (<1 mm) and a microprobe and thus a logical SPE-probe volume match was not achieved. In paper [95], the SPE-NMR transfer was performed manually, and the authors argue that with this approach 1 mm I.D. SPE cartridges are compatible with micro coil NMR, which is the opposite conclusion of what is made in this thesis.

Unknown compounds: Metal complexing agents in *Blepheris Aspera*

Blepharis aspera is a plant that grows in Botswana, Africa and can survive in soils with metal concentration which are usually toxic for plants. The plant is therefore considered to be a potential mineral indicator. The reasons for why *B. Aspera* can survive are not understood, but it is likely that the presence of complexing compounds or antioxidants likely reduces the deteriorating processes associated with high metal concentrations [96]. In **Paper IV**, the goal

was to identify compounds in *B. Aspera* that could contribute to survival mechanisms of the plant in soils with high mineral concentrations.

Defining target compounds

As seen in the chromatogram of a methanol extract of *B. Aspera,* (**Figure 9**), the plant contains a large number of compounds of various concentrations. A comprehensive NMR analysis would literally take years to perform. Thus, the selection was narrowed down to *main compounds* that could *complex with metals* that are present at high concentrations in the soil which the plants grew, e.g. nickel and copper [97]. Therefore, compounds 1-5 were manually collected, spiked with metals Cu, Fe and Ni and subjected to UV-Vis spectroscopic analysis. Compounds 1-3 in Figure 9 showed significant UV-vis spectrum differences when added metals, and were thus focused upon.

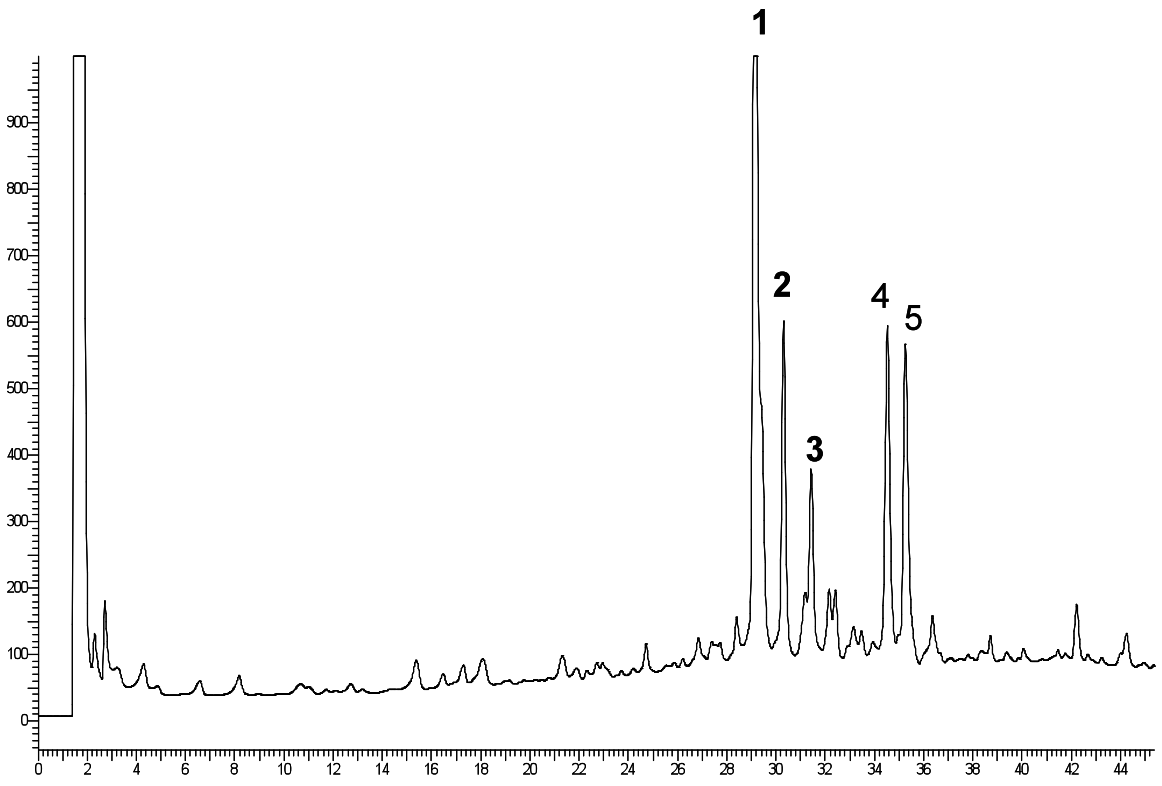


Figure 9: Chromatogram of a 5-20 % (0-35 min) ACN + 0.1 % f.a. gradient of 350 µL methanol extract of *B. aspera* **(Paper IV).**

Target compound determination

Compounds 1-3 were subject to MS^n analysis by ion trap mass spectrometry. The MS data revealed that compounds 1 and 2 have identical MS^n spectra, and compound 3's spectrum were virtually identical to that of compound 1 and 2, with the exception of the presence of a 647→ 479 fragment, and the absence of a 647 →471 fragment associated with compounds 1 and 2. However, although this was proof that the compounds were greatly related, MS^n information was insufficient for structural elucidation, and NMR spectroscopy was required. As mentioned in the introduction, structural analysis by NMR is usually preceded by off-line extractions and isolation steps. When HPLC is included in this process, compounds of interest are usually manually collected and undeuterated solvents must be removed by e.g. heat and/or nitrogen drying, and dissolved in a deuterated solvent prior to NMR analysis.

In contrast, on-line LC-SPE-NMR is a semi-automated approach that does not require the steps mentioned above. As discussed previously, LC-SPE-NMR can also be a robust and effective method, as long as the issues described in the chapter above are resolved, namely having full control of the SPE trapping step and the SPE-NMR transfer step. Therefore, on-line LC-SPE-NMR was employed for the structural analysis of compounds 1-3. The instrumentation employed was based on the developments made in **Paper II** and **Paper III**, with the exception of replacing two valves on each side of the column selector with one valve, making the instrumentation further resemble the simple instrumentation used in **Paper I (Figure 10)**.

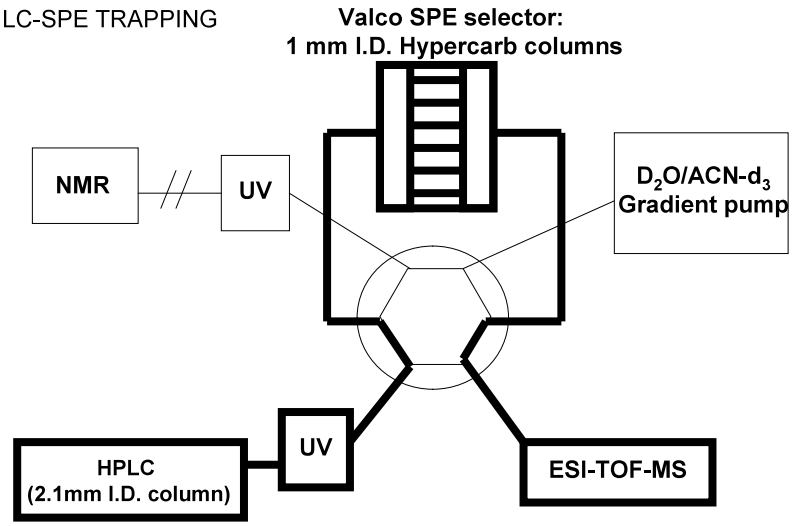


Figure 10: Scheme of the on-line LC-SPE-NMR instrumentation. Boxes and connections in bold illustrate the pathway of a compound during SPE trapping. A TOF-MS was connected to the outlet of the SPE selector to monitor an eventual compound breakthrough (**Paper VI**).

Compound 1 was identified on basis of the proton NMR spectrum (**Figure 11**) to be the known phenylpropanoid **verbascoside**. Compound 2 was identified as being a metal-verbascoside complex, and compound 3 was identified as being **isoverbascoside (Figure 12)**. This study was the first to identify these compounds in the genus *Blepharis*.
The verbascosides were quantified and found to be present at ~1 % of the dry mass of the plant. Verbascosides have earlier been associated with e.g. antioxidant and antimutagenic activity [98, 99]. The compounds have also been reported previously as being metal complexing, and found to inhibit lipid peroxidation by chelation of iron [100]. Thus, due to the relatively high levels present and the compounds' chemical traits, it is reasonable to hypothesize that verbascosides aid the survival of *Blepheris Aspera* in mineral-rich soils.

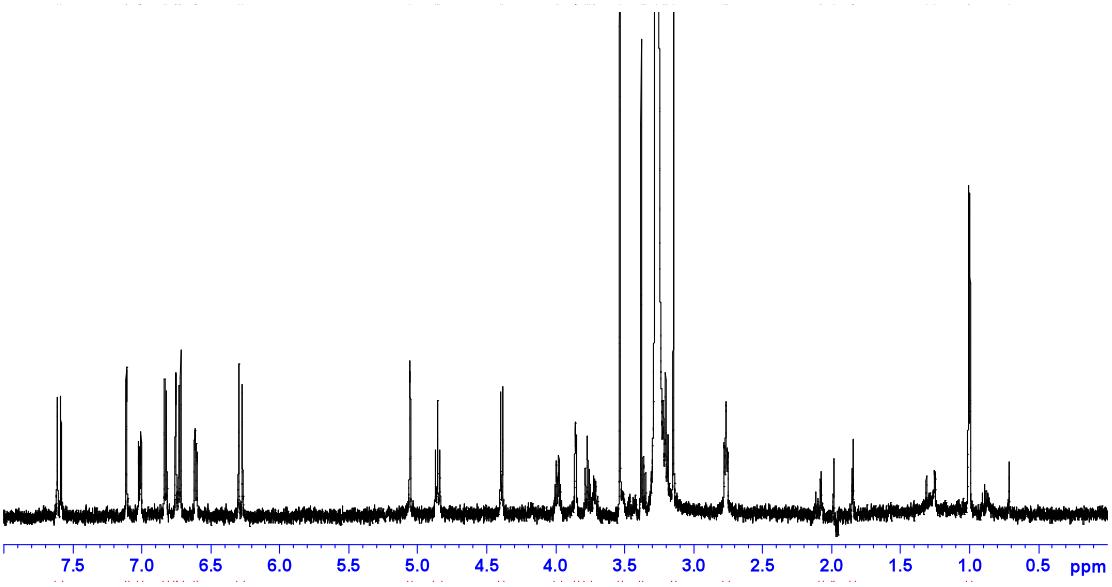


Figure 11: Proton NMR spectrum of verbascocide (**Paper IV**).

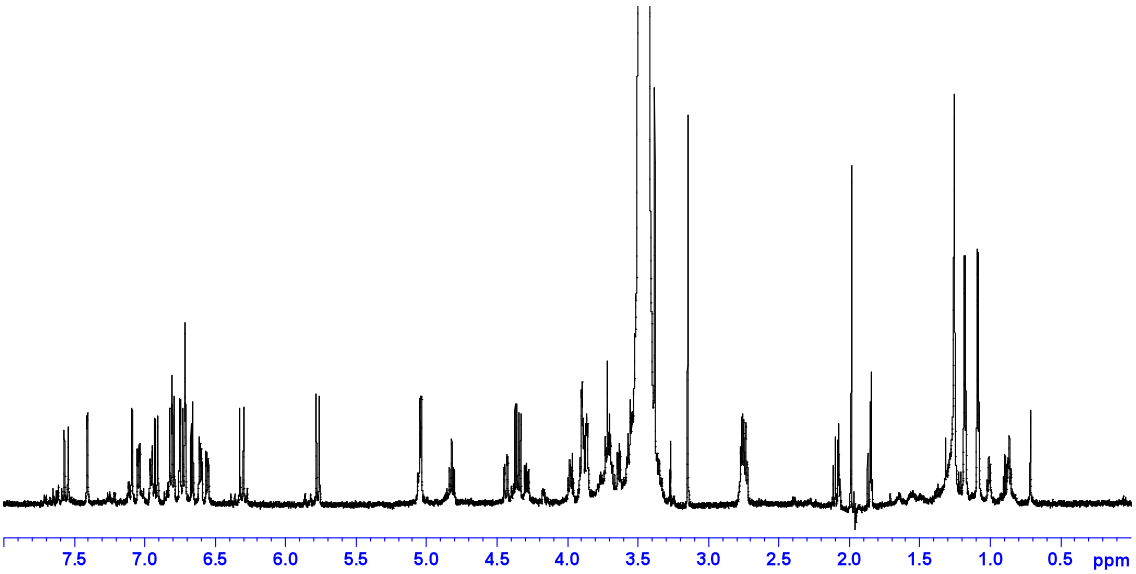


Figure 12: Proton NMR spectrum of isoverbascoside (**Paper IV**).

LC-SPE-NMR: Some conclusions

LC-SPE-NMR can be frustrating to use, but if the basic questions of SPE trapping are answered (e.g. *can the analyte(s) be focused on the SPE column? How large of a sample volume can be injected? How fast can this volume be introduced to the SPE column*?), an on-line LC-NMR method intended for identifying an unknown compound can be developed with a reasonable ease, with all the benefits of on-line coupling. However, commercial instrumentation is often so complicated that it hinders the user from addressing these issues in a thorough and realistic manner. Therefore, it can be highly beneficial to redesign their instrumentation as described in this thesis.

ON-LINE 2D LC

In the previous chapters, an on-line SPE-LC column switching system was employed for enabling whole microdialysate samples on to a narrow cap LC column. Also, an on-line LC-SPE was further developed to enable the separation of relatively large amounts of compound for NMR analysis.

In this chapter, the approaches of SPE-cap LC and LC-SPE are combined for injecting large samples on to a cap LC system, and separating large *numbers* of compounds with 2D LC.

Choice of 2D LC dimensions

As mentioned in the Introduction, a key to successful 2D LC is to use two separation modes which are orthogonal. With 2D LC peptide separations, it is common that the 1st dimension is an SCX column, while the 2nd dimension is an RP column. In preliminary studies, this combination for peptide separation was considered first. However, several problems aroused: difficulty in achieving repeatable retention times/peak shapes with the SCX column (and difficulty in acquiring remedies from manufacturers and literature), difficulty in monitoring 1st dimension chromatography of the SCX separation (i.e. single ion monitoring of compounds in a complex mixture) and limitations regarding the injection of salty samples. In addition, a study [19] showed that SCX and RP chromatography were not as orthogonal as previously

assumed. Thus, alternative 1st dimension separation modes were considered, i.e. HILIC chromatography [101]. The zwitterionic (ZIC) HILIC stationary phase is attached to porous silica particle, as shown in **Figure 13**.

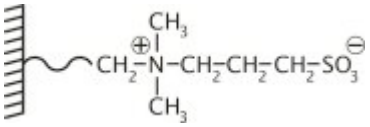


Figure 13: Structure of HILIC stationary phase [102].

Although the exact separation mechanism is not entirely clear, it is generally accepted that an aqueous layer is formed around the hydrophilic functional groups of the HILIC material. Separation of peptides can be explained by a partitioning mechanism between the aqueous layer and the hydrophobic buffer [103], by hydrogen bonding with the HILIC material [104], or a mechanism somewhere in between, with both partitioning and hydrogen bonding [105]. A self-packed HILIC column was tested with mobile phases ACN and 10 mM ammonium acetate in accordance with [19].
The column was tested regarding repeatability, and was found to be satisfactory, regarding peak shape and retention times.
HILIC-MS and RP-MS separations of tryptic digest peptides of protein Apo-I was performed, and a plot of 23 randomly chosen peptides revealed a random pattern (**Figure 14**), supporting the evidence of good orthogonality between the two separation modes [19].

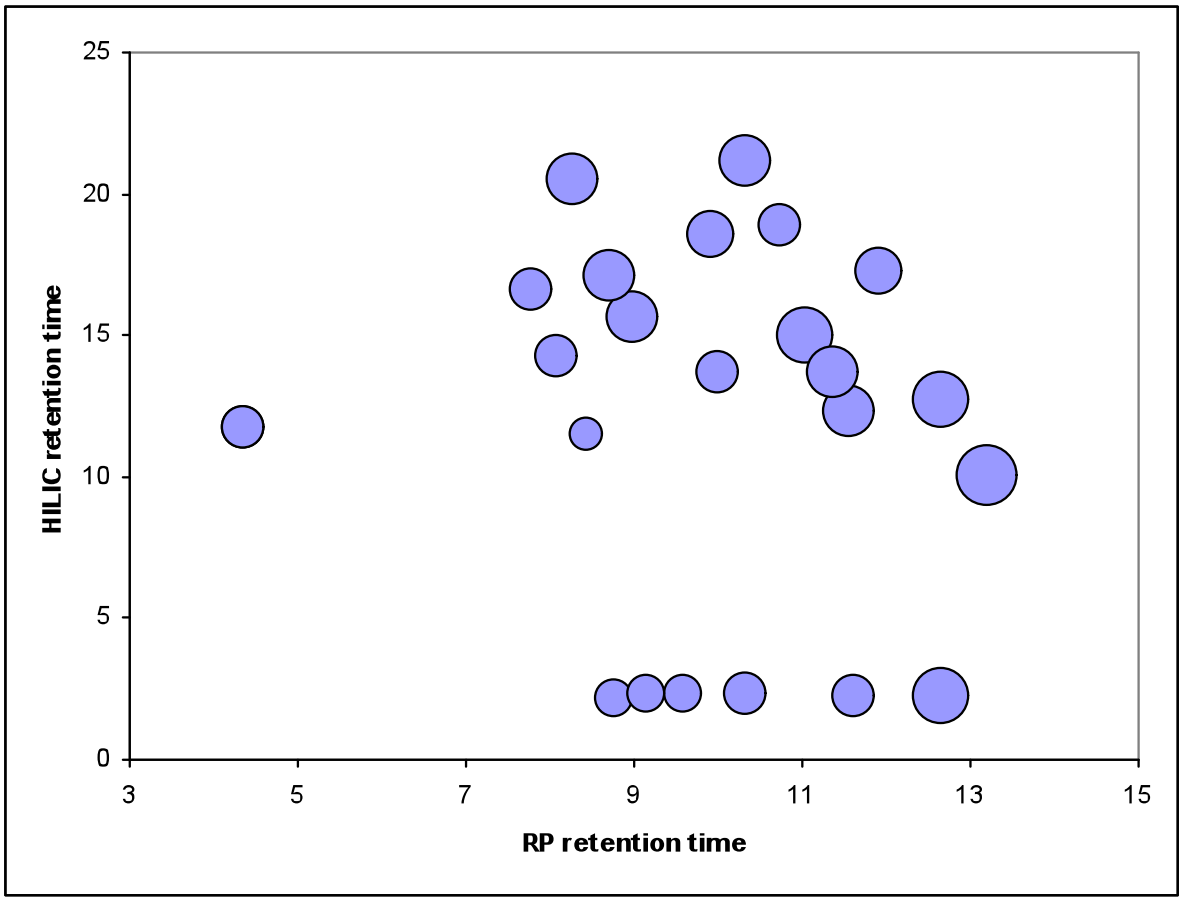


Figure 14: Plot of HILIC and RP retention times of tryptic digest peptides of Apo A-I. Samples were injected on to separate 1D RP-MS and 1D HILIC-MS systems. The bubble size corresponds to molecular weight (343-1677 Da) (**Paper V**).

Optimal HILIC conditions are obtained when dissolving a sample in a weak HILIC solvent, e.g. ACN. However, this approach may not be practical with some samples (due to e.g. precipitation in biological samples). However, placing a switching system in front of the HILIC column (as used in **Paper I**), allows for injecting large, aqueous samples on to the HILIC system without deterioration of chromatographic performance.

Eluent compatibility

As mentioned in the Introduction, chromatographic problems can occur if the mobile phases from the two 2D separation modes are not compatible. Therefore, instrumental schemes employing SPE trapping columns have been designed [52]. However, the success of such

systems depends on having a fast 2nd dimension separation, which may not always be practical. Instead, the SPE unit concept employed in **Paper III** was employed (**Figure 15)**. This system allowed for 18 1st dimension fractions to be trapped on individual SPE columns, and separated from each other in space and time, allowing for lengthened 2nd dimension separations. To the author's knowledge, this system is the first on-line HILIC-RP coupling. A detailed description of the operation of the system is found in the *Experimental* chapter in **Paper V**.

Initially, it was planned to employ a larger 1st dimension column than the 2nd dimension columns (to be able to inject larger samples), as is common in many 2D LC systems. However, the use of a wide column could cause problems with the SPE trapping step, as described above and in **Paper II**. Therefore, a cap LC column was also used for the 1st dimension. Besides, since a switching system was placed in front of the 1st dimension, there were really no practical limitations regarding injection volumes. Also, employing a cap LC column allowed for a more sensitive 1st dimension UV-monitoring.

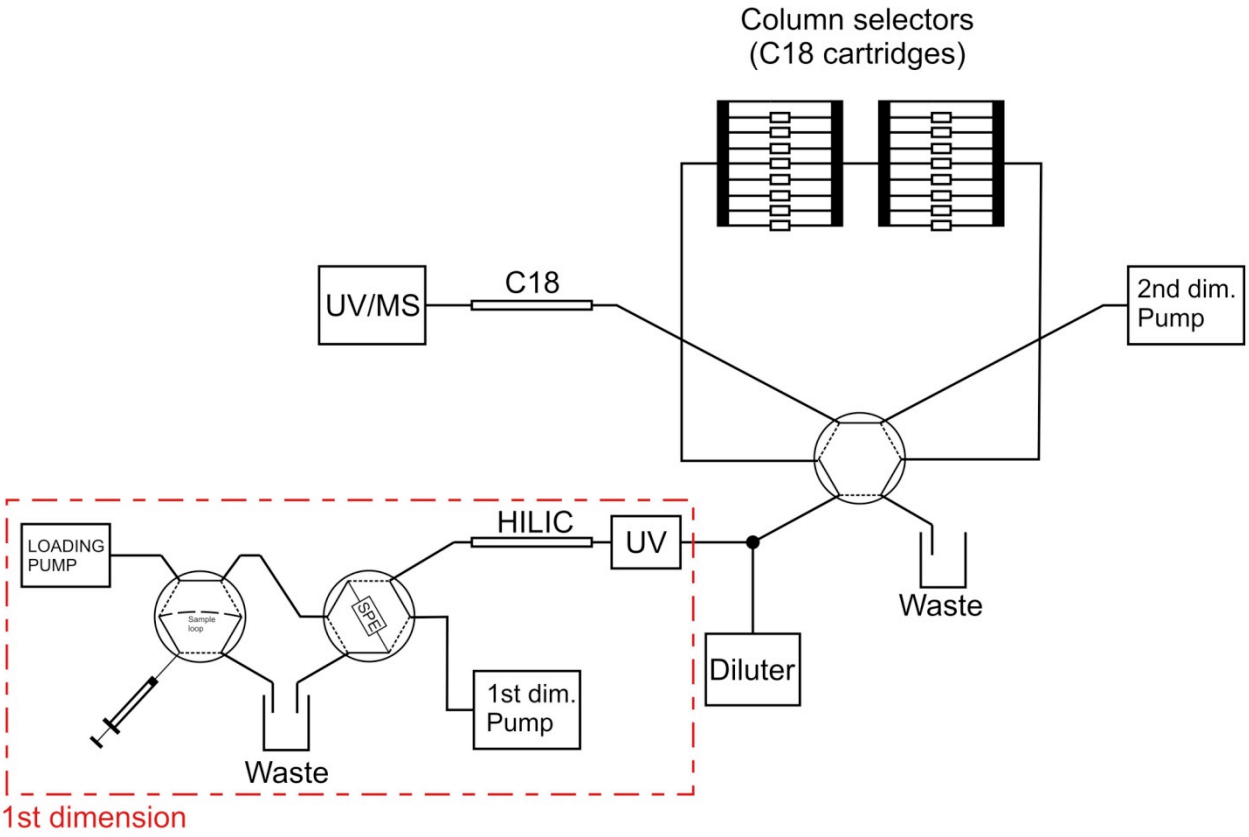


Figure 15: Scheme of the SPE-HILIC-SPE-RP system. Samples are loaded on to the (RP) SPE attached to valve 2, and back-flushed on to a HILIC column for 1st dimension separation and UV detection. HILIC fractions are diluted and trapped on separate (RP) SPE trapping columns attached to the column selectors. Trapped HILIC fractions are subsequently back-flushed on to a RP column for 2nd dimension separation and UV/MS detection (**Paper V**).

LC × LC of tryptic digest of three proteins

The high resolving 2D LC technique can give a unique insight of the entire sample when all the 2nd dimension chromatograms are presented in a "bird's eye view" plot, as in **Figure 16**, which shows a separation of tryptic digest peptides of a mixture of albumin, hemoglobin and transferrin executed with the on-line SPE-HILIC-SPE-RP system.

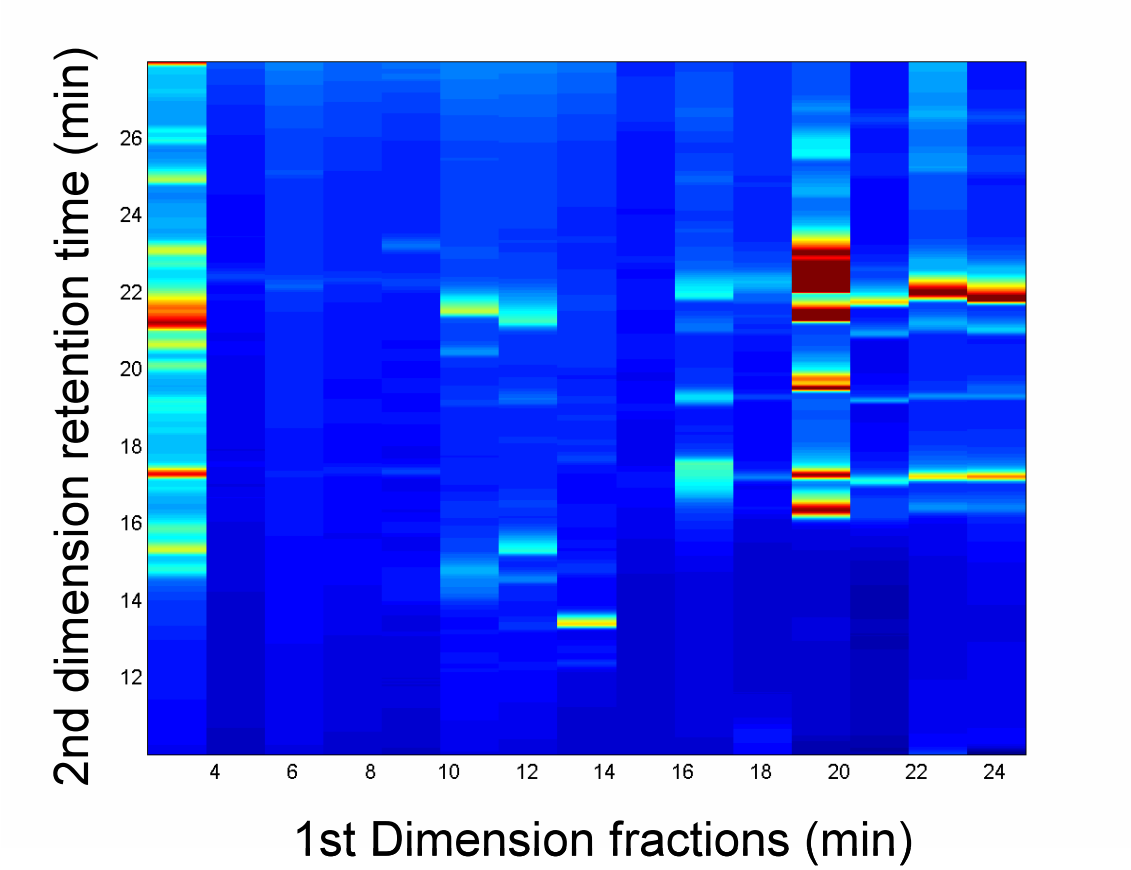


Figure 16: Plot of an on-line SPE-HILIC-SPE-RP-UV separation of a tryptic-digest mixture of albumin, hemoglobin and transferring (**Paper V**).

Application: bradykinin monitoring in muscle dialysate

A set of microdialysate samples of rat muscle tissues, where the rats were subject to various conditions that were expected to vary the levels of kinins, were to be investigated. Since the goal of this project was to monitor differences in kinin levels, rather than verifying the presence of these (as done in **Paper I**), it was considered necessary to strengthen the quantitative strength of the method. Therefore, the 2D LC system described above was employed for heart-cut 2D LC analysis of these samples (**Figure 17**). 1st dimension fractions containing the target compounds were identified by MS, and only these target fractions were subsequently analyzed. Analysis of the MS spectra of the target compound peaks showed

virtually no co-eluting peaks, thus the chance of quantification-distortion by suppression was considered to be minimal. In addition, an "in-line" standard (bradykinin) was post-column injected to correct eventual drift in the MS. This approach gave a satisfactory precision of ~4 %.

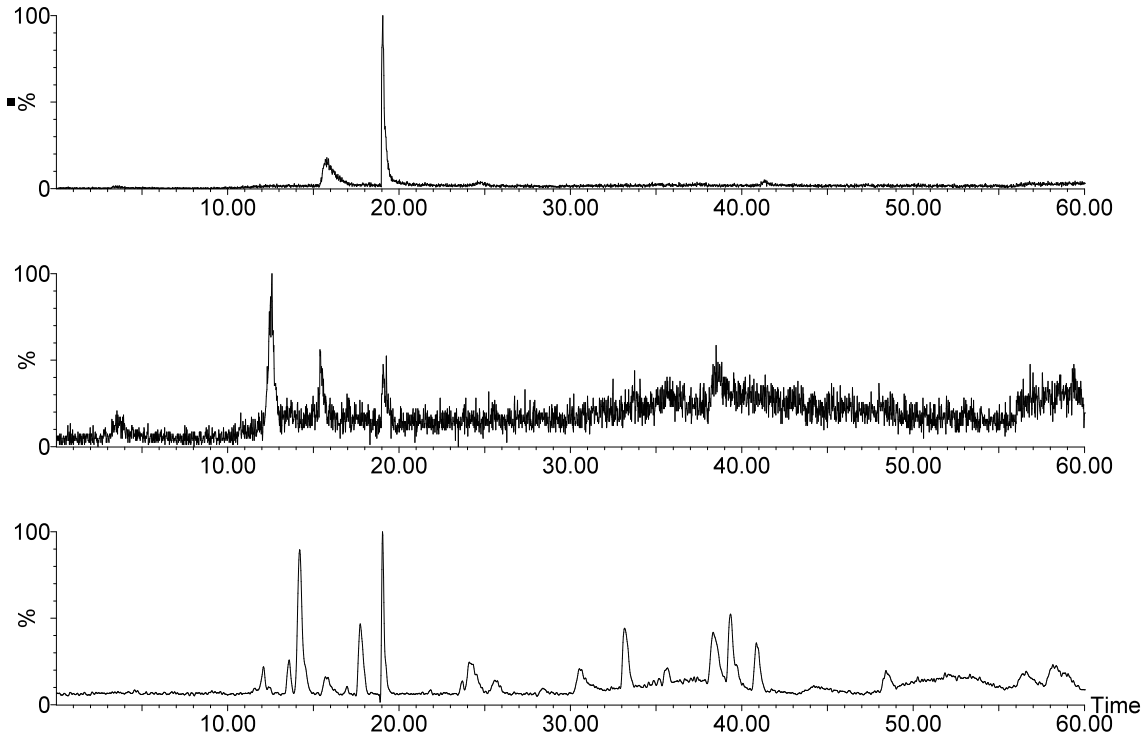


Figure 17: EIC of bradykinin in sample + post column injected bradykinin internal standard; EIC of arg-bradykinin in sample; BPI of RP separation of the combined fractions CS2-7 and CS2-8 (**Paper V**).

CONCLUSIONS

In this thesis, the usefulness of employing easily automatable and sample saving on-line SPE-LC techniques for the analysis of limited, complex and/or unknown mixtures has been discussed and demonstrated. SPE-capLC is well suited for analyzing limited samples where the target analytes are present at low levels. However, it is important to develop a well functioning SPE trapping methodology to ensure that a compound breakthrough will not occur. Specific issues that must be investigated are SPE material, injection size and loading flow rate.

On-line LC-SPE-NMR is an exiting, multidimensional technique with a lot of potential, but the technique is weakened by often being prone to poor SPE trapping. However, this can be remedied if the issues described above are addressed. In this context, it can also be wise to critically evaluate the diameter of the HPLC column. For instance, in the work presented here, the conclusion was ironically that employing an HPLC column with a reduced diameter will increase the capacity of the system. In this thesis, ways have been described to simplify LC-SPE-NMR by using alternative instrumentation and removing unnecessary SPE drying steps. However, it is of the author's opinion that LC-NMR will not be a major technique before NMR probes are made more sensitive.

2D LC is a technique that is becoming more and more popular, and variations of the technique are being published on a monthly basis. In this thesis an on-line 2D LC system has been described, and was based on the instrumentation and knowledge acquired in the work done with SPE-cap LC and LC-SPE-NMR. The system is robust, the dimensions are orthogonal and the system is easily handled. Also, it is (to the authors' knowledge) the first online coupling of HILIC and RP chromatography.

REFERENCES

1. Huber, J.F.K., *High-efficiency, high-speed liquid chromatography in columns.* Journal of Chromatographic Science, 1969. **7**(2): p. 85-90.
2. Horvath, C.G. and S.R. Lipsky, *Peak capacity in chromatography.* Analytical Chemistry, 1967. **39**(14): p. 1893.
3. Giddings, J.C., R.A. Keller, and Editors, *Advances in Chromatography, Vol. 2*. 1966. 377 pp.
4. Giddings, J.C., *Dynamics of Chromatography, Part I: Principles and Theory (Chromatographic Science Series, Vol. 1)*. 1965. 336 pp.
5. Saito, Y., K. Jinno, and T. Greibrokk, *Capillary columns in liquid chromatography: Between conventional columns and microchips.* Journal of Separation Science, 2004. **27**(17-18): p. 1379-1390.
6. Horvath, C.G. and S.R. Lipsky, *Rapid analysis of ribonucleosides and bases at the picomole level using pellicular cation exchange resin in narrow bore columns.* Analytical Chemistry, 1969. **41**(10): p. 1227-34.
7. Vissers, J.P.C., *Recent developments in microcolumn liquid chromatography.* Journal of Chromatography, A, 1999. **856**(1 + 2): p. 117-143.
8. Ishii, D. and Editor, *Introduction to Microscale High-Performance Liquid Chromatography*. 1988. 208 pp.
9. Sojo, L.E., G. Lum, and P. Chee, *Internal standard signal suppression by co-eluting analyte in isotope dilution LC-ESI-MS.* Analyst (Cambridge, United Kingdom), 2003. **128**(1): p. 51-54.
10. Jessome, L.L. and D.A. Volmer, *Ion suppression: a major concern in mass spectrometry.* LCGC North America, 2006(Suppl.): p. 83-89.
11. Holm, A., S.R. Wilson, P. Molander, E. Lundanes, and T. Greibrokk, *Determination of perfluorooctane sulfonate and perfluorooctanoic acid in human plasma by large volume injection capillary column switching liquid chromatography coupled to electrospray ionization mass spectrometry.* Journal of Separation Science, 2004. **27**(13): p. 1071-1079.

12. W. Yu, Y. Li, C. Deng, X. Zhang, Xiangmin. *Comprehensive two-dimensional separation in coupling of reversed-phase chromatography with capillary isoelectric focusing followed by MALDI-MS identification using on-target digestion for intact protein analysis.* Electrophoresis, 2006. **27**(11): p. 2100-2110.
13. Shellie, R.A. and P.R. Haddad, *Comprehensive two-dimensional liquid chromatography.* Analytical and Bioanalytical Chemistry, 2006. **386**(3): p. 405-415.
14. Erni, F. and R.W. Frei, *Two-dimensional column liquid chromatographic technique for resolution of complex mixtures.* Journal of Chromatography, 1978. **149**: p. 561-9.
15. Bushey, M.M. and J.W. Jorgenson, *Automated instrumentation for comprehensive two-dimensional high-performance liquid chromatography/capillary zone electrophoresis.* Analytical Chemistry, 1990. **62**(10): p. 978-84.
16. Giddings, J.C.i., *Multidimensional high-performance liquid chromatography, Cortes (ed).* 1990. **50**: p. 1-27.
17. Schoenmakers, P., P. Marriott, and J. Beens, *Nomenclature and conventions in comprehensive multidimensional chromatography.* LC-GC Europe, 2003. **16**(6): p. 335-336, 338-339.
18. Giddings, J.C., *Concepts and comparisons in multidimensional separation.* HRC & CC, Journal of High Resolution Chromatography and Chromatography Communications, 1987. **10**(5): p. 319-23.
19. Gilar, M., P. Olivova, A.E. Daly, and J.C. Gebler, *Orthogonality of Separation in Two-Dimensional Liquid Chromatography.* Analytical Chemistry, 2005. **77**(19): p. 6426-6434.
20. David, F., G. Vanhoenacker, B. Tienpont, I. Francois, and P. Sandra, *Coupling columns and multidimensional configurations to increase peak capacity in liquid chromatography.* LC-GC Europe, 2007. **20**(3): p. 154-158, 160-162.
21. Dachtler, M., T. Glaser, K. Kohler, and K. Albert, *Combined HPLC-MS and HPLC-NMR on-line coupling for the separation and determination of lutein and zeaxanthin stereoisomers in spinach and in retina.* Analytical Chemistry, 2001. **73**(3): p. 667-674.
22. Wolfender, J.-L., K. Ndjoko, and K. Hostettmann, *Liquid chromatography with ultraviolet absorbance-mass spectrometric detection and with nuclear magnetic resonance spectrometry: a powerful combination for the on-line structural*

investigation of plant metabolites. Journal of Chromatography, A, 2003. **1000**(1-2): p. 437-455.

23. Shockcor, J.P., S.E. Unger, P. Savina, J.K. Nicholson, and J.C. Lindon, *Application of directly coupled LC-NMR-MS to the structural elucidation of metabolites of the HIV-1 reverse-transcriptase inhibitor BW935U83.* Journal of Chromatography, B: Biomedical Sciences and Applications, 2000. **748**(1): p. 269-279.
24. Schaller, F., J.-L. Wolfender, K. Hostettmann, and S. Mavi, *New antifungal \"quinone methide\" diterpenes from Bobgunnia madagascariensis and study of their interconversion by LC/NMR.* Helvetica Chimica Acta, 2001. **84**(1): p. 222-229.
25. Albert, K., G. Schlotterbeck, U. Braumann, H. Haendel, M. Spraul, and G. Krack, *Structure determination of vitamin A acetate isomers through coupled HPLC and 1H NMR spectroscopy.* Angewandte Chemie, International Edition in English, 1995. **34**(9): p. 1014-16.
26. Albert, K., M. Dachtler, T. Glaser, H. Haendel, T. Lacker, G. Schlotterbeck, S. Strohschein, L.-H. Tseng, and U. Braumann, *Online coupling of separation techniques to NMR.* Journal of High Resolution Chromatography, 1999. **22**(3): p. 135-143.
27. Wilson, I.D., E.D. Morgan, R. Lafont, J.P. Shockcor, J.C. Lindon, J.K. Nicholson, and B. Wright, *High-performance liquid chromatography coupled to nuclear magnetic resonance spectroscopy and mass spectrometry applied to plant products. Identification of ecdysteroids from Silene otites.* Chromatographia, 1999. **49**(7/8): p. 374-378.
28. Sandvoss, M., L.H. Pham, K. Levsen, A. Preiss, C. Mugge, and G. Wunsch, *Isolation and structural elucidation of steroid oligoglycosides from the starfish Asterias rubens by means of direct online LC-NMR-MS hyphenation and One- and Two-Dimensional NMR investigations.* European Journal of Organic Chemistry, 2000(7): p. 1253-1262.
29. Farrant, R.D., B.C. Cupid, J.K. Nicholson, and J.C. Lindon, *Investigation of the feasibility of directly-coupled HPLC-NMR with 2H detection with application to the metabolism of N-dimethylformamide-d7.* Journal of Pharmaceutical and Biomedical Analysis, 1997. **16**(1): p. 1-5.
30. Zang, X., R. Luo, N. Song, K. Chen Ta, and H. Bozigian, *A novel on-line solid-phase extraction approach integrated with a monolithic column and tandem mass*

spectrometry for direct plasma analysis of multiple drugs and metabolites. Rapid communications in mass spectrometry, 2005. **19**(22): p. 3259-68.

31. Rodriguez-Mozaz, S., J. Lopez de Alda Maria, and D. Barcelo, *Advantages and limitations of on-line solid phase extraction coupled to liquid chromatography-mass spectrometry technologies versus biosensors for monitoring of emerging contaminants in water.* Journal of chromatography. A, 2007. **1152**(1-2): p. 97-115.
32. Li, M., Y. Alnouti, R. Leverence, H. Bi, and I. Gusev Arkady, *Increase of the LC-MS/MS sensitivity and detection limits using on-line sample preparation with large volume plasma injection.* Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 2005. **825**(2): p. 152-60.
33. Huber, J.F.K., R. Van der Linden, E. Ecker, and M. Oreans, *Column switching in high-pressure liquid chromatography.* Journal of Chromatography, 1973. **83**: p. 267-77.
34. Dai, S., H. Song, G. Dou, X. Qian, Y. Zhang, Y. Cai, X. Liu, and Z. Tang, *Quantification of sifuvirtide in monkey plasma by an on-line solid-phase extraction procedure combined with liquid chromatography/electrospray ionization tandem mass spectrometry.* Rapid communications in mass spectrometry, 2005. **19**(10): p. 1273-82.
35. Choi, K.-J., S.-G. Kim, C.-w. Kim, and S.-H. Kim, *Determination of antibiotic compounds in water by on-line SPE-LC/MSD.* Chemosphere, 2007. **66**(6): p. 977-984.
36. Cantwell, F.F., *Pre-column reactions to eliminate interferents in the liquid chromatographic analysis of p-hydroxybenzoates in complex pharmaceuticals.* Analytical Chemistry, 1976. **48**(13): p. 1854-9.
37. Barrett, Y.C., B. Akinsanya, S.-Y. Chang, and O. Vesterqvist, *Automated on-line SPE LC-MS/MS method to quantitate 6beta-hydroxycortisol and cortisol in human urine: Use of the 6beta-hydroxycortisol to cortisol ratio as an indicator of CYP3A4 activity.* Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences, 2005. **821**(2): p. 159-165.
38. Little, J.N. and G.J. Fallick, *New considerations in detector-application relationships.* Journal of chromatography, 1975. **112**: p. 389-97.
39. Dolphin, R.J., F.W. Willmott, A.D. Mills, and L.P.J. Hoogeveen, *Column switching techniques in the liquid chromatographic analysis of organochlorine pesticides in milk.* Journal of Chromatography, 1976. **122**: p. 259-68.

40. Holm, A., P. Molander, E. Lundanes, S. Ovrebo, and T. Greibrokk, *Fast and sensitive determination of urinary 1-hydroxypyrene by packed capillary column switching liquid chromatography coupled to micro-electrospray time-of-flight mass spectrometry.* Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences, 2003. **794**(1): p. 175-183.

41. Holm, A., P. Molander, E. Lundanes, and T. Greibrokk, *Determination of rotenone in river water utilizing packed capillary column switching liquid chromatography with UV and time-of-flight mass spectrometric detection.* Journal of Chromatography, A, 2003. **983**(1-2): p. 43-50.

42. Pasch, H. and P. Kilz, *Analysis of copolymers by online-coupled two-dimensional chromatography.* GIT Labor-Fachzeitschrift, 1999. **43**(3): p. 239-240,242-244.

43. Nagele, E., M. Vollmer, and P. Horth, *Improved 2D nano-LC/MS for proteomics applications: a comparative analysis using yeast proteome.* Journal of biomolecular techniques, 2004. **15**(2): p. 134-43.

44. Fujii, K., T. Nakano, H. Hike, F. Usui, Y. Bando, H. Tojo, and T. Nishimura, *Fully automated online multi-dimensional protein profiling system for complex mixtures.* Journal of Chromatography, A, 2004. **1057**(1-2): p. 107-113.

45. Dugo, P., O. Favoino, R. Luppino, G. Dugo, and L. Mondello, *Comprehensive Two-Dimensional Normal-Phase (Adsorption)-Reversed-Phase Liquid Chromatography.* Analytical Chemistry, 2004. **76**(9): p. 2525-2530.

46. van der Horst, A. and P.J. Schoenmakers, *Comprehensive two-dimensional liquid chromatography of polymers.* Journal of Chromatography, A, 2003. **1000**(1-2): p. 693-709.

47. Stoll, D.R. and P.W. Carr, *Fast, Comprehensive Two-Dimensional HPLC Separation of Tryptic Peptides Based on High-Temperature HPLC.* Journal of the American Chemical Society, 2005. **127**(14): p. 5034-5035.

48. Francois, I., A. de Villiers, and P. Sandra, *Considerations on the possibilities and limitations of comprehensive normal phase-reversed phase liquid chromatography (NPLC * RPLC).* Journal of Separation Science, 2006. **29**(4): p. 492-498.

49. Wolters, D.A., M.P. Washburn, and J.R. Yates, III, *An automated multidimensional protein identification technology for shotgun proteomics.* Analytical Chemistry, 2001. **73**(23): p. 5683-5690.

50. Evans, C.R. and J.W. Jorgenson, *Multidimensional LC-LC and LC-CE for high-resolution separations of biological molecules.* Analytical and Bioanalytical Chemistry, 2004. **378**(8): p. 1952-1961.

51. Schoenmakers, P.J., G. Vivo-Truyols, and W.M.C. Decrop, *A protocol for designing comprehensive two-dimensional liquid chromatography separation systems.* Journal of Chromatography, A, 2006. **1120**(1-2): p. 282-290.

52. Holm, A., E. Storbraten, A. Mihailova, B. Karaszewski, E. Lundanes, and T. Greibrokk, *Combined solid-phase extraction and 2D LC-MS for characterization of the neuropeptides in rat-brain tissue.* Analytical and Bioanalytical Chemistry, 2005. **382**(3): p. 751-759.

53. Watanabe, N. and E. Niki, *Direct-coupling of FT-NMR to high performance liquid chromatography.* Proceedings of the Japan Academy, Series B: Physical and Biological Sciences, 1978. **54**(4): p. 194-9.

54. Hansen, S.H., A.G. Jensen, C. Cornett, I. Bjornsdottir, S. Taylor, B. Wright, and I.D. Wilson, *High-Performance Liquid Chromatography On-Line Coupled to High-Field NMR and Mass Spectrometry for Structure Elucidation of Constituents of Hypericum perforatum L.* Analytical Chemistry, 1999. **71**(22): p. 5235-5241.

55. Louden, D., A. Handley, S. Taylor, E. Lenz, S. Miller, I.D. Wilson, and A. Sage, *Reversed-Phase High-Performance Liquid Chromatography Combined with On-Line UV Diode Array, FT Infrared, and 1H Nuclear Magnetic Resonance Spectroscopy and Time-of-Flight Mass Spectrometry: Application to a Mixture of Nonsteroidal Antiinflammatory Drugs.* Analytical Chemistry, 2000. **72**(16): p. 3922-3926.

56. Roberts, J.K. and R.J. Smith, *Use of liquid chromatography-nuclear magnetic resonance spectroscopy for the identification of impurities in drug substances.* Journal of Chromatography, A, 1994. **677**(2): p. 385-9.

57. Shockcor, J.P., S.H. Unger, I.D. Wilson, P.J.D. Foxall, J.K. Nicholson, and J.C. Lindon, *Combined HPLC, NMR Spectroscopy, and Ion-Trap Mass Spectrometry with*

Application to the Detection and Characterization of Xenobiotic and Endogenous Metabolites in Human Urine. Analytical Chemistry, 1996. **68**(24): p. 4431-4435.

58. Cavin, A., O. Potterat, J.-L. Wolfender, K. Hostettmann, and W. Dyatmyko, *Use of On-flow LC/1H NMR for the Study of an Antioxidant Fraction from Orophea enneandra and Isolation of a Polyacetylene, Lignans, and a Tocopherol Derivative.* Journal of Natural Products, 1998. **61**(12): p. 1497-1501.
59. Garo, E., J.-L. Wolfender, K. Hostettmann, W. Hiller, S. Antus, and S. Mavi, *Prenylated flavanones from Monotes engleri. Online structure elucidation by LC/UV/NMR.* Helvetica Chimica Acta, 1998. **81**(4): p. 754-763.
60. Hostettmann, K. and J.-L. Wolfender, *Rapid determination of plant glycosides by LC/MS and LC/NMR.* Studies in Plant Science, 1999. **6**(Advances in Plant Glycosides, Chemistry and Biology): p. 233-260.
61. Benfenati, E., P. Pierucci, R. Fanelli, A. Preiss, M. Godejohann, M. Astratov, K. Levsen, and D. Barcelo, *Comparative studies of the leachate of an industrial landfill by gas chromatography-mass spectrometry, liquid chromatography-nuclear magnetic resonance and liquid chromatography-mass spectrometry.* Journal of chromatography. A 1999. **831**(2): p. 243-56.
62. Godejohann, M., M. Astratov, A. Preiss, K. Levsen, and C. Muegge, *Application of Continuous-Flow HPLC-Proton-Nuclear Magnetic Resonance Spectroscopy and HPLC-Thermospray-Mass Spectroscopy for the Structural Elucidation of Phototransformation Products of 2,4,6-Trinitrotoluene.* Analytical Chemistry, 1998. **70**(19): p. 4104-4110.
63. Levsen, K., A. Preiss, and M. Godejohann, *Application of high-performance liquid chromatography coupled to nuclear magnetic resonance and high-performance liquid chromatography coupled to mass spectrometry to complex environmental samples.* TrAC, Trends in Analytical Chemistry, 2000. **19**(1): p. 27-48.
64. Preiss, A., U. Saenger, N. Karfich, K. Levsen, and C. Muegge, *Characterization of Dyes and Other Pollutants in the Effluent of a Textile Company by LC/NMR and LC/MS.* Analytical Chemistry, 2000. **72**(5): p. 992-998.
65. Webb, A.G., *Nuclear magnetic resonance coupled microseparations.* Magnetic Resonance in Chemistry, 2005. **43**(9): p. 688-696.

66. Smith, R.M., O. Chienthavorn, I.D. Wilson, and B. Wright, *Superheated deuterium oxide reversed-phase chromatography coupled to proton nuclear magnetic resonance spectroscopy.* Analytical Communications, 1998. **35**(8): p. 261-263.
67. Smallcombe, S.H., S.L. Patt, and P.A. Keifer, *WET solvent suppression and its applications to LC NMR and high-resolution NMR spectroscopy.* Journal of Magnetic Resonance, Series A, 1995. **117**(2): p. 295-303.
68. Styles, P., N.F. Soffe, C.A. Scott, D.A. Cragg, F. Row, D.J. White, and P.C.J. White, *A high-resolution NMR probe in which the coil and preamplifier are cooled with liquid helium.* Journal of Magnetic Resonance (1969-1992), 1984. **60**(3): p. 397-404.
69. Spraul, M., A.S. Freund, R.E. Nast, R.S. Withers, W.E. Maas, and O. Corcoran, *Advancing NMR Sensitivity for LC-NMR-MS Using a Cryoflow Probe: Application to the Analysis of Acetaminophen Metabolites in Urine.* Analytical Chemistry, 2003. **75**(6): p. 1536-1541.
70. Olson, D.L., T.L. Peck, A.G. Webb, R.L. Magin, and J.V. Sweedler, *High-resolution microcoil 1H-NMR for mass-limited, nanoliter-volume samples.* Science (Washington, D. C.), 1995. **270**(5244): p. 1967-70.
71. Exarchou, V., M. Godejohann, T.A. Van Beek, I.P. Gerothanassis, and J. Vervoort, *LC-UV-Solid-Phase Extraction-NMR-MS Combined with a Cryogenic Flow Probe and its Application to the Identification of Compounds Present in Greek Oregano.* Analytical Chemistry, 2003. **75**(22): p. 6288-6294.
72. Godejohann, M., L.-H. Tseng, U. Braumann, J. Fuchser, and M. Spraul, *Characterization of a paracetamol metabolite using on-line LC-SPE-NMR-MS and a cryogenic NMR probe.* Journal of Chromatography, A, 2004. **1058**(1-2): p. 191-196.
73. Griffiths, L. and R. Horton, *Optimization of LC-NMR. III. Increased signal-to-noise ratio through column trapping.* Magnetic Resonance in Chemistry, 1998. **36**(2): p. 104-109.
74. Pukalskas, A., T.A. van Beek, and P. de Waard, *Development of a triple hyphenated HPLC-radical scavenging detection-DAD-SPE-NMR system for the rapid identification of antioxidants in complex plant extracts.* Journal of Chromatography, A, 2005. **1074**(1-2): p. 81-88.

75. Clarkson, C., D. Strk, S.H. Hansen, P.J. Smith, and J.W. Jaroszewski, *Identification of Major and Minor Constituents of Harpagophytum procumbens (Devil's Claw) Using HPLC-SPE-NMR and HPLC-ESIMS/APCIMS.* Journal of Natural Products, 2006. **69**(9): p. 1280-1288.

76. Lam, S.-H., C.-Y. Wang, C.-K. Chen, and S.-S. Lee, *Chemical investigation of Phyllanthus reticulatus by HPLC-SPE-NMR and conventional methods.* Phytochemical Analysis, 2007. **18**(3): p. 251-255.

77. Lee, S.-S., Y.-C. Lai, C.-K. Chen, L.-H. Tseng, and C.-Y. Wang, *Characterization of Isoquinoline Alkaloids from Neolitsea sericea var. aurata by HPLC-SPE-NMR.* Journal of Natural Products, 2007. **70**(4): p. 637-642.

78. Seger, C., M. Godejohann, M. Spraul, H. Stuppner, and F. Hadacek, *Reaction product analysis by high-performance liquid chromatography-solid-phase extraction-nuclear magnetic resonance.* Journal of Chromatography, A, 2006. **1136**(1): p. 82-88.

79. Vasilev, N.P., M.K. Julsing, A. Koulman, C. Clarkson, H.J. Woerdenbag, I. Ionkova, R. Bos, J.W. Jaroszewski, O. Kayser, and W.J. Quax, *Bioconversion of deoxypodophyllotoxin into epipodophyllotoxin in E. coli using human cytochrome P450 3A4.* Journal of Biotechnology, 2006. **126**(3): p. 383-393.

80. Sharman, G.J. and I.C. Jones, *Critical investigation of coupled liquid chromatography-NMR spectroscopy in pharmaceutical impurity identification.* Magnetic Resonance in Chemistry, 2003. **41**(6): p. 448-454.

81. Xu, F. and A.J. Alexander, *The design of an on-line semi-preparative LC-SPE-NMR system for trace analysis.* Magnetic Resonance in Chemistry, 2005. **43**(9): p. 776-782.

82. Alexander, A.J., F. Xu, and C. Bernard, *The design of a multi-dimensional LC-SPE-NMR system (LC2-SPE-NMR) for complex mixture analysis.* Magnetic Resonance in Chemistry, 2006. **44**(1): p. 1-6.

83. Liu, Y.P. and C.S. Tung, *Introduction and application of microdialysis.* Yixue Yanjiu, 1993. **14**(2): p. 71-80.

84. Boix, F., C. Roe, L. Rosenborg, and S. Knardahl, *Kinin peptides in human trapezius muscle during sustained isometric contraction and their relation to pain.* Journal of Applied Physiology, 2005. **98**(2): p. 534-540.

85. Joseph, K. and A.P. Kaplan, *Formation of bradykinin: A major contributor to the innate inflammatory response.* Advances in Immunology, 2005. **86**: p. 159-208.
86. Hagiwara, Y., M. Kojima, T. Kurashi, I. Hayashi, T. Miyata, and S. Oh-ishi, *Identification of rat urinary kinin as bradykinin.* Life Sciences, 1995. **57**(10): p. 997-1002.
87. Boix, F., L. Rosenborg, U. Hilgenfeldt, and S. Knardahl, *Contraction-related factors affect the concentration of a kallidin-like peptide in rat muscle tissue.* Journal of Physiology (Cambridge, United Kingdom), 2002. **544**(1): p. 127-136.
88. Harvey, D.T., *The Essence of Chromatography by Colin F. Poole.* Journal of Chemical Education, 2003. **80**(8): p. 883-884.
89. Poole, C.F., S.K. Poole, D.S. Seibert, and C.M. Chapman, *Determination of kinetic and retention properties of cartridge and disk devices for solid-phase extraction.* Journal of Chromatography, B: Biomedical Applications, 1997. **689**(1): p. 245-259.
90. Kiss, G., Z. Varga-Puchony, and J. Hlavay, *Determination of polycyclic aromatic hydrocarbons in precipitation using solid-phase extraction and column liquid chromatography.* Journal of Chromatography, A, 1996. **725**(2): p. 261-72.
91. Liska, I., J. Krupcik, and P.A. Leclercq, *The use of solid sorbents for direct accumulation of organic compounds from water matrixes - a review of solid-phase extraction techniques.* Journal of High Resolution Chromatography, 1989. **12**(9): p. 577-90.
92. Hennion, M.-C., V. Coquart, S. Guenu, and C. Sella, *Retention behavior of polar compounds using porous graphitic carbon with water-rich mobile phases.* Journal of Chromatography, A, 1995. **712**(2): p. 287-301.
93. Godejohann, M., A. Preiss, C. Muegge, and G. Wuensch, *Application of Online HPLC-1H NMR to Environmental Samples: Analysis of Groundwater near Former Ammunition Plants.* Analytical Chemistry, 1997. **69**(18): p. 3832-3837.
94. Djukovic, D., S. Liu, I. Henry, B. Tobias, and D. Raftery, *Signal Enhancement in HPLC/Microcoil NMR Using Automated Column Trapping.* Analytical Chemistry, 2006. **78**(20): p. 7154-7160.

95. Lambert, M., J.-L. Wolfender, D. Strk, S.B. Christensen, K. Hostettmann, and J.W. Jaroszewski, *Identification of Natural Products Using HPLC-SPE Combined with CapNMR.* Analytical Chemistry, 2007. **79**(2): p. 727-735.
96. El Sedef, N. and S. Karakaya, *Radical scavenging and iron-chelating activities of some greens used as traditional dishes in Mediterranean diet.* International Journal of Food Sciences and Nutrition, 2004. **55**(1): p. 67-74.
97. Nkoane, South Africal Journal of Science, 2007.
98. Zhao, C., G. Dodin, C. Yuan, H. Chen, R. Zheng, Z. Jia, and B.-T. Fan, *\"In vitro\" protection of DNA from Fenton reaction by plant polyphenol verbascoside.* Biochimica et Biophysica Acta, General Subjects, 2005. **1723**(1-3): p. 114-123.
99. Deepak, M., D.C. Umashankar, and S.S. Handa, *Verbascoside-a promising phenylpropanoid.* Indian Drugs, 1999. **36**(6): p. 336-345.
100. Li, J., Y. Zheng, Z. Hong, B.N. Su, and R.L. Zheng, *Differentiation of human gastric adenocarcinoma cell line MGc80-3 induced by verbascoside.* Planta Medica, 1997. **63**(6): p. 499-502.
101. Hemstroem, P. and K. Irgum, *Hydrophilic interaction chromatography.* Journal of Separation Science, 2006. **29**(12): p. 1784-1821.
102. www.sequant.se (April 2007).
103. Alpert, A.J., *Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds.* Journal of Chromatography, 1990. **499**: p. 177-96.
104. Yoshida, T., *Peptide Separation in Normal Phase Liquid Chromatography.* Analytical Chemistry, 1997. **69**(15): p. 3038-3043.
105. Berthod, A., S.S.C. Chang, J.P.S. Kullman, and D.W. Armstrong, *Practice and mechanism of HPLC oligosaccharide separation with a cyclodextrin bonded phase.* Talanta, 1998. **47**(4): p. 1001-1012.