Thesis for the Master's degree in chemistry

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Determination of peptides in microdialysates using 2-dimensional capillary liquid chromatography coupled to electrospray time-of-flight mass spectrometry (2D-capLC-ESI-TOF-MS)

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Preface

This graduate study was carried out at the Department of Chemistry at the University of Oslo, in the period from August 2005 to March 2007 under the supervision of Professor Tyge Greibrokk, Professor Elsa Lundanes and Ph.D. Steven Ray Wilson.

I would like to thank my supervisors for giving me the opportunity to conduct this interesting and challenging project. I want to thank my supervisors for the all invaluable help and guidance throughout this study. Special thanks to Steven Ray Wilson for providing me with help at any time.

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Many thanks to my family for their love, support, motivation and patience. Thank you Linda for all your love and patience.

Oslo, Norway, March 2007

Mikolaj Jankowski

ABSTRACT

In the present work, a 2D-capLC-ESI-TOF-MS method for fractionation and separation of peptides in microdialysates is demonstrated. Both comprehensive and quantitative analysis was conducted. The microdialysates samples (335 µL) were loaded onto a 1 mm x 5 mm loading column packed with 5 µm Kromasil C_{18} particles by a carrier solution of 0.1 % formic acid in ACN/H_2O (5/95, v/v) at a flow rate of 100 µL/min. Back-flushing onto a fractionating 0.32 mm x 150 mm ZIC-HILIC column packed with 5 µm particles was performed using a decreasing linear solvent ACN/H_2O gradient containing 10 mM ammonium acetate at pH 6.8. Dilution of the effluent was done using a solution containing 0.1 % formic acid prior to on-line collection of fractions onto multiple 1 mm x 5 mm Kromasil C_{18} column packed with 5 µm particles. Back-flushed elution of the fractions onto a 0.3 mm x 150 mm PLRP-S column packed with 3 µm particles was performed using an increasing stepped solvent ACN/H_2O gradient containing 0.1 % formic acid. Positive ESI was performed in the *m/z* range of 200-1500. A post-column standard was introduced to MS detection when performing target compound analysis. The estimated concentration limit of detection (cLOD) was 0.15 ng/mL.

ABBREVIATIONS

µLC	Micro liquid chromatography
ACN	acetonitrile
BPI	base peak intensity
capLC	capillary liquid chromatography
cLOD	concentration limit of detection
cLOQ	concentration limit of quantification
EIC	extracted ion chromatogram
ESI	electrospray ionization
f.a.	formic acid
f.s.	fused silica
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
I.D.	inner diameter
LC	liquid chromatography
LLE	liquid-liquid extraction
m/z	mass to charge ratio
mLOD	mass limit of detection
mLOQ	mass limit of quantification

MP	mobile phase
MS	mass spectrometry
nanoLC	nanocolumn liquid chromatography
NP	normal phase
PEEK	polyetheretherketone
RP	reversed phase
RPC	reversed phase chromatography
RSD	relative standard deviation
S/N	signal to noise ratio
SCX	strong cation exchange
SPE	solid phase extraction
THF	tetrahydrofuran
TFA	trifluoro acetic acid
TIC	total ion current
TOF	time-of-flight
UV	ultraviolet
WCX	weak cation exchange
ZIC	zwitterionic

1. INTRODUCTION

1.1 Background

Neurotransmitters constitute a group of compounds that mediate signaling between neurons and non-neuronical cells. Living organisms depend on a finely tuned balance between these compounds. Even a slight variation can cause diseases like depression, schizophrenia and other disorders. Neurotransmitters are divided in three main groups; amino acids, biogenic acids, and neuropeptides [1]. Studying the activity of these compounds much knowledge about the nature of different physiological conditions can be obtained. Bradykinin is a neuropeptide from a group called kinins. Kinins are peptide hormones that play a role in inflammation, blood pressure regulation, coagulation and pain [2]. Bradykinin is a kinin which besides the mentioned properties also increases glucose metabolism [3-6] and capillary vessel permeability [3]. Release is known to happen under high muscle activity [5]. As increased levels of bradykinin tell that an inflammation is taking place it may be an indicator of ill medical conditions such as chronic congestive heart-failure [7, 8]. Monitoring of kinins is useful for study of the various mechanisms in which they occur. Figure 1 illustrates the structure of bradykinin.

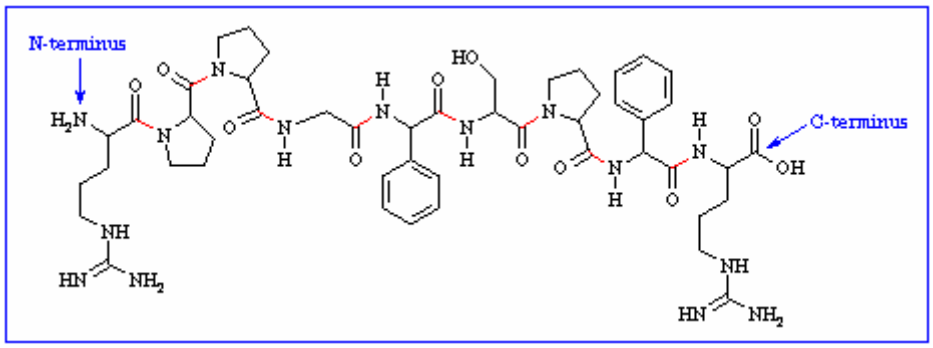

Figure 1. Structure of bradykinin [9]

Several methods have been developed for the determination of peptides in mice [10], rats [11], humans [5, 12] and protein digest and peptide standard solutions [13, 14]. Saliva [15, 16], urine [12], plasma [17] and microdialysates [4, 11, 18, 19] are the most common matrices. In a recent study [4] bradykinin and kallidin was detected in microdialysis samples collected from human muscle tissue using an immunoassay technique with specific antibodies for each analyte. A similar study [11] used capillary column-switching liquid chromatography (LC) coupled with mass spectrometry (MS) to detect arg-bradykinin and bradykinin in microdialysates. Due to the complexity of biological samples it is often required to employ methods with high resolving power such as two dimensional liquid chromatography (2DLC) to obtain satisfactory separation. Several 2DLC methods have been developed for peptide [11, 12, 20, 21] and protein [15] separation. In essence, this type of methodology enables two types of chromatography to resolve the compounds in a sample. Fractions are collected from the first dimension, and the compounds contained in each fraction are then separated using a different type of chromatographic principle. As analyte concentrations are low, sensitive detection like mass spectrometry (MS) must be employed. For the same reason a preconcentration step

is often a necessity. This has been done using liquid-liquid extraction (LLE) [10], but solid phase extraction (SPE) is more commonly used in LC applications. For peptide separation in the first dimension, size exclusion chromatography (SEC) [22, 23], strong cation exchange (SCX) [24-27] and hydrophilic interaction liquid chromatography (HILIC) [20, 28-30] have been mainly applied. Mostly reversed phase (RP) chromatography [12, 15, 20, 21, 27, 31] is used in the second dimension. 2D-RP-RP peptide separation has also been conducted [32]. RP is the most common applied separation principle.

1.2 Separation by SCX, HILIC and RP

In SCX chromatography analytes are retarded by ionic interactions. The analytes are separated by charge. Higher charge yields greater retention. The functional group on the stationary phase (SP) in a SCX column is a strong acid residue chemically bound to a polymer or silica particles. Mostly sulfonic acids are used and they remain charged over the entire pH range. To attain retention, the pH of the mobile phase (MP) must be chosen as to maintain charge of the analytes. The retention of the analytes also depends on the ion strength of the buffered MP decreasing with increasing MP ion strength. For peptides the pH of the MP should be in the acidic range. The analytes are introduced in a low salt concentration MP. At such conditions the analytes change place with the counter ion, thus ion exchange, and are retained. Elution is performed by increasing pH, the salt content or a combination of both. It is more common to apply a salt gradient (20 to 500 mM) as pH gradients are more difficult to reproduce. An organic modifier is often added

to the MP to suppress hydrophobic interactions between the analytes and the hydrophobic sites on the SP.

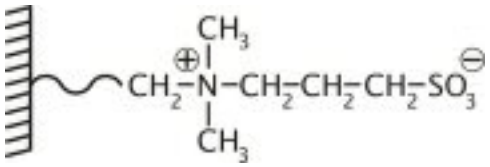

Figure 2. The functional group on the ZIC-HILIC packing material [33]

HILIC is variant of normal phase (NP) chromatography where an aqueous MP containing a high percentage of organic solvent is used. The SP is hydrophilic with zero net charge. In this study a zwitterionic (ZIC)-HILIC column was employed. The SP consists of a zwitterionic group chemically bound to silica particles. Figure 2 depicts the functional group of a ZIC-HILIC column. As the name states, analytes are retarded by hydrophilic interactions. Because the column has zero net charge only a low amount of salt or buffer is used in the MP. Normally 10 mM will suffice for peptides. Elution can be performed with a decreasing organic gradient or increasing salt/buffer content. The elution order is from less to more hydrophilic analytes. The most important consideration regarding HILIC is the orthogonality with reversed phase chromatography (RPC). This simply means that the elution order is the opposite of each other. Analytes that are not retained on a HILIC column will be retained on a RP column, and vice versa. If a 2D-plot is made from HILIC and RPC separations of a sample, true orthogonality is obtained if the plot reveals no correlation [34]. The concept of orthogonality is described in figure 3.

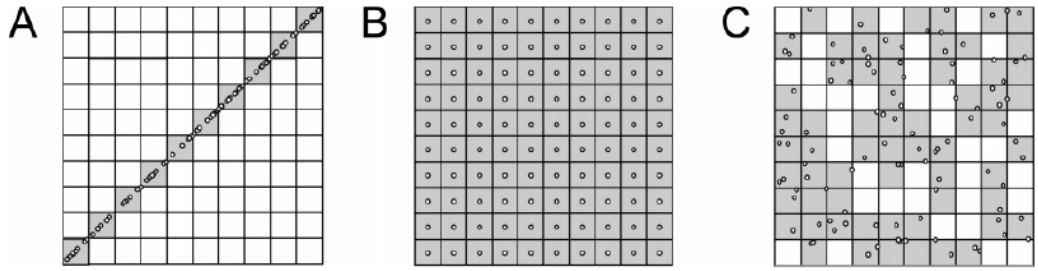

Figure 3. Geometric orthogonality concept. Hypothetical separation of 100 analytes in a 10 x 10 normalized separation space. (A) Nonorthogonal system, 10 % area coverage represents 0 % orthogonality. (B) Hypothetical ordered system, full area coverage. (C) Random, ideally orthogonal, system. Area coverage is 63 % representing 100 % orthogonality. [34]

RPC is the most common type of chromatography and is frequently used for separating peptides in both one dimensional (1D)LC [11] and 2DLC [12, 15, 20, 21, 31]. Retention on a RP column is due to hydrophobic interactions between the analytes and the SP. The most common SP consists of long alkyl chains chemically bonded to a polymer or silica particles. Various chain lengths exist (C_1 to C_{18}) whereas C_{18} materials are of the most frequently applied. Increasing organic content in the MP decreases retention. Elution order is from less to more hydrophobic. Samples are subjected to the RP column at low organic conditions, and typically an increasing organic gradient is used to elute the analytes. The organic solvents used must be water miscible [35]. Typical solvent used are acetonitrile (ACN), methanol and tetrahydrofuran (THF) [35]. Additives, such as trifluoro acetic acid (TFA) and formic acid (f.a.), are added to the MP in small amounts (< 1%, v/v) for several reasons. Adding a modifier increases retention and peak shape for ionic compounds by the formation of ion-pairs [36], and suppresses ionic interactions with residual silanol groups on silica based columns.

1.3 Microdialysis

The samples analyzed in this study were collected microdialysates. It is a miniaturized form of dialysis where a probe is inserted directly into the tissue [6]. The probe only collects the compounds that pass through a membrane. The samples for this project were collected using a membrane with a cut-off value of 15 kDa. But the cut-off value is not accurate in the sense that all the compounds with higher masses than 15 kDa are excluded. The cut-off is defined as the molecular mass at which 80 % of the molecules are prevented from passing through the membrane [18]. The probe is perfused with a physiological solution consisting of e.g. Ringer acetate. The analytes distribute equally between the carrier solution and the surrounding tissue. Due to the extremely low concentration of analytes [5, 6, 17] and complexity of the sample, methods with high separating power and sensitivity must be utilized in order to obtain satisfactory detection of the analytes in the microdialysates.

1.4 Capillary liquid chromatography (capLC)

In analytical LC the amounts of analytes is often so low that detection is difficult to achieve unless extensive sample preparation must be performed to obtain detection. Using a narrower column presents several advantages over a larger bore column. There are several classes of column depending on their internal diameter (I.D.) presented in table 1. The most important advantage of downscaling the column I.D. is the increased mass sensitivity due to less volumetric dilution of the chromatographic band in the column. If the same amount of analyte is introduced into a conventional column (4.6 mm

I.D.) and a capillary column (0.32 mm I.D.), the capillary will have a theoretical mass limit of detection (mLOD) ~200 times lower than the conventional column [37]. The dilution *D* of an injected sample is given by;

$$D = \frac{\varepsilon \pi r^2 (1+k)(2\pi L H)^{\frac{1}{2}}}{V_{inj}} = \frac{C_{end}}{C_{inj}} \quad (1.1)$$

Equation 1.1 contains parameters where ε is the column porosity, r is the column internal radius, k is the retention factor, L is the column length, H is the plate height, and V_{inj} is volume injected. The reduction of column size requires lesser amounts of sample, which is convenient in biological analysis when samples often are in limited amount. The lower flow in a capillary (down to 1 μL/min) simplifies the use MS detection because of the small amount of solvent required to evaporate. Lower solvent and stationary phase consumption is a profitable affair.

Table 1. Classification of LC columns according to the internal diameter (I.D.)

Column class	Internal diameter [mm]
Conventional	3 - 5
Narrow-bore	2
Micro bore	0.5 - 1
Capillary	0.1 - 0.5
Nano	0.01 - 0.1
Open tubular	0.005 - 0.05

1.5 Electrospray ionization mass spectrometry (ESI-MS)

For compounds to be detected in the MS, they must first be ionized. In this project electrospray ionization (ESI) was utilized since peptides are easily ionized to cations due

to their basic residues. ESI is a so-called soft-ionization technique, which provides little or no fragmentation of the analytes. Voltage is applied over the tip of an f.s. capillary needle. An additional gas flow axially with the needle is needed to assist the nebulization. An ideal spray has the shape of a Taylor cone [38]. A volatile salt/buffer should be present in the MP to provide conductivity, which in turn assists in the formation of charged droplets. The existence of organic solvent reduces the surfaces tension to aid droplet formation and solvent evaporation. The fine jet of solvent produced at the spray capillary disintegrates into charged droplets with a diameter of circa 1 μm. Solvent evaporation from the droplets occur to a point where the droplets reach the Rayleigh limit, which specifies the maximum charge a spherical droplet can hold before the Coulomb repulsion overcomes the surface tension. The droplets then undergo an uneven fission where they eject about 20 offspring droplets each. The offspring droplets are about one order of magnitude smaller than their mother droplets and include about 15 % of their charge and about 2 % of their mass. Subsequent evaporation and uneven fission of the droplets continues, creating second-generation droplets [38]. As the droplets continue into the MS, they are further desolvated until gas phase ions are formed and detected by the MS.

1.6 Aim of study

The purpose of this study was to develop a sensitive on-line 2D-LC method with high resolving power for determination of peptides in complex matrices, both for comprehensive and target compounds. The method will be optimized in microdialysis

samples. One of the goals, despite the intricacy of 2D systems, is to create a system that is robust and relatively easy in operation.

2. EXPERIMENTAL

2.1 MATERIALS AND REAGENTS

HPLC grade acetonitrile (ACN) was obtained from Rathburn Chemicals Ltd. (Walkerburn, UK). Grade 1 water was provided by a Milli-Q ultrapure water purification system (Millipore, Bedford, MA, USA). Ammonium acetate (NH_4Ac, purum), ammonium formate (NH_4formate, MS grade) and formic acid (f.a. 50%, HPLC grade) were purchased from Fluka (Buchs, Switzerland). NaCl (≥ 99.5 %, analytical grade) was purchased from Sigma Aldrich (St.Louis, MO, USA). AnalR grade ammonia (25 % NH_3 solution, 13.4 M) was acquired from BDH Chemicals Ltd. (Poole, UK). Hydrogen chloride (HCl) 37 % solution (analytical grade) was purchased from Merck KGaA (Darmstadt, Germany). Purum quality sodium hydrogen carbonate (> 99.3 % $NaHCO_3$) was obtained from KEBO Lab AB (Spånga, Sweden). Bovine albumin (≥ 97 %, gel electrophoresis), chicken egg albumin (99 %, gel electrophoresis), rabbit hemoglobin (Hb), human transferrin (≥ 98 %) and Apolipoprotein A (Apo A) were purchased from Sigma Aldrich GmbH (Steinheim, Germany). Bradykinin, angiotensin II, oxytocin and thyrotropin-releasing hormone (TRH) were acquired from Fluka. Fused silica capillaries were purchased from Polymicro Technologies Inc. (Phoenix, AZ, USA). Helium (≥ 99.996 %) and nitrogen (99.99 %) were purchased from AGA AS (Oslo, Norway). Microdialysis samples from rat muscle were obtained from the National Institute of Public Health, Oslo, Norway. Fractionating column materials were PolyLC

Polysulfoethyl Aspartamide (PolyLC Inc., CO, USA) and ZIC-HILIC (SeQuant, Umeå, Sweden), both with particle size of 5 µm. The pore size was 300 Å and 200 Å for PolyLC and ZIC-HILIC, respectively. The PolyLC (1 x 150 mm) column was purchased from G&T Septech (Kolbotn, Norway). The TSK-Gel Amide 80 (5 µm, 80 Å) column (1 x 50 mm) was obtained from Tosoh Bioscience GmbH (Stuttgart, Germany). Pre- and trap columns (1 x 5 mm) packed with Kromasil C_{18} (5 µm, 100 Å) and a PLRP-S polystyrene-divinylbenzene (PS-DVB) (3 µm, 300 Å) column (0.32 x 150 mm) were obtained from G&T Septech. A Jupiter Proteo C_{12} (4 µm, 90 Å) column (0.32 x 150 mm) was obtained from Phenomenex (Aschaffenburg, Germany). A Chromolith CapRod C_{18} (2 µm, 130 Å) monolithic column (0.1 x 150 mm) was purchased from Merck. Bulk Kromasil C_{18} (3.5 µm, 100 Å) packing material was purchased from G&T Septech.

Stock solutions of angiotensin II and bradykinin (1 mg/mL) were made by dissolving 25 mg of each compound in 25 mL water, and transferred to 1.5 mL polypropylene micro centrifuge tubes (Brand GmbH, Wertheim, Germany) in 1 mL aliquots for storage at -18ºC. A mixture of five peptides containing 0.1 mg/mL angiotensin II, 0.05 mg/mL bradykinin, 0.1 mg/mL leucine enkephaline, 5 µg/mL oxytocin and 0.2 mg/mL, was used in preliminary testing of the fractionating columns.

2.2 SAMPLE PREPARATION

2.2.1 Protein digests

A 1 M solution of $NaHCO_3$ was prepared by dissolving 8.4014 g of the latter compound in 100 mL water. A 1 M NH_3 solution was made by diluting 7.462 mL (25 % / 13.4 M) NH_3 to a volume of 100 mL. A 25mM ammonium hydrogen carbonate (NH_4HCO_3) buffer was composed by mixing 2.5 mL 1 M NH_3 and 2.5 mL 1 M $NaHCO_3$ and dilution to a volume of 100 mL. The buffer was titrated to pH 7.5 using some drops of concentrated HCl. A 2 mg/mL trypsin solution was prepared by dissolving 4 mg trypsin (Promega, Mannheim, Germany) in 2 mL NH_4HCO_3. Separate protein digest solutions containing bovine albumin, chicken egg albumin, rabbit hemoglobin and human transferrin were made for column and system testing. 1 mg of each protein were weighed into individual 1.5 mL polypropylene tubes (Brand GmbH, Wertheim, Germany), then 250 µL NH_4HCO_3 buffer and 25 µL 2 mg/mL trypsin solution was added. The tubes were incubated at 37°C for 24 hours and then stored at -18°C as stock protein trypsinates. These trypsinates were thawed and 750 µL NH_4HCO_3 buffer was added before use, resulting in a total volume of 1 mL. Additionally a mixture of three trypsinated proteins (bovine albumin, human transferrin, rabbit hemoglobin) was prepared by mixing 250 µL of each protein trypsinate solution and adding 250 µL NH_4HCO_3 buffer. This solution will further be referred to as XXX.

2.2.2 Microdialysis samples

Fifteen samples were collected by microdialysis in rat thigh muscle in aliquots of 600 µL into 1.5 mL polypropylene tubes. Two of the delivered samples had a volume of ~300 µL. The carrier solution was Ringer acetate and the membrane in the microdialysis probe had a cut-off value equal to 15 kDa. The microdialysates were stored at -18°C. Each sample was thawed at ambient temperature prior to analysis. The sample material to be analyzed was collected by Fernando Boix from the National Institute of Public Health (Oslo, Norway). Prior to injection each sample was centrifuged at 3000 rpm for 10 minutes, using a Biofuge centrifuge (Heraeus instruments, Hanau, Germany).

2.2.3 Calibration solutions

A 10 µg/mL bradykinin solution was made by transferring 1.0 mL of bradykinin (1.0 mg/mL) to a 100.0 mL volumetric flask and diluting with 0.1 % f.a. solution. An aliquot of this solution was transferred to a 20 mL polypropylene (VWR, Oslo, Norway) vial and used to prepare bradykinin calibrations solutions containing 0.025, 0.25, 2.5 and 5 µg/mL. All solutions contained 0.05 % formic acid. A calibration curve was obtained by injecting 2 µL with 0.025, 0.25, 2.5 and 5 µg/mL solutions corresponding to 0.05, 0.5, 2.5 and 10 ng of bradykinin, respectively.

2.3 COLUMN PREPARATION

The fractionating capillary columns were slurry packed using a downward high pressure method developed in-house [39]. Carbon tetrachloride was used as solvent for the slurry. The stationary phases used were PolyLC, ZIC-HILIC and Kromasil C_{18}. Fused silica capillaries, 320 μm internal diameter (I.D.) and 450 μm outer diameter (OD), and 0.5 mm I.D. steel tubing, served as column bodies, all of 15 cm length. PolyLC was also packed in 0.5 mm I.D. x 12.5 cm steel tubing. Valco (Valco Instruments, Houston, TX, USA) ZU1C unions, Valco FS1.4 polyimide ferrules and Valco 2SR1 steel screens were used for the end fittings.

2.4 CHROMATOGRAPHIC SYSTEM

2.4.1 Preliminary test system

A preliminary test system was assembled to examine the PolyLC (0.5 mm x 10 cm, 1 mm x 15 cm) columns. Loops of 1, 2, 5, and 50 μL were used for sample introduction using a 6-port Valco valve (Val.1). Sample was loaded onto a C_{18} SPE (1 x 5 mm). The load MP (ACN/H_2O [5/95, v/v] containing 0.1 % f.a.) was delivered by a Hitachi L-7100 (Hitachi High-Technologies Corporation, Tokyo, Japan) isocratic pump. Elution was performed by switching a second 6-port Valco valve (Val.2) and back-flushing the SPE using a linear salt gradient from 2 to 100 % B in 10 minutes. Solvent A was constituted of

H_20/ACN (75/25, v/v) containing 0.1 % TFA. Solvent B constituted of A + 500 mM NaCl. Both solvents had a pH of 2. The gradient MP was delivered using an Agilent 1100 Series capillary gradient pump (Agilent, Palo Alto, CA, USA) with an incorporated on-line vacuum degasser. UV detection was done with a 100 UVIS detector (Spectra-Physics, Fremont, CA, USA) equipped with a 100 µm I.D. on-capillary flow cell. Data acquisition was done at 220 nm and 254 nm using Totalchrom 6.2.1. All connections were done using fused silica (f.s.) with I.D. of 75 µm. Figure 4 shows the instrumental arrangement.

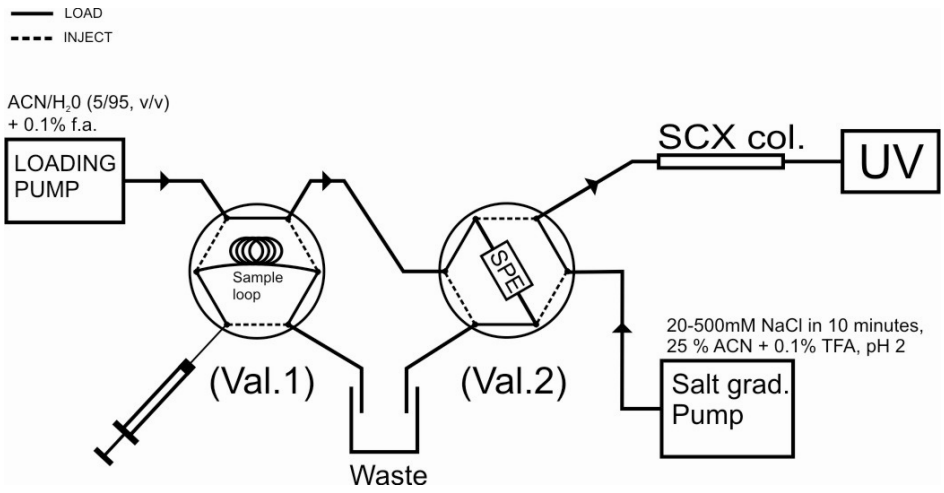

Figure 4. The preliminary instrumental setup

2.4.2 First dimension (µSPE-capLC-UV)

Figure 5 depicts the entire instrumental arrangement. The dashed line points out the first dimension, which is a column switching system. The sample was loaded onto a Kromasil C_{18} SPE (1 x 5 mm) column by use of a Hitachi L-7100 isocratic pump (PUMP1). The

loading mobile phase (ACN/H_2O [5/95, v/v] containing 0.1 % f.a.) was delivered, at a flow rate of 100 µL/min. Back flushing of the SPE column was performed using a linear gradient delivered at 5 µL/min by an Agilent 1100 Series capillary gradient pump with an incorporated on-line vacuum degasser (PUMP2). The gradient ran from 85 % to 45 % B in 30 minutes followed by a 10 minute wash with 45 % B. Solvent A consisted of 10 mM NH_4Ac. Solvent B consisted of ACN/H_20 (98/2, v/v) and 10 mM NH_4Ac. The pH in both solvents was 6.8. Two Valco Cheminert 6-port two-position valves were used for sample introduction and column switching. A 335 µL sample loop (0.5 mm I.D. steel) was connected to valve 1 (V1). The C_{18} SPE column was coupled to valve 2 (V2). Pump 1 and pump 2 were connected to valve 1 (V1) and 2 (V2), respectively. Detection was done using a Spectra 100 UVIS detector equipped with a 100 µm I.D. on-capillary flow cell. Data acquisition was performed at 220 nm using Totalchrom 6.2.1. The fractionating fused silica capillary column (0.32 x 150 mm) was packed with ZIC-HILIC stationary phase. Remaining connections were made with 75 µm I.D. fused silica, except for the capillary in the UV detector which was 100 µm I.D. due to the requirement of detection path length. Fused silica connections were made using Valco ZU1C unions and Valco FS1.4 polyimide ferrules.

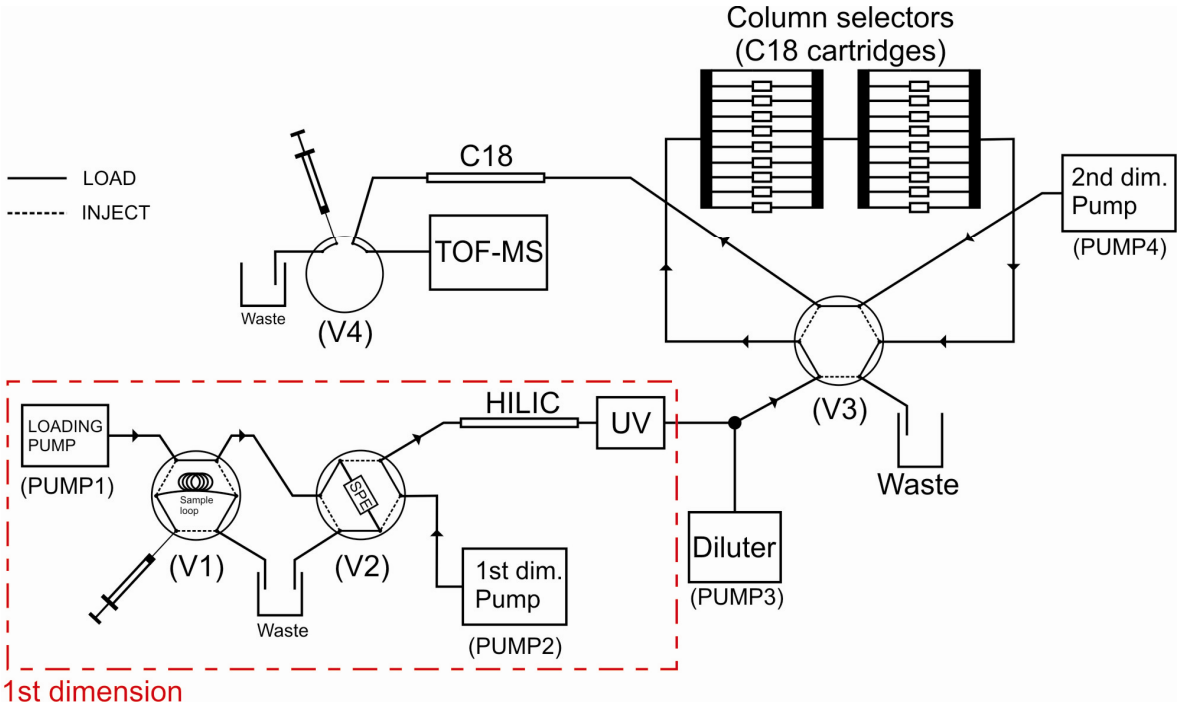

Figure 5. Diagram of the instrumental setup

2.4.3 Second dimension (μSPE-capLC)

The effluent from the UV detector was diluted with 0.1 % f.a. delivered by a Hitachi L-7100 isocratic pump (PUMP3), using a Valco T-connection. Pump 3 delivered the diluting solution (0.1% f.a.) at a flow rate of 70 μL/min, resulting in a 15x dilution. The resulting dilute was transferred to the C_{18} SPE columns. This was achieved by coupling two column carousels in series, equipped with 9 C_{18} SPE columns each. That gives 18 SPE columns in total for collection of fractions. See figure 5 for graphical explanation. The two carousels were coupled to a Valco 6-port two position valve (V3). The latter valve and carousels were mounted on a metal plate. Each carousel consisted of two interconnected Cheminert C5 10-port stream selector valves mounted on one Cheminert

high torque (EMT) microelectric actuator (see Figure 6 for picture of the carousels). One port on each carousel was fitted with a bypass loop (75 µm I.D. f.s.). Switching valve 3 permits back-flushing of the sample from the carousel(s) onto the analytical PLRP-S column (0.32 x 150 mm) with a stepped gradient delivered by an Agilent 1100 Series (PUMP4). Flow of the mobile phase was 5 µL/min. Solvent A and B consisted of grade 1 water and ACN, respectively. Both reservoirs contained 0.1 % f.a. The gradient ran from 5 % to 15 % B in 3 minutes, 15 % to 30 % B in 15 minutes and then from 30 % to 50 % B in 5 minutes. Finally the column was washed for 10 minutes with 90 % B (see Table 2). Column conditioning was done using 5 % B for 20 minutes. Protein digests and UV detection was used in preliminary testing of the performance of the system, otherwise mass spectrometric (MS) detection was performed.

Table 2: RP gradient timetable

Time [min]	% B
0.01	5
3.00	15
18.00	30
23.00	50
23.01	90

For sample screening purposes all the SPE columns on the carousels were used. When performing target analyte quantification only one of the SPE columns was used.

2.4.4 Target analyte quantification and comprehensive analysis

For target analyte quantification a standard was introduced after the analytical column. This was achieved with a Valco 4-port valve with 500 nL internal loop just prior to MS detection. The standard (5 µg/mL bradykinin containing 0.05 % f.a.) was introduced into the MS, using V4, shortly after detection of the analyte peak. The last connection from V4 to the TOF-MS instrument was done using 63 µm I.D. polyetheretherketone (PEEK) tubing (Upchurch Scientific, Oak Harbour, WA, USA).

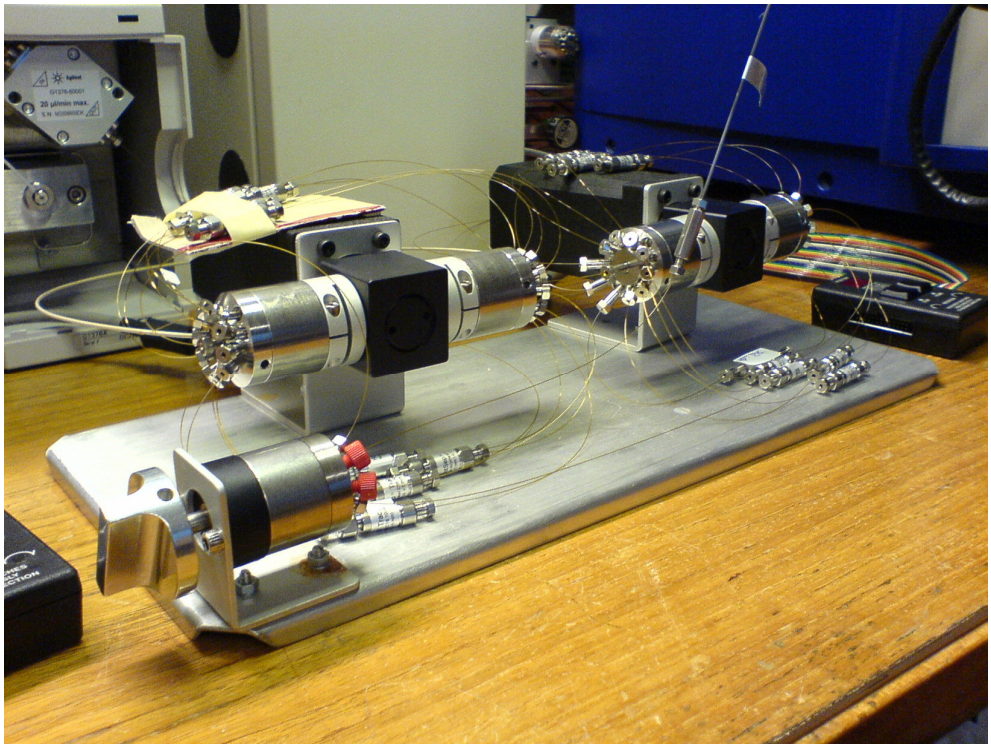

Figure 6. Picture of the two column carousels in series, coupled to V3. Everything was mounted on a metal plate for added robustness and mobility.

As soon as the analyte peak front, from the first dimension, was detected by the UV detector, the effluent flow was directed through one of the C_{18} SPE columns on the

carousels. Dilution of the effluent was necessary to achieve retention. The dilution pump diluted the effluent 15 times so that the ACN percentage was 5.7 to 3 throughout the HILIC gradient. Kromasil C_{18} (5 μm particles) trapcolumns were employed to accommodate the high flow and avoid too high back-pressure. The smaller particles in the analytical RP (3 μm particles) column provided refocusing when back-flushing the trap columns onto the analytical RP column. 50 μL of XXX solution was introduced onto the C_{18} SPE and back flushed onto the ZIC-HILIC column. The sample was divided into 8 parts using only one carousel, thus collecting each fraction for 3 minutes before switching the flow over to a new SPE. For comparison a similar screening was done but instead using all 19 SPE columns, thus collecting each fraction for 1.5 minutes. Each fraction was then eluted onto the analytical RP using a stepped gradient described in section 2.4.2. UV detection at 220 nm was used in both cases.

2.4.5 Detection (ESI-TOF-MS)

Detection in the second dimension was done using a Micromass LCT TOF-MS (Waters, Manchester, UK) equipped with a Z-spray atmospheric pressure electrospray ionization (ESI) ion source. The electrospray was operated in positive mode. The voltages applied over the capillary, sample- and extraction cone are shown in table 3. See appendix 6.5 for more parameter details.

Table 3. Applied MS-voltages

	Voltage [V]
Capillary	3200
Sample Cone	11
Extraction Cone	3
RF Lens	500

Desolvation and source temperature were both set at 110°C. The desolvation gas flow rate was adjusted to ~300 L/h. Data were acquired in the mass-to-charge (*m/z*) interval 200 – 1500 using MassLynx 4.0 (Micromass). Calibration of the instrument was done using a sodium/caesium iodide solution (2 µg/mL) dissolved in isopropanol/water (50/50, v/v).

3. RESULTS AND DISCUSSION

In 2DLC there are two main techniques, comprehensive chromatography and heart-(/front-/end-) cutting. A 2D separation is considered comprehensive if every part of the sample is subjected to two different separations, each with a different characteristics, whereas in heart cutting only one fraction from the first dimension is subjected to separation in the second dimension [40, 41]. The amounts of peptides present in microdialysis samples are relatively low [11]. 2DLC in combination with column switching provides the required sensitivity and selectivity to detect peptides present at very low concentrations. The use of large volume injection further lower the detections limits [11], given there is sufficient amount of sample.

3.1 Comprehensive analysis

a. Method development

1. First dimension

The columns used in a 2DLC system should provide orthogonal separation for obtaining the best resolution of complex samples. UV and TOF-MS detection were both used for testing the columns in the first dimension. Two different types of SP were employed for fractionation; SCX (PolyLC) and HILIC (ZIC-HILIC and TSK-Gel). The choice of mobile phase for the SCX column was based upon a previous study [42]. A linear salt

gradient (20 to 500 mM NaCl) at pH 2 was used. A mixture of peptides was introduced onto the PolyLC column in preliminary experiments (Appendix 6.4). Angiotensin II and bradykinin standards were introduced in the preliminary experiments. Narrow peaks (~ 0.5 min) of both angiotensin II and bradykinin appeared, but the compounds were not fully separated. Exchanging NaCl with NH_4-formate did not provide separation. However peak broadening and severe peak fronting, due to SP collapse, were observed after some time. The column collapse could be due to bad packing of the SP. Hence different slurries were explored for packing of the SCX column. Solvents used for slurries were carbon tetrachloride, H_20/MeOH (50/50, v/v) and MeOH. However, SP collapse was observed for all slurries. A commercial column also exhibited serious SP collapse. Instability of this particular SP has also been observed in a previous study [43]. No reasonable explanation was found for this behavior therefore the use of SCX columns was discontinued from use.

The types of HILIC columns that were tested for the first dimension were ZIC-HILIC (0.32 x 150 mm) and TSK-Gel Amide 80 (1 x 5 mm). Elution on both HILIC columns was carried with a linear gradient from 85 to 45 % ACN containing 10 mM NH_4Ac. Despite the fact that NH_4Ac is not UV transparent at 220 nm, the low concentration does provide detection of peptides at concentrations which do not overload the column. The volatile salt is fully compatible with electrospray ionization (ESI)-MS detection.

The TSK-Gel HILIC column displayed varying retention times and peak sizes. Reproducibility could not be achieved even after extended periods of column

conditioning. Air spikes were initially observed while using the TSK-Gel HILIC column. The latter types of columns are very vulnerable to air passing through them. Air bubbles will most certainly create channels in the column bed and render it chromatographically destroyed, which may have been the case in this situation. After this discovery the TSK-Gel HILIC column was discontinued from use.

The ZIC-HILIC column demonstrated adequate retention of peptides. Angiotensin II and bradykinin was well separated but tailing occurred to some extent (see Figure 7).

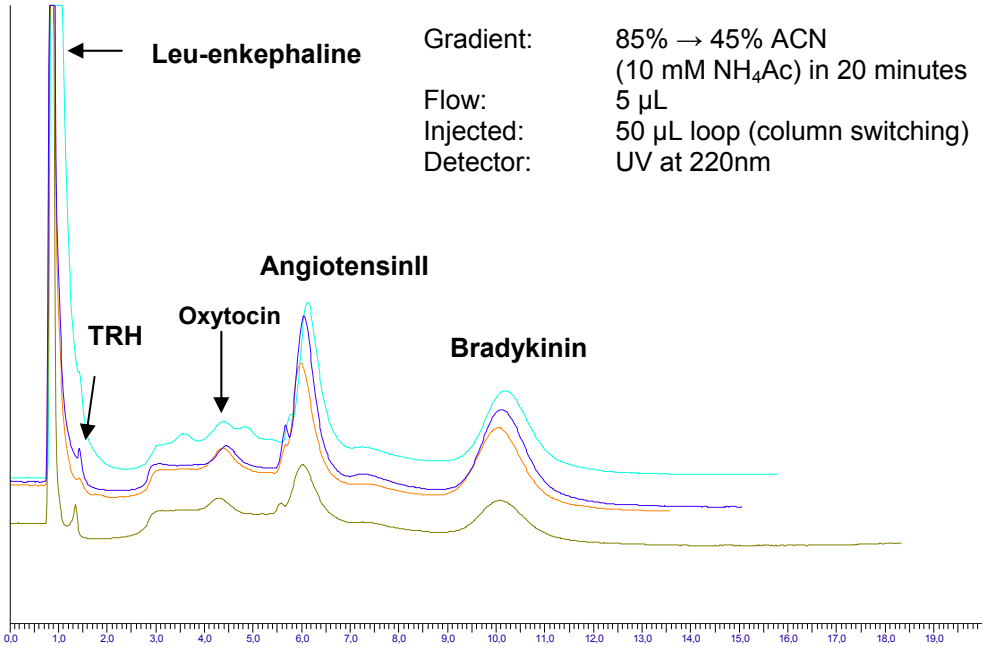

Figure 7. Chromatogram of a solution of different peptides injected onto the ZIC-HILIC column (0.32 x 125 mm). The solution contained 0.1 mg/mL angiotensin II, 0.05 mg/mL bradykinin, 0.1 mg/mL leucine enkephalin, 5 µg/mL oxytocin and 0.2 mg/mL TRH. 50 µL sample was loaded onto a C_{18} SPE (1 x 5 mm) using ACN/water (5/95, v/v) containing 0.1 % f.a. delivered at 50 µL/min. Elution was done with a gradient running from 85 to 45 % ACN containing 10 mM NH_4Ac, in 20 minutes. Detection with UV at 220 nm.

To attain stable retention times on the ZIC-HILIC column several cycles of gradients and injections of samples had to be completed. The retention of the peptides then increased to a constant value after several injections. Small amounts of the injected compounds tend to irreversibly adsorb to the metal frits in the column end fittings as well as on the column itself. Basic analytes in particular tend to bind irreversibly to the acidic rest-silanol groups on the silica particles. Thus several injections must be performed to saturate the chromatographic system before stable operation is achieved. Operation of the ZIC-HILIC packing material in HILIC mode required a high amount of ACN in the MP, and therefore a dilution step was necessary prior to collection of fractions on the second dimension RP trap columns. In preliminary experiments a long column (23 cm) was utilized, but the length was reduced to 12.5 cm in order to obtain lower analysis time. Trypsinated albumin, apo A and the pre made XXX solution (trypsinated; bovine albumin, human transferrin, rabbit hemoglobin) were injected to establish a picture of the column performance with more complex samples. The goal was to achieve an even distribution of the sample content throughout the separation to attain adequate fractionation. Gradient times were varied to find the best distribution versus time (see figure 8).

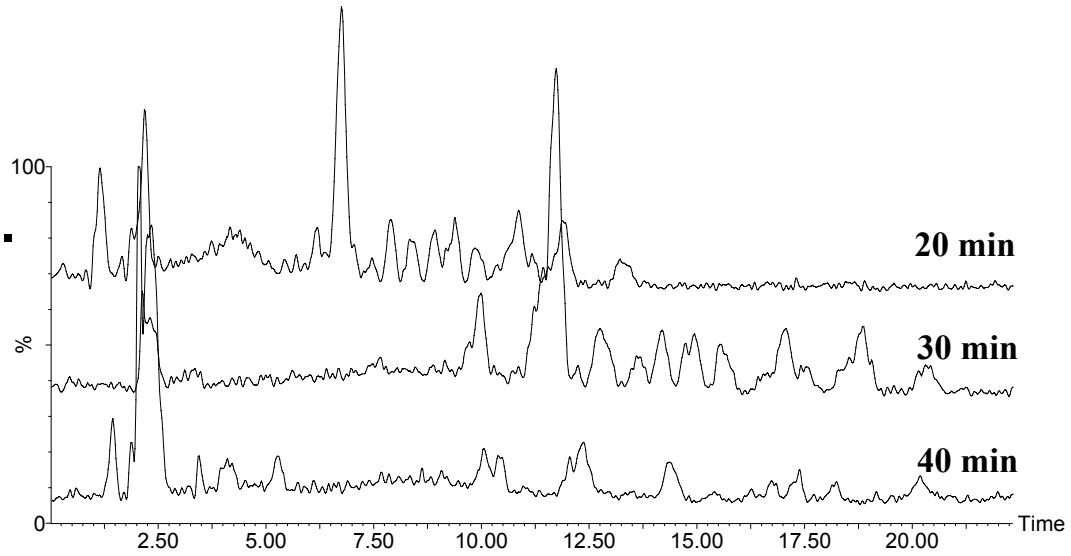

Figure 8. Overlay BPI chromatograms of trypsinated apo A on a ZIC-HILIC column (0.32 x 125 mm). Sample introduction using column switching with Kromasil C_{18} operculum (1 x 5 mm). Loading MP was 95/5 (ACN/H_20, v/v) containing 0.1 % f.a. Loop size was 5 µL (upper chromatogram: 10 µL). Elution with linear gradient from 85 to 45 % ACN containing 10 mM NH_4Ac delivered at 5 µL/min. Gradient time from top to bottom; 20, 30 and 40 minutes. Detection was performed with ESI-TOF-MS in positive mode. Acquisition was done from *m/z* 200 to 1500.

A gradient time of 30 minutes proved to be the best compromise between resolution and analysis time. The retention of polar compounds on the ZIC-HILIC column was relatively high. In an effort to decrease retention and improve peak shape, elution at elevated column temperatures was assessed (see Figure 9). This did lower the retention slightly, but also the peak height did. The peak broadened as the temperature increased. Because no preheating of the MP was done, a temperature gradient in the cross section of the column (f.s. I.D. 0.32 mm) may have been created at the column inlet. In such a case

analytes travels faster near the column wall thus explaining the peak broadening [44]. Therefore further experiments were performed at ambient temperatures.

The repeatability of the ZIC-HILIC column over a period of two subsequent days was tested using angiotensin II and bradykinin. The results are represented in Figure 10 and Table 4.

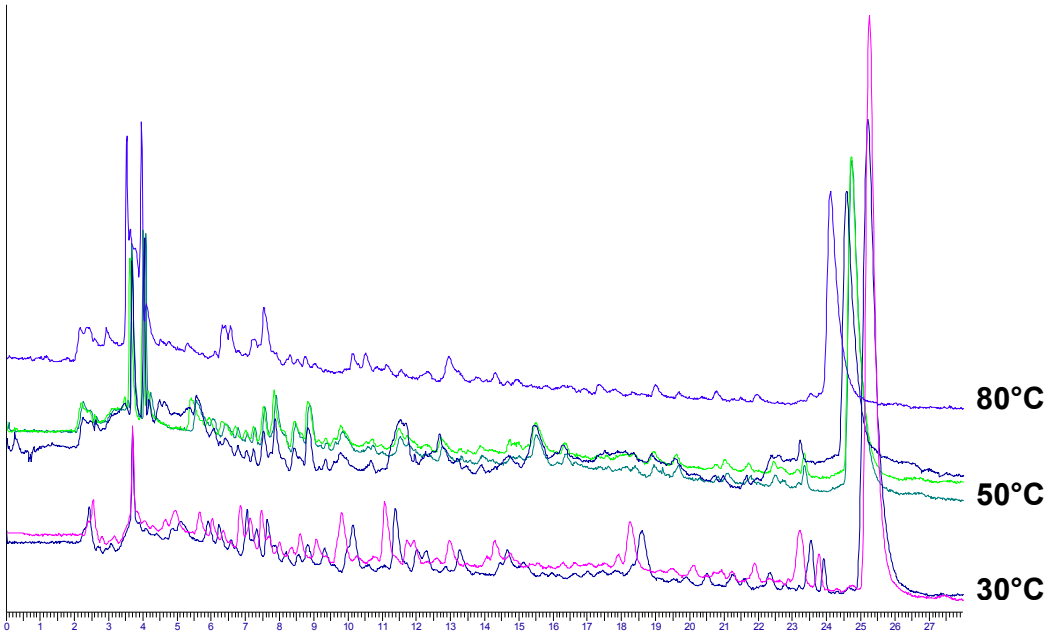

Figure 9. Chromatogram of albumin trypsinate. Variations in retention times at equal temperatures are due to insufficient column conditioning. Gradient elution as described in figure 7 and 8. Injection loop with a volume of 500 nL was used. Gradient time was 30 minutes. Detection was performed with UV at 220 nm.

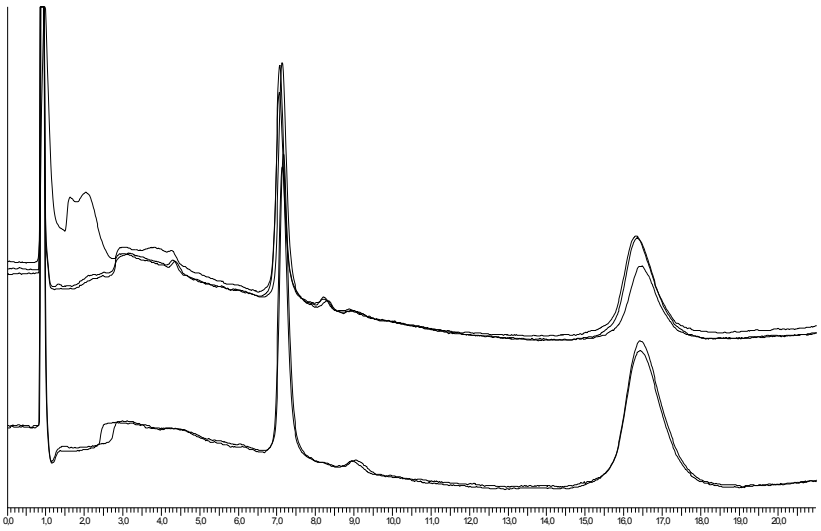

Figure 10. 0.83 µL of a 1 mg/mL solution of angiotensin II and bradykinin separated, in that order, on a ZIC-HILIC (5 µm, 200 Å) column (0.32 mm I.D. x 23 cm) using a decreasing organic gradient (ACN/water) containing 10 mM ammonium acetate in 20 minutes. Mobile phase flow at 10 µL/min. Two (lower traces) and three (upper traces) replicates were performed on two subsequent days.

Table 4. Retention times and areas of angiotensin II and bradykinin from the chromatograms in Figure 10.

	Angiotensin II		Bradykinin	
Run	R_t [min]	Area	R_t [min]	Area
1	7.1	22471	16.3	32074
2	7.0	22849	16.3	29086
3	7.0	20651	16.4	21867
4	7.1	30752	16.4	53821
5	7.1	29457	16.4	52544
Average	7.1	25236	16.4	37878
STD	0.055	4544	0.055	14462
RSD (%)	0.01	0.18	0.00	0.38

2. Second dimension

UV and TOF-MS detection was used in examination of the column for the second dimension. RPC was decided to be employed in the second dimension since it provides good orthogonality with HILIC, as explained in section 1.2. Initially, a nano-RP column (100 μm I.D.) was supposed to be employed for the second dimension, but the TOF-MS used in this study was not fully optimized to function with nanoflow (>500 nL/min). An issue that could arise was the lower capacity of such a narrow column. Despite their lower capacity, a nanocolumn was tested. It was a monolithic silica based endcapped C_{18} column (100 μm I.D.), providing low back pressure at elevated flow rates compared with packed columns with the same internal diameter. The flow through the monolith was set to 1 μL/min. The other columns examined were PLRP-S (0.3 mm I.D.) and Jupiter Proteo (0.3 mm I.D.). The PLRP-S is a PS-DVB polymer based column. The Jupiter Proteo is a silica based endcapped C_{12} column. Performance assessment was done by introducing tryptic apo A and comparing four closely eluting peaks viewed in an extracted ion chromatogram (EIC). Elution was done using a linear gradient running from 5 to 50 % ACN containing 0.1 % formic acid. In preliminary experiments TFA was used an additive, but TFA bind so strongly to the analytes that ion-suppression is likely to occur in the MS [45]. Instead formic acid was used as an additive. The three RP columns were examined by comparison of four closely eluting in a tryptic apo A solution (see Figure 11).

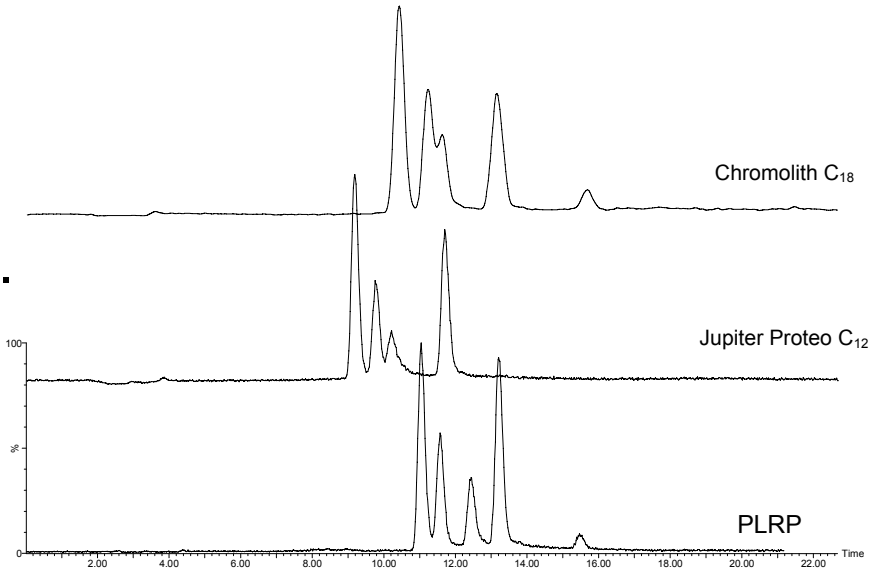

Figure 11. EIC chromatograms of four closely eluting peaks in a tryptic apo A eluted through three different columns. Elution with a linear gradient from 5 to 50 % ACN in 15 minutes, containing 0.1 % f.a.

The gradient described in section 2.4.2, was based on an earlier in-house study [15]. Since most peptides elute in the interval 15 – 30 % ACN in a RP system the gradient slope in this area is the lowest with a rise at 1 % ACN/min.

3. Sample introduction

A sample loop with the proper volume had to be used. It was desirable to install a sample loop with a volume as large as possible, but using a too large loop could result in precolumn breakthrough and fractionation column overloading. For a loop to be homogeneously filled it should be overfilled at least three times its volume. Considering the rather limited amount of sample solution available, using a loop size to fulfill the latter statement would require a loop with a volume of 100 µL. This would compromise sensitivity, therefore a loop volume of 335 µL was employed. To introduce samples of

such a volume, column switching was utilized with the use of a Kromasil C_{18} SPE column (1 x 5 mm). For sample loading an ACN/H_2O (5/95, v/v) solution containing 0.1 % f.a. was used, providing on-line sample desalting, enrichment and cleanup since many polar compounds were not retained on the C_{18} SPE. The combination with a HILIC column provides excellent sample refocusing prior to gradient elution in the first dimension. No other material was tested as Kromasil already possesses the qualities of providing low backpressure, permission of high loading rates and loadability. No breakthrough testing was performed as this has been performed in a similar previous study [11].

b. Application

The full function of the instrumentation shown in figure 5 was tested using the conditions as described in section 2.4. In preliminary experiments the HILIC gradient was run from 85 to 45 % B in 20 minutes. The decision to increase the gradient time to 30 minutes was taken after the functionality tests. At first the XXX solution was used to examine the resolution of the method. Selected fractions from the XXX solutions were collected at different fraction lengths (30 sec, 1.5 min, 3 min) (see Figure 12). Splitting one or more compounds of low relative abundance into two fractions may result in the peak(s) to be under their respective detection limits, with respect to the second dimension analysis. This is especially an important note regarding broadened peaks. The front peak from the first dimension contained compounds of such hydrophobic character that virtually no retention was reached in the first dimension. This peak had a width of ~1.5 minutes

which made up the first fraction. In an effort to split it up capturing was done every 30 seconds, splitting the peak into three fractions (see Figure 12).

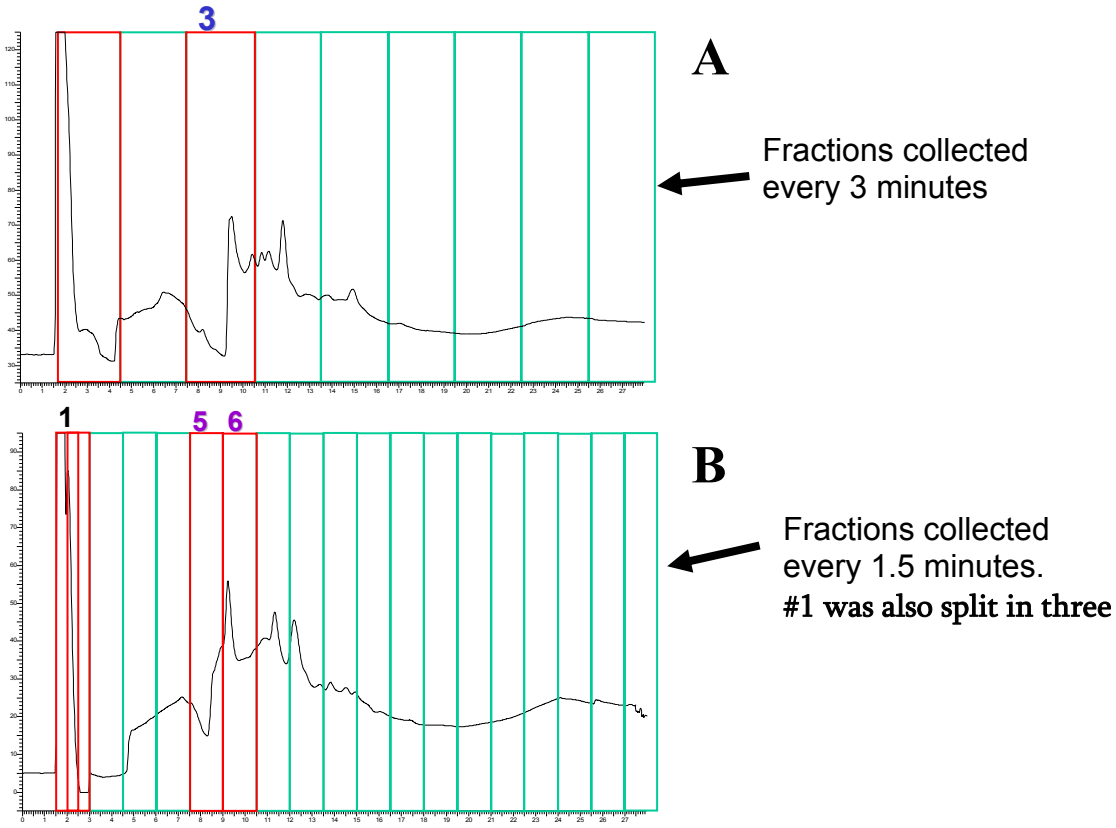

Figure 12. Two individual separations of XXX (trypsinated; bovine albumin, human transferrin, rabbit hemoglobin) in the first dimension. One (**A**) was fractionated every 3 minutes and the other (**B**) every 1.5 minutes. Peak #1 was split into fractions á 30 seconds. Chromatographic conditions as explained in section 2.4.2 and 2.4.3. Sample loop was 50 µL. UV detection done at 220 nm.

The second dimension RP separation of the fractions marked in red are shown in Figure 13. The observed time differences between the two chromatograms in figure 12 originate from too short column conditioning. At this stage it is of no particular importance as the objective was to examine the separation power of the method. Figure 14 shows the effect of splitting a peak (**A**: fraction 3) into two parts (**B**: fractions 5 and 6).

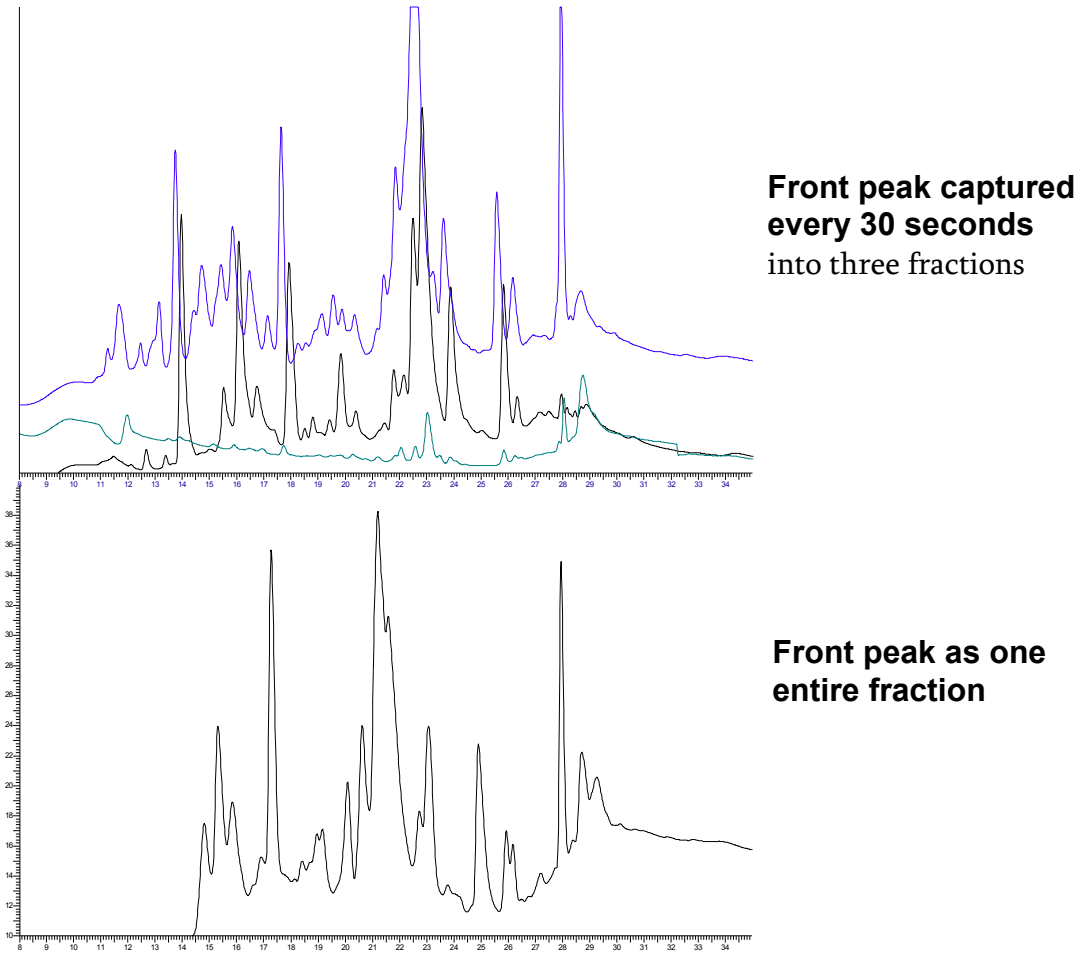

Figure 13. Fraction 1 from figure 12 chromatographed in the second dimension. Upper: Fraction 1 after splitting in three. Lower: Fraction 1 without any splitting. Chromatographic conditions as described in section 2.4.2. and 2.4.3. UV detection done at 220 nm.

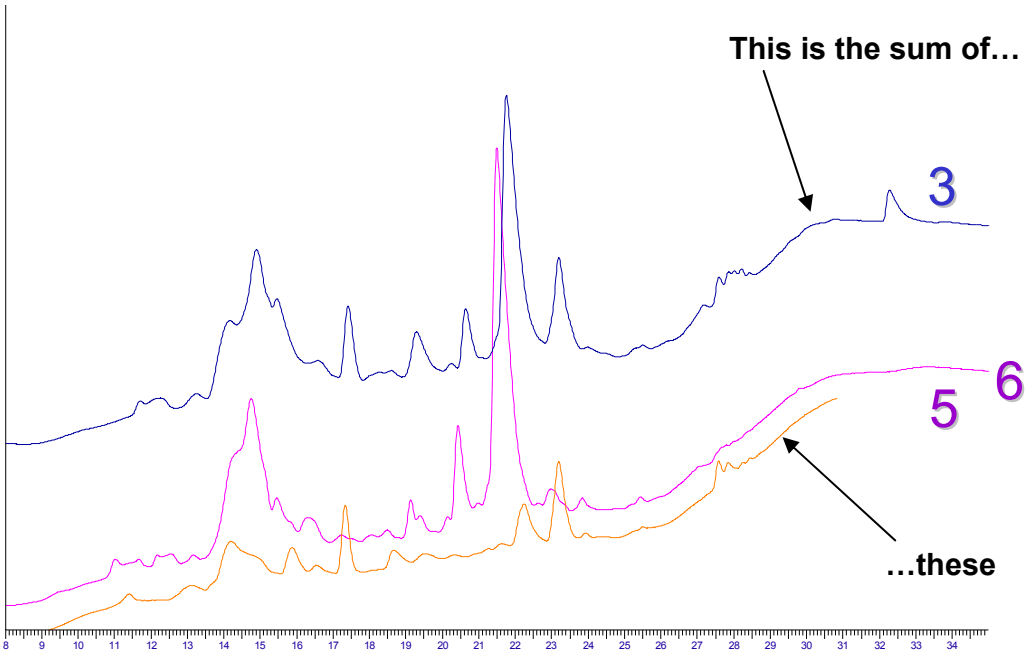

Figure 14. Second dimension of the fractions corresponding with the numbering in figure 12. Chromatographic conditions same as in figure 11.UV detection at 220 nm.

The chromatographed XXX divided into 1.5 minute fractions were combined in a 2D-plot to graphically illustrate the sample content (see Figure 15). The second dimension in the plot starts at 10 minutes because the gradient delay is ~10 minutes.

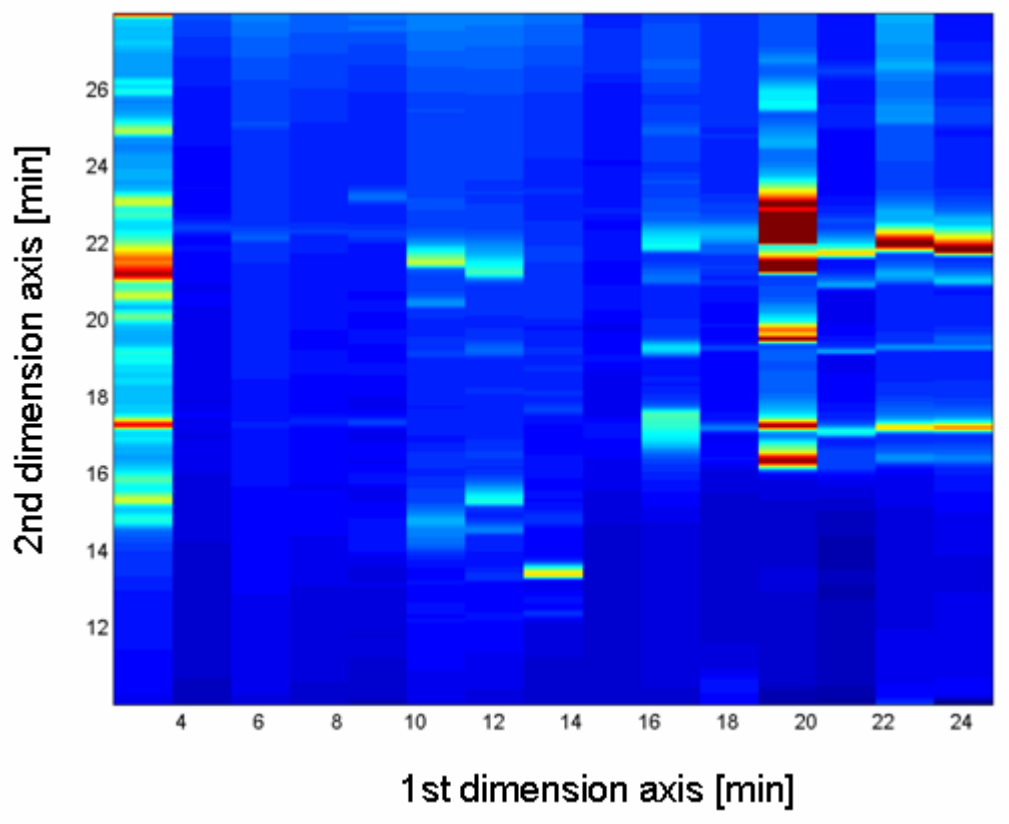

Figure 15. 2D-plot (HILIC-RP) of a XXX sample fractionated at 1.5 minute intervals. The x- and y-axis represent the retention time in the first and second dimension, respectively. The second dimension is plotted from 10 minutes due to the 10 minute gradient delay. Detection performed with UV at 220 nm. (Figure prepared by Gabriel Vivo Truyols, University of Amsterdam)

Figure 15 illustrates the distribution of the compounds in the sample. The first HILIC fraction contains the most hydrophobic compounds, not retained in the first dimension. The common bands that appear in more than one fraction may or may not be the same compound or group of compounds. By utilizing a MS detector in the second dimension the content in the bands can be determined. A rat brain extract was subjected to comprehensive analysis utilizing ESI-TOF-MS was used for detection in the second dimension, but was not completed due to instrumental difficulties (results not shown). Tryptic apo A was separated on both HILIC and RP columns to examine the

orthogonality. A number of representative *m/z* values from a tryptic apo A were chosen and ion masses were calculated to confirm that no *m/z* values originated the same ion. The *m/z* values were plotted in 2D-plot (Figure 16) which reveals that the orthogonality of the method is satisfactory. The numbers in figure 16 represent the mass of the respective compounds. The charge of the compound is shown in figure 17.

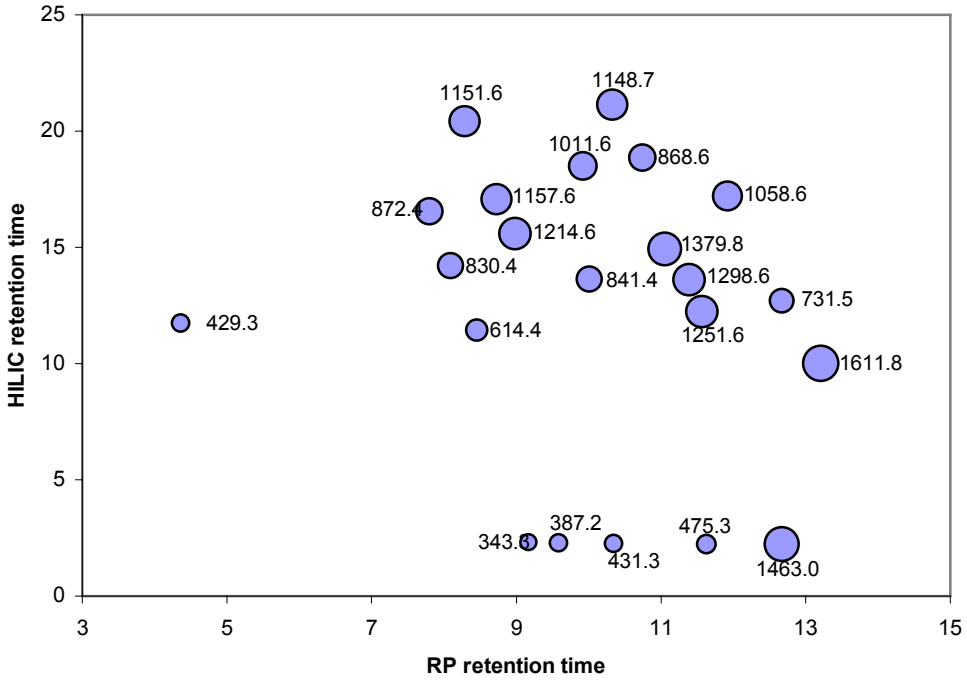

Figure 16. 2D-plot of selected masses from a tryptic apo A solution eluted through a ZIC-HILIC column and a PLRP column. The bubble sizes are relative to the respective ion masses calculated from table 9 (Appendix 6.1).Sample introduced by column switching using 5 µL loop and ACN/H_20 (5/95, v/v) containing 0.1 % f.a. as loading MP. Chromatographic conditions, 1st dimension; as in figure 8 using 30 minute gradient, 2nd dimension; Gradient ran from 5 to 50 % ACN in 15 minutes. Detection for both dimensions was ESI-TOF-MS.

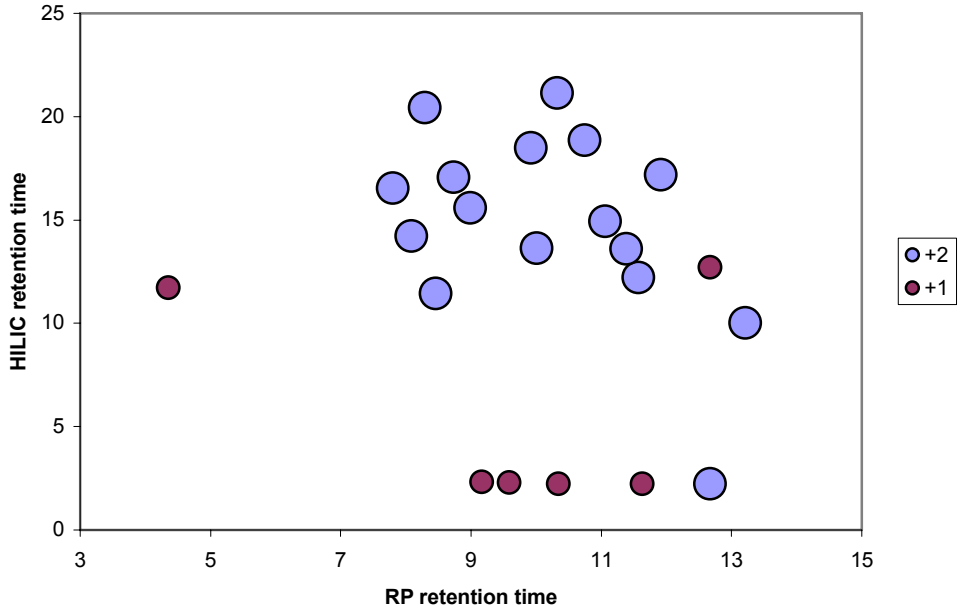

Figure 17. The distribution of charge. Small dots: charge +1, big dots: charge +2. Same conditions as in figure 16.

3.2 TARGET COMPOUND ANALYSIS

a. Method development

1. First and second dimension

When performing a quantification analysis either an internal or external standard is normally used. Standards are necessary to establish a calibration curve from which the amount of target analyte(s) may be calculated. External standards are used when the

chromatographic conditions are constant when performing sample analysis and that the injected volumes are precise. A calibration curve is established by plotting the standard area, of the target analyte(s), as a function of amount or concentration. An internal standard (I.S.) is a known amount of a compound that is added to the sample matrix prior to analysis. The area ratios between the analyte and I.S. in the sample is compared to the ratios from a calibration curve established with known amount of the target analyte and I.S. Using an I.S. will correct for sample loss, changes in injected volumes and other variations in the chromatographic conditions. In this study a combination of both techniques was used, further referred to as post-column standard (PC.STD.). Using this method, any variations in the response in the MS was compensated for as both the analyte and PC.STD. were introduced to the MS in a narrow time interval. The PC.STD. was introduced shortly after the standard was detected in the MS. The linearity of the MS was examined along with the establishment of the calibration curve. The calibration standard with the highest concentration level (5 μg/mL) appeared to exceed the linear range of the MS and was therefore removed from the calibration curve (see Figure 18). Raw data for the calibration curve is presented in Appendix 6.2

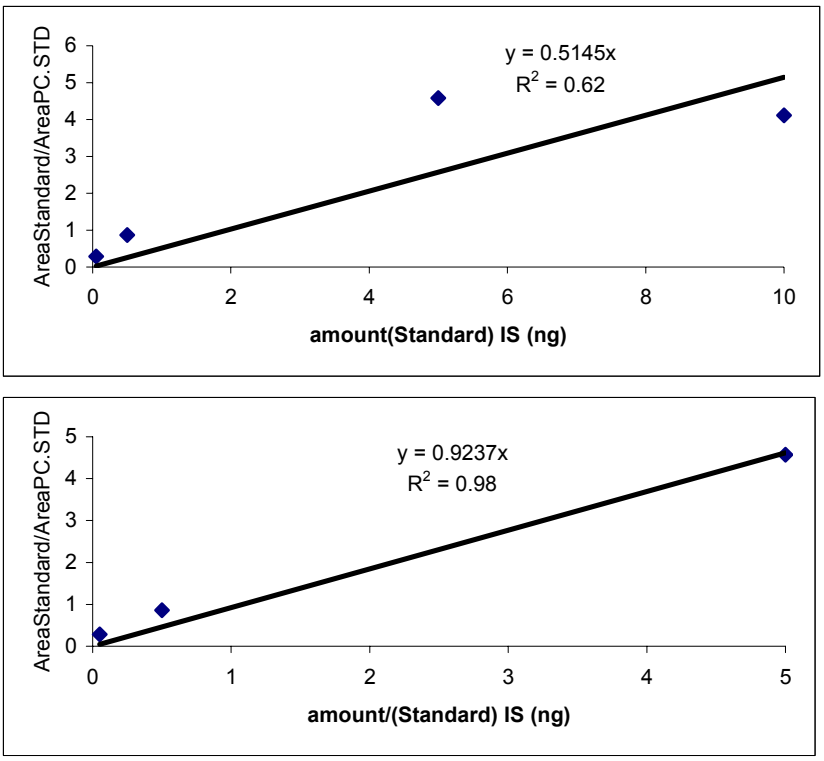

Figure 18. Plots of $Area_{Standard}/Area_{PC.STD.}$ as a function of ng standard injected. Upper: All four standard solutions included. Lower: The solution with the highest concentration excluded

The calibrations curve is expressed as $Area_{Standard}/Area_{PC.STD}$ as a function of absolute mass of standard. To ensure that the method using PC.STD. was stable, bradykinin (2 µL 5 µg/mL) was injected onto the PLRP column along with the PC.STD. (see table 5). The results in table 5 show that the peak areas of the standards fluctuate. Comparing the ratio between the standards reveals that any variations in response in the MS are compensated and that the technique is robust.

Table 5. Four injections of 2 µL 5 µg/mL bradykinin on the PLRP column, and 500 nL 5 µg/mL with valve 4 (V4) each time. Gradient ran from 5 to 50 % ACN in 15 minutes. Detection was done using ESI-TOF-MS.

Replicate	**Standard (STD)**		**Post-column standard (PC.STD)**		**Area ratio (STD/PC.STD)**
	Area	**R_t [min]**	**Area**	**R_t [min]**	
1	1985	12.49	1397	14.91	1.4209
2	2056	12.51	1406	15.15	1.4623
3	2700	12.42	1764	15.21	1.5306
4	2828	12.42	1977	14.95	1.4305
AVG	2392	12.46	1636	15.06	1.4611
STD	433	0.05	284	0.15	0.0496
RSD (%)	**18**	**0.4**	**17**	**0.98**	**3.4**

2. Quantification

The length of the f.s. tubing to the SPE column on the carousels varied somewhat for each SPE. Therefore only one of the SPE columns was used to quantify the analyte. The analysis of the target analyte was performed by heart-cutting a fractions from the first dimension. Prior to analysis of the microdialysates, bradykinin was injected onto the HILIC column to confirm the retention and determine in which time internal to perform the collection of the heart-cut fraction (see figure 19). The heart-cut was done from 19 to 24.5 minutes. To examine that the SPE on the carousel could handle large loading volumes bradykinin was heart-cut performed using a 4.5 and 10 minute capturing time (table 6).

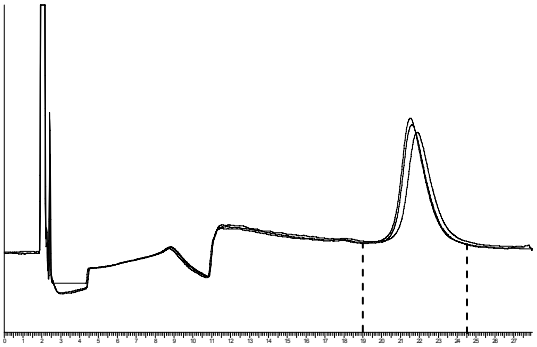

Figure 19. Chromatography of 2 µg bradykinin in the first dimension using the final instrumental arrangement and gradient program (n=3), as described in section 2.4.2. Heart-cutting from 19 to 24.5 minutes was performed.

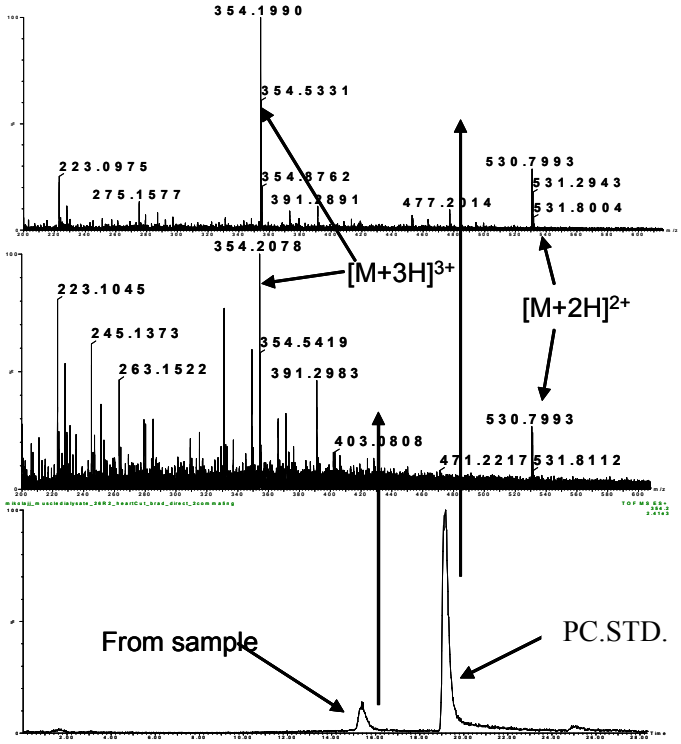

Figure 20. Second dimension separation of one of the heart-cut fractions from a microdialysate. EIC and mass spectra of bradykinin mass spectrum originating from the sample and standard are presented. Chromatographic conditions as described in section 2.4.3.

The +3 ion $[M+3H]^{3+}$ with *m/z* 354.2 was uses for quantification of bradykinin as it had a S/N three times the +2 ion $[M+2H]^{2+}$ with *m/z* 530.8 (see Figure 20).

Table 6. 2 µL 1 mg/mL bradykinin eluted through the first dimension and captured over different time interval and then eluted through the second dimension. Gradient (85 to 45 % ACN containing 10 mM NH_4Ac) time in the first dimension was 30 minutes. Gradient (linear 5 to 50 % ACN containing 0.1 % f.a.) in second dimension was 15 minutes. Detection performed with UV at 220 nm.

	Area 1st dim	Area 2nd dim	Recovery	n
RUN #1 (4.5 min)	3296332	3158316	0.9581	1
RUN #2 (10 min)	3082878	3030081	0.9829	1
Ratio (#2 / #1)	0.9352	0.9594		

b. Application

The microdialysates were subjected to analysis as described in section 2.4. The samples were centrifuged prior to analysis. Filtering was avoided because of potential adsorption of sample to the filter medium. The analyte mass and concentration in the dialysates calculated from the acquired data is presented in table 7 (Appendix 6.3). Two of the samples (R3 and 26R3) had a volume of ~300 µL. Therefore the injected volume R3 and 26R3 was 160 µL and 230 µL, respectively.

Table 7. Absolute masses and concentration levels of bradykinin in the microdialysis samples. 300 µL of both R3 and 26R3 was available therefore lower volumes were injected.

Sample	Analyte mass [ng]	Analyte conc. in sample [ng/mL]	
CL1	**NA**	NA	
CL2	**0.5730**	0.1919	
CL3	**2.8682**	0.9609	
R1	**3.3651**	1.1273	
R2	**0.8674**	0.2906	
R3	**0.6182**	0.0989	← injected 160 µL
L1	**0.7752**	0.2597	
L2	**1.5501**	0.5193	
L3	**0.4260**	0.1427	
26L1	**0.2278**	0.0763	
26L2	**0.4499**	0.1507	
26L3	**0.2609**	0.0874	
26R1	**0.2040**	0.0683	
26R2	**0.1425**	0.0478	
26R3	**0.0752**	0.0173	← injected 230 µL

c. Method evaluation

The limit of detection (LOD) was defined as the concentration providing a signal which was three times the noise level (S/N = 3) (see Figure 21), and the limit of quantification (LOQ) providing S/N = 10. 50 pg (2µL, 25 ng/mL) bradykinin was introduced onto the PLRP column and detected in the MS yielding a response with approximately S/N = 3. The mass limit of detection (mLOD) was estimated to be 50 pg. With a 335 µL sample loop the concentration limit of detection (cLOD) of bradykinin in microdialysates is 0.15 ng/mL. Values for mLOD, cLOD, mLOQ and cLOQ are presented in table 8.

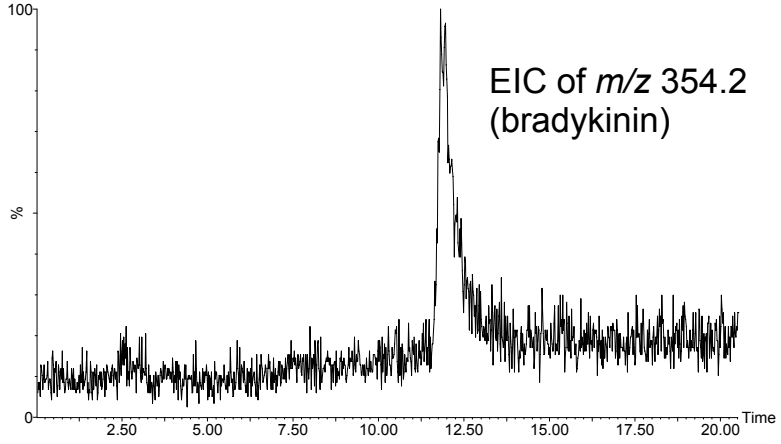

Figure 21. EIC of bradykinin at the approximate limit of detection (0.15 ng analyte/mL microdialysates), using a 335 µL loop.

Table 8. Limit of detection and limit of quantification.
Injection volume 335 µL.

Limits of detection		Value
mLOD	*[pg]*	50
cLOD	*[ng/mL]*	0.15
mLOQ	*[pg]*	167
cLOQ	*[ng/mL]*	0.50

Seven of the samples have calculated concentration values which fall below the cLOD. This is most probably a consequence of the fact that the calibration curve was established using only the second dimension, thus the calibration curve is valid only for the second dimension. The quantification method used is dependent on correct loop volumes. Since the 2 µL loop used for calibration represented the total sample volume of 335 µL, a small deviation from 2 µL gives an even greater deviation with respect to the 335 µL sample. Because all loop volumes have uncertainties, a greater loop than 2 µL would reduce the error. A more correct method would be to subject the calibration standards to both dimensions before recording the areas. This was not carried out due to time limitations. Although the applied quantification method yields inaccurate results, the relative values will be correct.

4. CONCLUSION

The present study has shown that the developed online μSPE-2D-capLC method coupled to ESI-TOF-MS detection provides high resolving power and potential to identity great number of peptides in a dialysates solution without extensive sample preparation. The flexibility to vary the fractionation according to the need is of great value in 2D comprehensive analysis. The employment of multiple online SPE columns for collecting fractions provides simplicity and flexibility as the entire sample can be captured in one operation and transported to other locations.

The developed quantification method was not suitable for accurate determination of target analytes due to the statements in section 3.2c, although values in relation to each other provided will be true. Future work with this method should include a more correct quantification technique and further optimization. A great variety of sample material can be analyzed using this method, and will hopefully be executed in future projects.

5. REFERENCES

1. http://faculty.washington.edu/chudler/chnt1.html#type *(05.03.2007)*.

2. Andreas Dendorfer, Sebastian Wolfrum, and Peter Dominiak, *Pharmacology and Cardiovascular Implications of the Kinin-Kallikrein System.* Jpn. J. Pharmacol., 1999. **79**: p. 403-426.

3. Edyta Madry, Teresa Torlinska, Pawel Mackowiak, Magdalena Perz, Tomasz Hryniewicki, and D. Rutowska, *Influence of bradykinin upon insulin - insulin receptor interaction in muscular tissue if a rat in vivo, in normo- and hypothermia.* Med. Sci. Monit., 1998. **4**: p. 408-412.

4. Fernando Boix, Cecilie Røe, Laila Rosenborg, and S. Knardahl, *Kinin peptides in human trapezius muscle during sustained isometric contraction and their relation to pain.* J. Appl. Physiol., 2005. **98**: p. 534-540.

5. Charles Balis Jr., Albert Adam, Denis Massicotte, and F. Peronnet, *Increase in blood bradykinin concetration after eccentric weight-training exercise in men.* J. Appl. Physiol., 1999. **87**: p. 1197-1201.

6. Fernando Boix, Cecilie Røe, Laila Rosenborg, and S. Knardahl, *Contraction-related factors affect the concentration of a kallidin-like peptide in rat muscle tissue.* J. Physiol., 2002. **554**: p. 127-136.

7. Adam C. Scotta, Roland Wensela, Constantinos H. Davosa, Panagiota Georgiadoua, L. Ceri Daviesa, Andrew J.S. Coatsb, Darrel P. Francisa, and Massimo F. Piepolia, *Putative contribution of prostaglandin and bradykinin to muscle reflex hyperactivity in patients on Ace-inhibitor therapy for chronic heart failure.* Eur. Heart J., 2004. **25**: p. 1806-1813.

8. Massimo Cugno, Piergiuseppe Agostoni, Hans R. Brunner, Marco Gardinali, Angelo Agostoni, and Jürg Nussberger, *Plasma bradykinin levels in human chronic congestive heart failure.* Clin. Sci., 2000. **99**: p. 461-466.
9. http://www.usm.maine.edu/~newton/Chy251_253/Lectures/BiopolymersIII/BiopolymersIII.html. *02.03.2007*

10. Oleg Chertov, Arya Biragyn, Larry W. Kwak, John T. Simpson, Tatiana Boronina, Van M. Hoang, DaRue A. Prieto, Thomas P. Conrads, Timothy D. Veenstra, and R.J. Fisher, *Organic solvent extraction of proteins and peptides from serum as an effective sample preparation for detection and identification of biomarkers by mass spectrometry.* Proteomics, 2004. **4**: p. 1195-1203.

11. S. R. Wilson, Fernando Broix, Anders Hols, Pål Molander, Elsa Lundanes, and T. Greibrokk, *Determination of bradykinin and arg-bradykinin in rat muscle tissue by microdialysis and capillary column-switching liquid chromatography with mass spectrometric detection.* J. Sep. Sci., 2005. **28**: p. 1751-1758.

12. Pedro R. Cutillas, Anthony G. W. Nordens, Rainer Cramer, Alma L. Burlingame, and R.J. Unwine, *Detection and analysis of urinary peptides by on-line liquid chromatography and mass spectrometry: application to patients with renal Fanconi syndrome.* Clin. Sci., 2003. **104**: p. 483-490.

13. Yan Wang, Bradley B. Schneider, Thomas R. Covey, and Janusz Pawliszyn, *High-Performance SPMEAP MALDI System for High-Throughput Sampling and Determination of Peptides.* Anal. Chem., 2005. **77**: p. 8095-8101.

14. Crimmins, D.L., *Strong cation-exchange high-performance liquid chromatography as a versatile tool for the charaterization and purification of peptides.* Anal. Chim. Acta, 1997. **352**: p. 21-30.

15. Milaim Pepaj, Anders Holm, Burkhard Fleckenstein, Elsa Lundanes, and Tyge Greibrokk, *Fractionation and separation of human salivary proteins by pH-gradient ion exchange and reversed phase chromatography coupled to mass spectrometry.* J. Sep. Sci., 2006. **29**: p. 519-529.

16. E.R. Vickers, C. Goebel, L.E. Mather, L. Mackay, and R.J. Wells, *High-performance liquid chromatographic determination of bradykinin in saliva: a critical review and a method.* J. Chromatogr., 2001. **755**: p. 101-110.

17. Massimo Cugno, Jürg Nussberger, Paolo Biglioli, Francesco Alamanni, Raffaella Coppola, and Angelo Agostini, *Increase of Bradykinin in Plasma of Patients Undergoing Cardiopulmonary Bypass The Importance of Lung Exclusion.* Chest, 2001. **120**: p. 1776-1782.

18. Martin H. Maurer, Christian Berger, Margit Wolf, Carsten D. Fütterer, Robert E. Feldmann Jr., Stefan Schwab, and W. Kuschinsky, *The proteome of human brain microdialysate.* Proteome Sci., 2003. **1**.

19. William E. Haskins, Ziqiang Wang, Christopher J. Watson, Rebecca R. Rostand, Steven R. Witowski, David H. Powell, and Robert T. Kennedy, *Capillary LC-MS2 at the Attomole Level for Monitoring and Discovering Endogenous Peptides in Microdialysis Samples Collected in Vivo.* Anal. Chem., 2001. **73**: p. 5005-5014.

20. Paul J. Boersem, A. Nullin Divecha, lbert J. R. Heck, and Shabaz Mohammed, *Evaluation and Optimization of ZIC-HILIC-RP as an Alternative MudPIT Strategy.* J. Proteome Res., 2006. **6**: p. 937-946.

21. Dwight R. Stoll and Peter W. Carr, *Fast, Comprehensive Two-Dimensional HPLC Separation of Tryptic Peptides Based on High-Temperature HPLC.* J. Am. Chem. Soc., 2005. **127**: p. 5034-5035.

22. Gregory J. Opiteck and James W. Jorgenson, *Two-Dimensional SECRPLC Coupled to Mass Spectrometry for the Analysis of Peptides.* Anal. Chem., 1997. **69**: p. 2283-2291.

23. Michael T. Davisa, Jill Beierlea, Edward T. Buresa, Michael D. McGinleya, Jessica Mort, John H. Robinson, Chris S. Spahr, Wen Yu, Roland Luethy, and Scott D. Patterson, *Automated LC-LC-MS-MS platform using binary ion-exchange and gradient reversed-phase chromatography for improved proteomic analyses.* J. Chromatogr., 2001. **752**: p. 281-291.

24. Josip Blonder, Maria C. Rodriguez-Galan, King C. Chan, David A. Lucas, T. Li-Rong Yu, homas P. Conrads, Haleem J. Issaq, Howard A. Young, and Timothy D. Veenstra, *Analysis of Murine Natural Killer Cell Microsomal Proteins Using Two-Dimensional Liquid Chromatography Coupled to Tandem Electrospray Ionization Mass Spectrometry.* J. Proteome Research, 2004. **3**: p. 862-870.

25. Goran Mitulovic, Christoph Stingl, Marek Smoluch, Remco Swart, Jean-Pierre Chervet, Ines Steinmacher, Christopher Gerner, and Karl Mechtler, *Automated, on-line two-dimensional nano liquid chromatography tandem mass spectrometry for rapid analysis of complex protein digests.* Proteomics, 2004. **4**: p. 2545-2557.

26. Edgar Nägele, Martin Vollmer, and Patric Hörth, *Two-dimensional nano-liquid chromatography–mass spectrometry system for applications in proteomics.* J. Chromatogr., 2003. **1009**: p. 197-205.

27. Hongji Liu, Jeffrey W. Finch, Joseph A. Luongo, Guo-Zhong Li, and John C. Gebler, *Development of an online two-dimensional nano-scale liquid chromatography/mass spectrometry method for improved chromatographic performance and hydrophobic peptide recovery.* J. Chromatogr, 2006. **1135**: p. 43-51.

28. Wen Jiang, Gerd Fischer, Yohannes Girmay, and Knut Irguma, *Zwitterionic stationary phase with covalently bonded phosphorylcholine type polymer grafts and its applicability to separation of peptides in the hydrophilic interaction liquid chromatography mode.* J. Chromatogr., 2006. **1127**: p. 82-91.

29. Alan R. Oyler, Barbara L. Armstrong, Jessica Y. Cha, Marilyn X. Zhou, Qing Yang, Robin I. Robinson, Richard Dunphy, and David J. Burinsky, *Hydrophilic interaction chromatography on amino-silica phases complements reversed-phase high-performance liquid chromatography and capillary electrophoresis for peptide analysis.* J. Chromatogr., 1996. **724**: p. 378-383.

30. Eva Hartmanna, Yuxin Chenb, Colin T. Mantc, Alois Jungbauera, and Robert S. Hodgesa, *Comparison of reversed-phase liquid chromatography and hydrophilic interactioncation-exchange chromatography for the separation of amphipathic α-helical peptides with L-and D-amino acid substitutions in the hydrophilic face.* J. Chromatogr., 2003. **1009**: p. 61-71.

31. Winnik, W.M., *Continuous pH/salt Gradient and Peptide Score for Strong Exchange Chromatography in 2D-Nano-LC/MS/MS Peptide Identification for Proteomics.* Anal. Chem., 2005. **77**: p. 4991-4998.

32. James A. Dowell, William Vander Heyden, and Lingjun Li, *Rat Neuropeptidomics by LC-MS/MS and MALDI-FTMS: Enhanced Dissection and Extraction Techniques Coupled with 2D RP-RP HPLC.* J. Protreome. Res., 2006. **5**: p. 3368-3375.

33. *http://www.sequant.com/sn/p_intro.php?id=7 (05.03.2007).*

34. Martin Gilar, Petra Olivova, Amy E. Daly, and J.C. Gebler, *Orthogonality of Separation in Two-Dimensional Liquid Chromatography.* Anal. Chem, 2005. **77**: p. 6426-6434.

35. Colin F. Poole, *The Essence of Chromatography.* 2003: p. 305.
36. *http://www.sigmaaldrich.com/Brands/Supelco_Home/TheReporter/Liquid_Chromatography/reporter_20_3_article1.html (09.03.2007).*

37. Y. Saito, K. Jinno, and T. Greibrokk, *Cappilary columns in liquid chromatography: between conventional and microchips.* J. Sep. Sci., 2004. **27**: p. 1379-1390.

38. M. Karas, U. Bahr, and T. Dülcks, *Nano-electrospray ionization mass spectrometry: addressing analytical problems beyond routine.* J. Anal. Chem., 1999. **366**: p. 669-676.

39. Kasper Solbu, *The thesis for the degree of Candidatus Scientiarum.* 2003.

40. K.M. Namara, R. Leardi, and A. Hoffmann, *Developments in 2D GC with Heartcutting*, in *LCGC Europe, vol 16, Dec*. 2003. p. 35-44.

41. P. Dugo, G. Dugo, and L. Mondello, *On-line Coupled LC–GC Theory and Application*, in *LCGC Europe, vol.16, Dec*. 2003. p. 14-23.

42. Espen Storbråten, *Thesis for the degree of Candidata Scientarium*, in *Department of Chemistry*. 2004, University of Oslo.

43. Albena Mihailova, Elsa Lundanes, and Tyge Greibrokk, *Determination and removal of impurities in 2-D LC-MS of peptides.* J. Sep. Sci., 2006. **29**: p. 576-581.
44. Dolan, J.W., *The Importance of Temperature*, in *LC.GC Europe*. 2002.

45. Claude R. Mallet, Ziling Lu, and Jeff R. Mazzeo, *A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts.* Rapid Commun. Mass Spectrum, 2004. **18**: p. 49-58.

6. APPENDIX

6.1 Comprehensive analysis

Table 9. Apo A separated individually on a PLRS (3µm, 300 Å) column (0.3 x 150 mm) and a ZIC-HILIC (5 µm, 200 Å). Sample introduced by column switching using 5 µL loop and loading MP ACN/H_20 (5/95, v/v) containing 0.1 % f.a. Chromatographic conditions, HILIC; as in figure 8 using 30 minute gradient, PLRP; Gradient ran from 5 to 50 % ACN in 15 minutes. Detection for both dimensions was ESI-TOF-MS. The marked *m/z* values originate from the compound not retained on the HILIC column.

ion mass	charge	*m/z*	RP R_t [min]	HILIC R_t [min]
1463	2	732.5	12.67	2.22
1611.8	2	806.9	13.21	10
1251.6	2	626.8	11.57	12.22
841.4	2	421.7	10.01	13.63
830.4	2	416.2	8.09	14.2
1379.8	2	690.9	11.06	14.93
1214.6	2	608.3	8.99	15.57
868.6	2	435.3	10.75	18.84
1151.6	2	576.8	8.29	20.42
429.3	1	430.3	4.36	11.72
731.5	1	732.5	12.67	12.69
1298.64	2	650.32	11.39	13.61
1157.6	2	579.8	8.73	17.06
NA	NA	386.87	8.79	2.13
1058.6	2	530.3	11.92	17.19
1011.628	2	506.814	9.92	18.49
1148.668	2	575.334	10.33	21.13
872.4	2	437.2	7.8	16.54
614.4	2	308.2	8.46	11.44
387.2	1	388.2	9.59	2.29
343.3	1	344.3	9.17	2.31
431.3	1	432.3	10.35	2.24
475.3	1	476.3	11.63	2.22

6.2 Calibration curve

Table 10. Areas of standards when eluted through PLRS-S using the stepped gradient used in the second dimension, as described in section 2.4.2. The values +2 and +3 correspond to bradykinin ions detected with *m/z* 530.8 and 354.2, respectively.

Standard		ion charge, STD [Area]		
STD.	PC.STD.	+2	+3	+2 and +3
10	2.5	428	2130	2558
5	2.5	644	3249	3893
0.5	2.5	81	538	619
0.05	2.5	33	182	215
		Ratio [STD/PC.STD]		
STD.	PC.STD.	+2	+3	+2 and +3
10	2.5	3.479675	4.111969	3.990640
5	2.5	4.025000	4.576056	4.474713
0.5	2.5	0.653226	0.863563	0.828648
0.05	2.5	0.235714	0.284375	0.275641

6.3 Target analysis

Table 11. Areas of standards and microdialysis samples and ratios, together with calculated bradykinin mass amount and dialysis solution concentration. Integration of the EIC peaks of the +3 ion with *m/z* 354.2 was done.

	Sample (S) [Area]	PC.STD. [Area]	Ratio [S/PC.STD]	Mass amount [ng]	Concentration [ng/mL]
CL1					
CL2	**337**	**624**	**0.54006**	**0.5730**	0.1919
CL3	**1942**	**733**	**2.64939**	**2.8682**	0.9609
R1	2583	831	**3.10830**	**3.3651**	1.1273
R2	939	1172	**0.80119**	**0.8674**	0.2906
R3	691	1210	**0.57107**	**0.6182**	**0.0989**
L1	**681**	**951**	**0.71609**	**0.7752**	0.2597
L2	**945**	**660**	**1.43182**	**1.5501**	0.5193
L3	**373**	**948**	**0.39346**	**0.4260**	**0.1427**
26L1	182	865	**0.21040**	**0.2278**	**0.0763**
26L2	293	705	**0.41560**	**0.4499**	0.1507
26L3	134	556	**0.24101**	**0.2609**	**0.0874**
26R1	**111**	**589**	**0.18846**	**0.2040**	**0.0683**
26R2	**111**	**843**	**0.13167**	**0.1425**	**0.0478**
26R3	**79**	**1137**	**0.06948**	**0.0752**	**0.0173**

6.4 Preliminary experiments

Chromatogram of a solution of different peptides injected onto the SCX PolyLC column (0.5 x 125 mm). The solution contained 0.1 mg/mL Angiotensin II, 0.05 mg/mL bradykinin, 0.1 mg/mL leucine enkepahline, 5 µg/mL oxytocin and 0.2 mg/mL TRH.

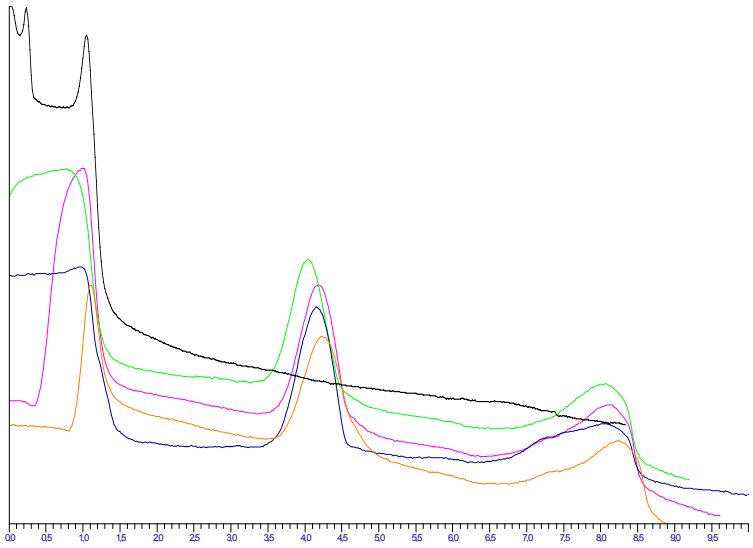

Figure 22. Uracil, leucine enkephaline and angiotensin II. Increasing injection volume: 5µL (ORANGE), 15µL (PINK), 30µL (BLUE), 100µL (GREEN) in water and 100µL (BLACK) in 90% Ringer acetate. A gradient (Solvent A 75% water, 25% ACN, 0.1% TFA at pH 2, Solvent B A containing 500mM NaCl) was run from 2% to 100% B in 10 minutes. Mobile phase delivered at a flow of 20 µL/min. UV detection at 254nm.

6.5 TOF-MS operating parameters

Table 12. Screen dumps from MassLynx tunepage showing the MS parameters.