Thesis for the degree
of Candidatus Scientiarum

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Determination of neuropeptides in rat brain tissue using 2-dimensional capillary liquid chromatography coupled to electrospray ion-trap mass spectrometry (2-D capLC ESI-IT-MSn)

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UNIVERSITY OF OSLO 09/2004

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PREFACE

This graduate study has been carried out at the Department of Chemistry at the University of Oslo, in the period of January 2003 to September 2004. All my work is carried out with supervision by Professor Tyge Greibrokk, Professor Elsa Lundanes and Ph.D. Anders Holm.

This thesis is mainly based on method development of an analytical system which will be used for future routine analyses of rat brain tissue samples. Some test runs have certainly been performed to check the systems functionality. No validation was included in this work because the compounds to be analysed were unknown and a matching matrix was unavailable. However, the repeatability of the system is proved by repeatability tests with angiotensin as test substance. Some of the method development was presented on a poster at the 16. Norwegian Chromatographic Symposium in Sandefjord, January 2004. The poster is to be found at page 48 in this thesis. All results obtained during this work are represented in the "Results and discussion"-section and will be submitted for publication as soon as possible.

I would like to thank my supervisors for all the inspiration and knowledge they have contributed with during my period as a Cand. Scient.-student. I would also like to thank the Instrumental- and electronics workshops at the department of Chemistry for good help with my technical problems. A special thank directed to Hege Lynne, senior research technician, for her great kindness and helpfulness by supplying lab equipment when needed. Also a great thank directed to my study mates for all the interesting discussions in the lab regarding chemistry and other topics.

Finally I would like to thank my family for all the encouragement they have given me through my ups and downs.

Oslo, Norway, September 2004

Espen Storbråten

"The heights by great men reached and kept,
were not attained by sudden flight, but they,
while their companions slept,
were toiling upward in the night."

Henry Wadsworth Longfellow

ABSTRACT

To monitor chemical changes in brain occurring during stress, several rats were put to death in both stressed and non-stressed situations. According to age it was desirable to see which effects stress constitutes in a rat brain. By using rats as experimental animals, the difference in the level of neurotransmitters was studied and compared in a stressed and non-stressed situation for different aged rats.

Sample preparation, including solid phase extraction (SPE) on a CBA (carboxy propyl phase) weak cation exchanger (WCX) and a reversed phase (RP) C18 column, was initially performed on rat brain parts to extract the compounds of interest. The extraction resulted in two fractions, A and B, with hydrophobic- and ionic compounds, respectively.

The samples were subsequently analysed using the developed 2D capillary LC method employing a strong cation exchanger (SCX) in the first dimension, and a reversed phase C18 column for focusing and separating the analytes in the second dimension. 50 µL of brain tissue extracts were initially loaded and focused on the SCX column and elution was performed by a buffered salt step gradient ranging from 20 – 500 mM ammonium formate. Subsequently, the fractions were trapped on reversed phase trap columns prior to back flushed elution onto the RP analytical column. The separation was conducted using a linear ACN/H_2O gradient where the mobile phase contained 10mM acetic acid. Detection was performed by an electrospray ionization ion trap mass spectrometer (ESI-IT-MS^n) in the positive mode.

The experiments proved abnormal activity in a rat brain during stress, and some compounds with bigger abundance differences in the stressed and non-stressed occasions were found and identified. The system evaluation revealed some fluctuations in the analyte area. With angiotensin II as a test substance, the area had an RSD (%) value of 16 % while the retention time had a corresponding value of 1 %.

ABBREVIATIONS AND DEFINITIONS

µLC	micro liquid chromatography
ACN	acetonitrile
BPC	base peak chromatogram
CBA	carboxy propyl phase
cLOD	concentration limit of detection
EIC	extracted ion chromatogram
ESI	electrospray ionisation
I.D.	inner diameter
IT	ion trap
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
mLOD	mass limit of detection
MS	mass spectrometry
MS/MS or MS^2	tandem mass spectrometry
O.D.	outer diameter
Proteomics	the qualitative and quantitative comparison of proteomes under different conditions or further unravel biological processes
RP	reversed phase
S/N	signal to noise ratio
SCX	strong cation exchanger
SPE	solid phase extraction
TFA	trifluoro acetic acid
TIC	total ion chromatogram
UV	ultraviolet

1. Introduction

In this project the analyses were performed on brain tissue extracts from two different main groups of rats, dependent on how they were killed. One group was stressed when exposed to quick suffocation, while the other group were put to sleep by slow suffocation. The suffocation procedure was controlled by high (stress) and low (non-stress) influx of CO_2 (g). Outer influence affects the psychic state of an individual and can give physical reactions as increased heartbeat. A group of compounds existing in brain, neurotransmitters, control all the bodily functions in a human being. Depending on the situation, for example during stress as in this case, the levels of certain neurotransmitters change.

Neurotransmitters constitute a group of compounds responsible for signal transmission between neurons or non-neuronal cells that they innervate. Neurotransmitters play a major role in controlling our state of mind and even slight changes in neurotransmitter systems can contribute to the development of neurological diseases or brain disorders. Neurotransmitters are generally divided into three groups; amino acids, biogenic amines and neuropeptides. In this case, neuropeptides from rat brain tissue were the compounds of interest. Due to high receptor specificity, neuropeptides are normally found in very small amounts, requiring highly sensitive methodology for their determination. Due to the complex nature of the samples and the high number of potentially neurotransmitting peptides, high separation power is required for their determination. Below it is shown how neurotransmitters are moving from the presynaptic neurons to the postsynaptic neurons where the chemical signal is transformed to an electrical signal (Fig.1). A more descriptive figure of a neuron's function is shown in appendix 4.3.

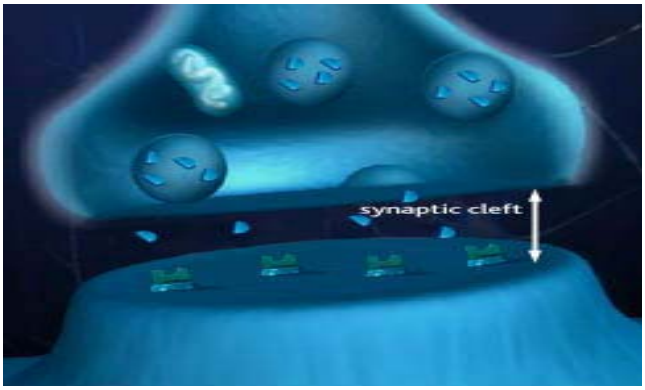

Fig. 1 Signal transmission between neurons in the synaptic cleft [1]

During recent years packed capillary liquid chromatography (LC) has become an important tool for analytical purposes, due to several advantages associated with the use of smaller dimension columns compared to the conventional size. One important advantage is the increased mass sensitivity due to less dilution of the chromatographic band in the column. If the same amount of analyte (absolute mass) is introduced into a capillary column and a conventional column, the capillary column will have a theoretical mass limit of detection (mLOD) approximately 200 times lower than the conventional one (Eq. 1) [2].

$$D = \frac{c_0}{c_{max}} = \frac{\varepsilon \pi r^2 (1+k)(2\pi L H)^{\frac{1}{2}}}{V_{inj}}$$

Eq. 1: Calculation of dilution in the column

The equation contains parameters where c_0 is sample concentration, c_{max} is final concentration at detector, ε is column porosity, r is radius of the column, k is retention factor, L is length of column, H is plate height and V_{inj} is volume injected. In biological analysis there are sometimes very small amounts of sample available and it is therefore convenient to use miniaturized techniques. Reduction in the consumption of mobile- and stationary phases and the possibility to use temperature programming are also benefits of importance. The former are of economical and environmental matter, while the latter can be exploited in retention control because of the low thermal mass of the capillary columns. Furthermore, the low flow rates (down to 1 µL/min) used in capillary LC makes it easier to use solvent evaporation based detectors as the mass spectrometer (MS) [3], the Fourier-transform-infrared-spectrophotometer (FT-IR) [4] and the evaporative light scattering detector (ELSD) [5].

To maintain the same efficiency in capillary LC as in conventional HPLC, the sample volume injected must be smaller and injectors with small internal loops are used. The increased mass sensitivity could only be exploited when injecting the same sample volumes as in conventional LC. When greater sample volumes are injected, techniques with external loops and sample enrichment are required, such as on-column focusing large volume injection or column switching systems with on-line solid phase extraction [6-8]. In a switching system the sample can be introduced in a non-eluting solvent, and solutes in large volumes can be concentrated on the trap column followed by elution through the analytical column without

any loss in resolution. These techniques have been used for quantification of peptides and proteins at low concentration in biological and environmental samples [9-10].

In recent years two dimensional (2-D) chromatographic systems have been subject to increased use in connection with biochemical analyses [11-14]. Combining two techniques with different separation principles makes it possible to analyse more complex samples in biochemical analyses. Because the separation is performed in two independent dimensions, the peak capacity is larger and can be estimated by multiplying the two dimensions peak capacities [15-16]. There are two different main techniques in 2-D LC, comprehensive chromatography and heart- (/front-/end-) cutting. In the comprehensive approach the entire sample is subjected to two different separations, each with different separation characteristics. By contrast, in heart cutting only a fraction from the first retention axis is transferred for separation on the second dimension axis [15-16].

Peptide and protein samples often consists of highly complex mixtures where high-resolution techniques are essential to get accurate information about their abundance and also, in some cases, information about their configuration. In some cases two dimensional systems are required to get sufficient resolution, but it has also been performed successful analyses by the use of one dimensional systems [17-18]. 2-D polyacrylamide gel electrophoresis (PAGE) has been the main separation technique for separation of proteins [19-23], but the last few years 2-D LC has become a popular supplement to this technique. Anyway, due to the peptides' low molecular weight they cannot be analysed by the use of PAGE [24]; therefore 2-D LC becomes a natural choice for peptide analysis. Combinations used in 2-D LC separations are reversed phase together with size exclusion chromatography (SEC)-RP [25-26] or strong cation exchange (SCX)-RP [27-30], the latter used in this study. In SCX-RP the sample is first separated with regard to the components ionic character into different fractions by the use of a SCX column and a buffered salt step gradient. The respective fractions from the first dimension are then subjected to linear gradient separation on a RP-column in the second dimension. The two dimensions of the LC-system used in this study have complementary selectivities that are similar to those of 2-D PAGE. Ion-exchange provides separations which are roughly equivalent to that of isoelectric focusing, while reversed-phase chromatography provides a quasi-molecular size separation in which retention tends to increase with increasing molecular weight. An advantage of this 2-D LC system is the solvent compability of the two dimensions; elution solvents utilized for the ion exchanger are weak solvents in the

reversed phase system. This leads to refocusing of the chromatographic band when the eluted compounds from the first dimension reach the second dimension. Another advantage is the compability of the solvents used in reversed phase with electrospray ionization (ESI) [36] and Matrix Assisted Laser Desorption Ionization (MALDI) [31-32].

1.1 Aim of study

The aim of this study was to develop a miniaturized two-dimensional capillary liquid chromatographic (2-D capLC) method for determination of several neurotransmitters (mainly neuropeptides) from rat brain tissue extracts. Because of the anticipated very high amount of unknown compounds in the samples it was not possible to identify them all. The scope of this project was therefore to map the differences between the most abundant compounds and determine which neurotransmitters (or neuropeptides) they constituted. From the results it was desirable to see, according to age, what effect stress constitutes on the production of neuropeptides in a brain.

2. Experimental

Six different regions of the rat brain were studied in this experiment; cerebellum, brain stem, rhinencephalon, hippocampus, prefrontal cortex and hypothalamus (fig. 2). Samples from the two former were used for testing and optimization of the 2-dimensional system. Samples from different main group (stressed / not stressed), but from the same brain region, were studied to establish the variations between the samples and identify the most abundant compounds.

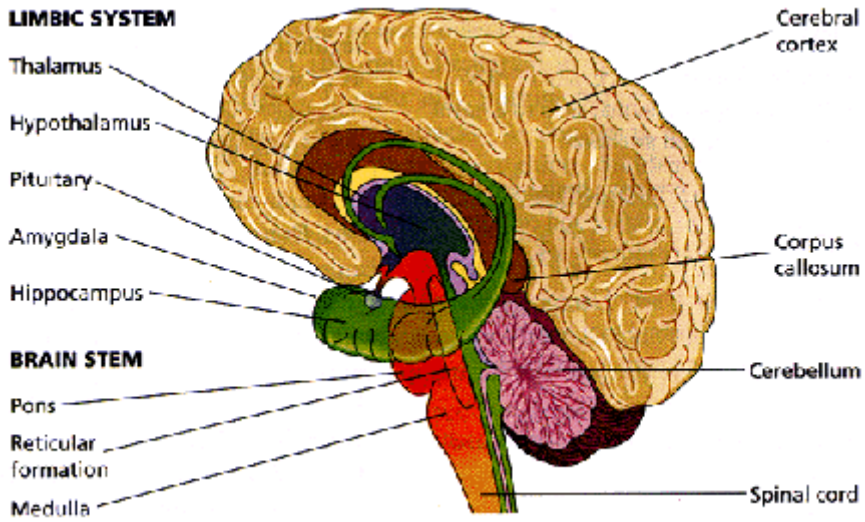

Fig.2 Illustration of the brain lobes in a human brain [33]

2.1 Materials and reagents

All samples which are analysed and represented in the results and discussion section are taken from male Wistar rat brains delivered from the Medical University of Gdansk (Gdansk, Poland). Grade 1 water used to prepare mobile phases was obtained from a Milli-Q ultrapure water purification system (MilliPore, Bedford, MA, USA). HPLC grade acetonitrile (ACN) was purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). Ammonium acetate (98%), formic acid (50%) and gradient grade water used in mobile phases for the real sample runs were purchased from Fluka (Buchs, Switzerland). Three internal standards were used in this experiment; propanolol, ibuprofen and L-Dopa-2,5,6-d_3. The two former were purchased from Sigma Aldrich (Oslo, Norway) while the third was purchased from C/D/N-Isotopes Inc. (Quebec, Canada). The peptides used as test substances, angiotensin II and bradykinin, were purchased from Sigma Aldrich. The SPE columns used during sample preparation, Isolute

reversed phase C18 and Isolute weak cation exchanger CBA, both with 1 mg sorbent and 1 mL bed volume, were delivered by International Sorbent Technology (Glamorgan, UK). Anopore filters with pore size 0.22 μm used during sample preparation were purchased from Anotop IC (Whatman plc, UK). BioBasic SCX stationary phase material with particle size 5 μm was obtained from Thermo El. Corp. (Cheschire, UK), while the packing material for the Partisil SCX-column with particle size 10 μm was obtained from SGE Europe Ltd (Potters Lane, UK). The Macrosphere column (10 cm x 0.3 mm I.D.) with particle size 7 μm was purchased from G&T SepTech A/S (Kolbotn, Norway). The polysulphoethyl aspartam stationary phase used for packing of the strong cation exchanger in first dimension was obtained from Teknolab A/S (Kolbotn, Norway). The stationary phase used in the Kromasil C18 trap columns (4 cm x 320 μm I.D.) with particle size 5 μm and in the Kromasil C18 analytical (10 cm x 320 μm I.D.) column with particle size 3.5 μm was purchased from Eka Nobel (Bohus, Sweden). The PLRPS polystyrene/divinylbenzene (Polymer Labs, Amherst, MA, USA) pre columns (0.5cm x 0.8mm I.D.) with a particle size 5 μm were delivered by Teknolab A/S. All fused silica capillaries were obtained from Polymicro Technologies Inc. (Phoenix, AZ, USA).

2.2 Sample preparation

The rat brain tissues in the mass range 20-450 mg were stored in a Cryo-Exchange Vapor Shipper (Taylor-Wharton, Husum, Germany) before and after the sample preparation. 10 μL of the three internal standards with a concentration of 1.5 ng/μL were added and are presented in table 1.

Table 1 Internal standards and their molecular weights

Internal standard	**Mw**
ibuprofen	206.28 $gmol^{-1}$
L-Dopa-2,5,6-d_3	197.19 $gmol^{-1}$
propranolol	259.35 $gmol^{-1}$

The rat brain was homogenized in a 3 mL propylene vial (Brand GMBH, Wertheim, Germany) in 0.5 mL 0.02 M ammonium acetate in 70 % acetonitrile and 30 % water. Homogenization was performed with a homogenizer (Kontes, New Jersey, USA) with a connected glass pistil (Fig. 3a). The procedure lasted exactly one minute, independent of sample size. The three internal standards were added before the homogenization started. The sample was then centrifuged (Jouan centrifuge, Kebo Lab, Sweden) for ten minutes with a speed of 4000 rpm. After the centrifugation the sample consisted of three layers; first layer was foam containing remnants from fats and proteins, second layer was liquid consisting of solvent with peptides and third layer was solid of mostly fat (Fig. 3b). After centrifugation the liquid was decanted from the vial into a 1 mL polypropylene syringe (Omnifix, Braun, Germany) (Fig. 3c). Before loading the sample onto two coupled SPE columns, the SPE columns was conditioned separately with the same conditioning agents. Two conditioning agents were used, first 0.5 mL acetonitrile (ACN) was added, then 1.0 mL of 0.02 M ammonium acetate (Fig.3d). During the sample preparation two different solid phase extraction (SPE) columns were used, an Isolute reversed phase (C18) followed by an Isolute weak cation exchanger (CBA). The sample solution was transferred from the syringe into the separation columns through a 0.22 μm filter (Fig. 3e). The columns containing the sample solution was stepwise filled with two eluting agents which were pushed through the columns with the use of a syringe. The first eluting agent, consisting of 0.02 M ammonium acetate in 70% acetonitrile, made an A-fraction, while the second eluting agent, consisting of 1.0 M formic acid in 50% acetonitrile, made the B-fraction (Fig. 3f). The eluting (0.5mL) and washing (0.3mL) procedure was carried out in the same step, that is 0.5mL + 0.3mL = 0.8mL total volume of the eluting agent. The two fractions were collected in two 1.5 mL polypropylene micro centrifuge tubes (Brand GMBH, Wertheim, Germany), A and B, and immediately frozen at -197 °C in a nitrogen dewar.

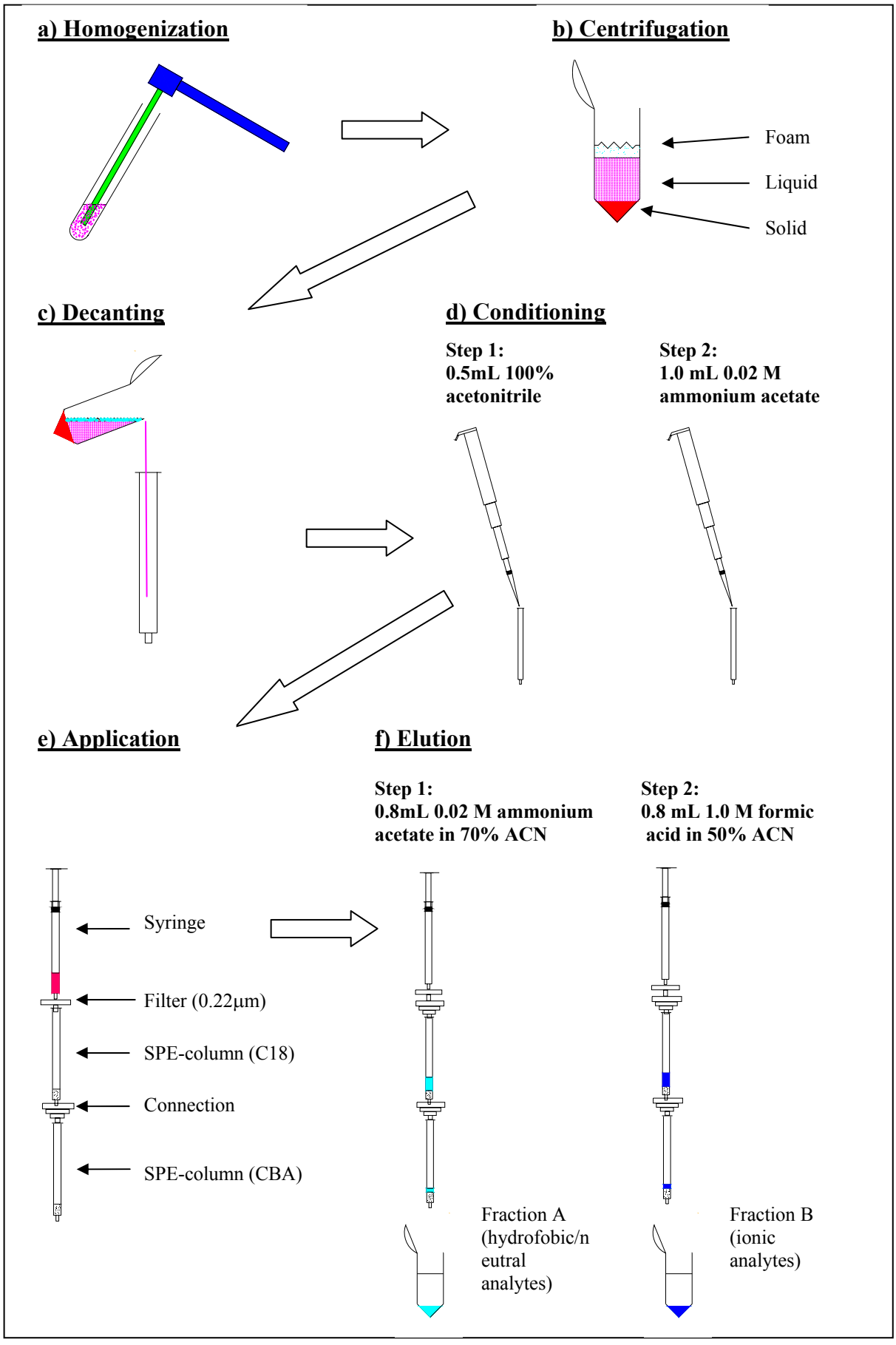

Fig. 3 Flow chart of the sample preparation

2.3 Column preparation

Except for the pre columns, all the columns used in this study were capillary columns made in-house. Polyimide coated fused silica was utilized as the column body due to this material's robustness and flexibility. As the diameter of the column is decreased, extra-column dead volumes become critical with regard to chromatographic performance, and dead volumes arising from e.g. connections, tubing cuts, filters, unions etc., were carefully considered and minimized. The capillary columns were packed in the laboratory using a technique where a suspension of stationary phase and the non-agglomerating solvent carbon tetrachloride introductorily were sonicated to get a homogenized slurry. Subsequently, the slurry was transferred to a stainless steel packing chamber connected to a syringe pump containing a mobile phase of ACN and water (70/30, v/v). The slurry was pumped through the fused silica column body by linearly increasing the mobile phase pressure to 650 bars. The separation columns of 320 μm I.D. and 450 μm O.D. were packed in lengths of 5 cm and 10 cm for the trap columns and the analytical (both SCX and RP) columns, respectively. Valco ZU1C unions in combination with Valco FS1.4 polyimide ferrules and Valco 2SR1 steel screens served as column end fittings (Valco Instruments, Houston, TX, USA) in all columns. The ferrules used in the column end fittings needed to be enlarged to 450 I.D. in order to fit the column O.D.

2.4 Chromatographic systems

Before the development of the two dimensional system took place; a temporary system used for column testing was set up. Only the strong cation exchanger columns were tested, while the choice of reversed phase columns was based on an unpublished report made by Sandra Rinne at the University of Oslo [34]. A detailed description of the chromatographic system used in the preliminary experiments is given in point 2.4.1 while the 2D-LC system used are described in 2.4.2.

2.4.1 Column testing system

The μLC-system used for the column testing consisted of an Eldex MicroPro Syringe (Eldex, Napa, CA, USA) gradient LC pump, and a Linear UVIS 200-detector from Spectra-Physics (Fremont, CA, USA) with an on capillary detection. A Valco Cheminert C4 (Cotati, MO, USA) injector valve with an inner loop of 50 nL was used for introduction of the sample. The detection was performed at 220 nm. Fused silica capillaries with an inner diameter of 50 μm were used to connect all the components in this instrumental setup except from the capillary connected between the injector and the detector. Due to the detection window needed in combination with the UV-detector the capillary had an inner diameter of 100 μm.

2.4.2 Two-dimensional system

The two-dimensional system (fig.4) used for the analysis of the rat brain extracts consisted of three pumps, two Eldex MicroPro Syringe gradient LC pumps and one micro LC isocratic pump (L-7100, Hitachi, Tokyo, Japan). The valves used were two electronic 10-ports Valco Cheminert C2-1030D valves (Vici AG, Valco International, Schenkon, Switzerland) where one of them, coupled to an outer loop of 50 μL, was used for introduction of the sample. Both the valves were two-position microelectronic valves, but of two different models, model E36-230 in the first dimension and the faster model EHMA in the second dimension. All the components of the 2-dimensional system were connected to the valves with 50 μm I.D. fused silica capillaries, except from the pumps that were connected with 75μm I.D. fused silica capillaries. Detection was performed by an Esquire 3000+ Ion-Trap Mass Spectrometer (IT-MS) (Esquire Series, Bruker).

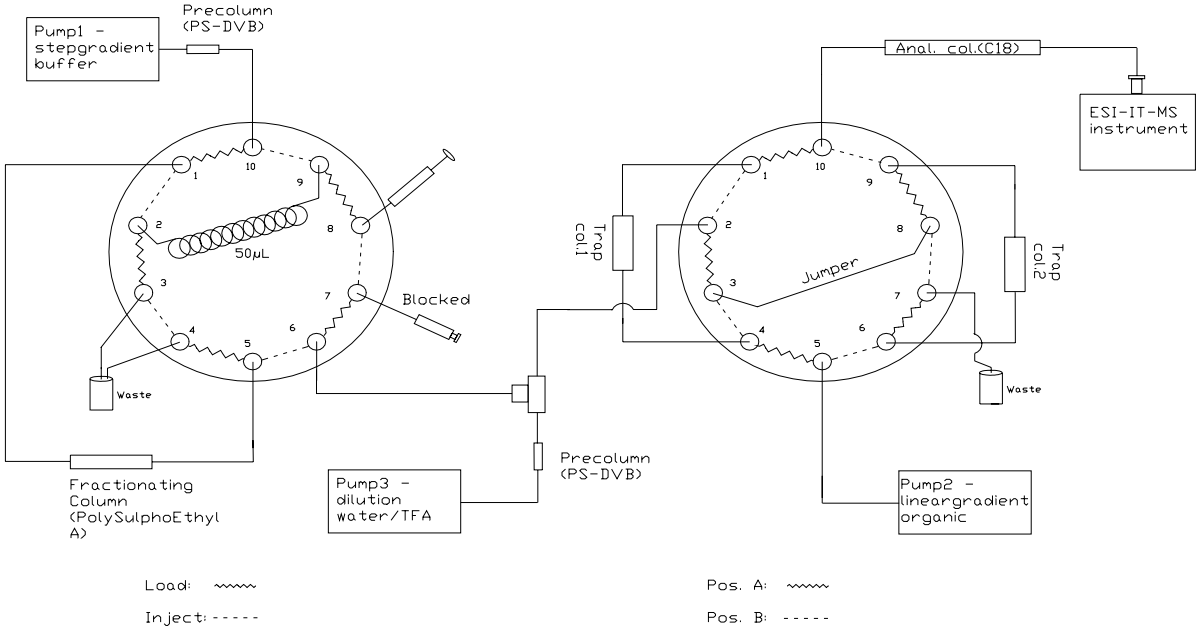

Fig.4 Illustration of the two-dimensional system. See text for description of the system.

2.4.2.1 Mobile phases

In the 2-dimensional system two different solvent gradients were used; a salt step gradient in the first dimension and a linear reversed phase gradient in the second dimension. The *salt step gradient* pump had two solvent reservoirs; reservoir A contained 1 mM ammonium formate in 10 % ACN with pH adjusted to 3, reservoir B contained 500 mM ammonium formate in 10 % ACN with pH adjusted to 3.5. The *linear gradient* used two solvent reservoirs; reservoir A contained 99.9 % water and 0.1 % acetic acid, and reservoir B contained 99.9 % ACN and 0.1 % acetic acid. An additional isocratic pump (pump 3) with a reservoir containing 99.9 % water and 0.1 % TFA was connected between the two dimensions.

2.4.2.2 Functionality

The same instrumentation and conditions were used for analyses of both the A- and B-fractions, except from the salt step gradient that differed in the two cases (Table 2). The sample prepared rat brain tissue extracts were introduced in the system by on-column focusing large volume injection. Initially the samples were loaded onto the strong cation

exchanger with a weak buffer solution of ammonium formate in 10 % acetonitrile. Further the remaining analytes were released from the cation exchanger in fractions by increasing the salt concentration stepwise. Each fraction was trapped on one of the two trap columns every other time and while the organic linear gradient was back flushing one trap column, the next fraction was trapped on the other. Finally, the back flushed analytes from the trap columns was transferred to the reversed phase analytical column. The elution on the analytical column was performed by the same gradient that back flushed the analytes from the trap-columns. The reversed phase linear gradient ranged from 5 to 70 % in 30 min followed by 10 min equilibration for each of the fractions from the first dimension. With six fractions from the first dimension the total run time for the analyses of B-samples was 255 min, while the five fractions from the A-samples had duration of 215 min. After separation in the chromatographic system the sample was introduced in an ion-trap mass spectrometer by electrospray ionization (ESI).

Table 2a: LC time table for salt step gradient for the A-samples

Time (min):	% B:	Conc. ammonium formate (mM):	Fraction no.
0.01 – 15	0	1	1
15.01 – 55	1	5	2
55.01 – 95	2	10	3
95.01 – 135	4	20	4
135.01 – 175	10	50	5

Table 2b: LC time table for salt step gradient for the B-samples

Time (min):	% B:	Conc. ammonium formate (mM):	Fraction no.
0.01 – 15	4	20	1
15.01 – 55	10	50	2
55.01 – 95	20	100	3
95.01 – 135	40	200	4
135.01 – 175	70	350	5
175.01 – 215	100	500	6

2.4.2.3 Automation

Three actions were done manually in every run; loading of sample, start of pump in first dimension and start of acquisition. At the same time the pump in first dimension was started, the first valve was automatically triggered by the pump and so injected the sample. After the fifteen minutes long injection the first pump triggered the second pump which switched the second valve and started the linear gradient. At this moment the data acquisition was manually started. Every fortieth minute (after the linear gradient described in previous page) the second valve was switched by the linear gradient pump to elute the previous fraction from the first dimension.

2.4.2.4 Detection

The peptides eluting from the second dimension were detected by an Esquire 3000+ ITMS instrument by Bruker Daltonics (Bremen, Germany) equipped with a low-flow nebulizer to accommodate flows in the low µl/min range. The ITMS instrument was operated in the positive mode with a capillary voltage of 3700 V, end plate offset at 500 V, skimmer at 40 V and capillary exit at 128.5 V. Nebulizer and drying gas were applied at 15.0 psi and 6 L/min, respectively, in order to enhance mobile phase evaporation and spray stability. Data was acquired in the *m/z* range 250 – 2000 using the Esquire Control v 3.5 by Bruker Daltonics both in the MS mode and in the Auto MS(n) mode. Determination of the compounds was performed by fragmenting the most abundant compounds in the ITMS. Fragmentation was only performed on compounds with a higher intensity than a preset threshold value that was decided by experimental trials. Two injections from each sample were performed, the first in single MS mode and the second in auto MS (MS/MS) mode. The single MS run was used to define the most abundant compounds while the corresponding fragment line spectra of the compound masses in the auto MS run was used for identification. The identification was performed by exporting one of the abundant peaks' fragment spectrum from Esquire Control into BioTools software followed by a MS/MS online search with the Mascot search engine from Matrix Science. The fragment spectrum was then compared with hypothetical fragment spectrums of peptides virtually derived from a limited amount of the proteins in the database at the National Center for Biotechnology Information (NCBInr). The search was limited by choosing a specific taxonomy, in this case mammals.

3. Results and discussion

Even though a two-dimensional system with high resolving power was used in this project, offline sample preparation was necessary to finally get sufficient resolution. Also, since the samples was solid brain tissue at the starting point, some extraction was necessary to get the analytes into an injectable liquid at the same time as enzymatic degradation needed to be reduced.

3.1 Sample preparation

In order to reduce the enzymatic degradation that always will take place to some extent during extraction, the tissue was homogenized/extracted with 70% ACN and then centrifuged, which is expected to halt further degradation. Due to the high concentration of acetonitrile in the sample none of the hydrophobic analytes were retained on the C18 column during the application, but rather eluted straight through the column. The reason for the C18 column to be there was to remove most of the fat in the sample. The fat has a very great retention on a C18-column and 70 % acetonitrile in the eluting agent assures the extraction of the more hydrophobic peptides, while at the same time extracting only a minor part of the fat of the brain tissue, as seen by a fatty residue which was removed by centrifugation. Consequently, the hydrophobic analytes/compounds were eluted through both the columns from the very beginning; while the ionic compounds were retarded on the CBA-column until the second eluting agent (formic acid in ACN) was added. Due to the 0.02 M ammonium acetate in the first eluting agent, weak cationic compounds could also elute in this fraction. Because the analytes in the A-fraction only are slightly ionic, the steps used for the A-fraction in the chromatographic systems step gradient are smaller than for the B-fractions.

3.2 Column testing

Three different SCX-columns were initially tested before the final 2-D LC system was put together; Partisil SCX (10cm x 0.32mm I.D., 10µm), Macrosphere SCX (10cm x 0.5 mm I.D., 7µm) and BioBasic SCX (10cm x 0.32mm I.D., 5 µm). All the SCX-columns required the

same amount of acetonitrile to avoid secondary interactions, and it was found that 20 % acetonitrile was requisite to make the analytes elute through the ion-exchange column (fig.5). To avoid break through on the trap-columns that concentrates the components in all the fractions from the first dimension, it was necessary to have only a few percent of acetonitrile in the mobile phase entering the RP trap-column. None of the tested SCX columns obtained good efficiency with an amount of 5 % ACN, but a later tested column, PolySulphoEthyl A, required only 10 % acetonitrile in the mobile phase to elute the compounds. This packing material was therefore used in the further study. The amount of ACN needed was still too large to focus the analytes on the trap columns inlet, and therefore a dilution pump was added to the system between the two dimensions. The dilution pump gave a flow twice the flow of the buffered salt step gradient pump (20 µL/min) which made a three times dilution of the mobile phase.

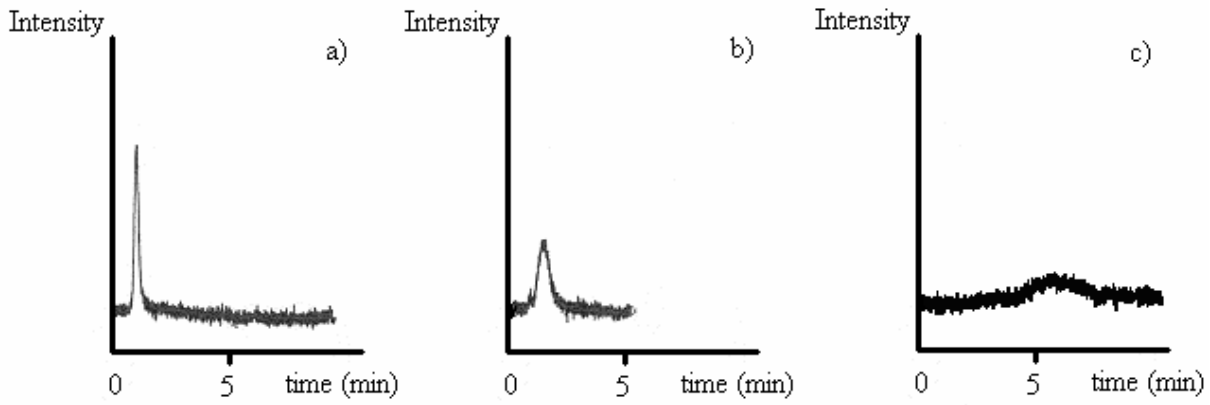

Fig. 5 With 0.5M ammonium chloride the analyte (AngII) elutes fast through the BioBasic SCX column but with big differences in efficiency according to the percentage of acetonitrile. a) 20%, b) 15%, c) 10%.

It was desirable to avoid the use of a diluting step between the two dimensions to decrease the instrumentation's complexity. Several different parameters were changed to make the analytes elute through the BioBasic SCX column by using only five percent acetonitrile: The pH of the mobile phase was first changed from pH 4 to pH 3 to make sure that the compounds had a total positive charge. The same was done with the sample solution to be sure that the analytes was not locally deionised, but no improvement was observed. A change in flow from 8 to 10 µl/min was performed to get faster elution and narrower peaks, but still no improvement. Mobile phases containing different buffer solutions were tested with the system, without any improvement in peak shape. NaCl had too weak eluting strength, and formic acid absorbed

too much UV-radiation. Ammonium chloride that has average eluting strength [35] and approximately no UV-absorption by 220 nm was used for the final testing of the SCX-columns.
With respect to the retention factors relatively large differences between the different reversed phase columns have been obeserved. As mentioned the RP columns were tested by others [34] and the conclusion was that the best efficiency was obtained by the Kromasil C18 material.

3.3 System evaluation

The 2-dimensional system used in this project was comprehensive with regard to the strong cation exchanger that fractionates the sample in the first dimension and the reversed phase column in the second dimension. The first dimension was originally planned to be a buffered salt step gradient with a pH of 3, but due to high buffer capacity this low level was not attainable in the B reservoir. The final system therefore also involved a small pH gradient from pH 3 (reservoir A) to pH 3.5 (reservoir B) in the first dimension. To get higher retention and better focusing on the C18 trap columns the analytes interacted with the ion-pairing agent TFA from the dilution mobile phase before reaching second dimension (Fig. 2). Unfortunately TFA interacts so strong with the analytes that it could cause ion-suppression in the MS [36], but this case was avoided by back flushing the analytes with the RP mobile phase containing acetic acid. Acetic acid makes weaker interactions with the analytes than TFA and simplifies ionization in the electrospray. As mentioned earlier the extent of ionization is also much affected by the mobile phase composition, and use of the combination water with acetonitrile and acetic acid fulfils the electrospray's demand for volatility [37].

Previous projects regarding peptides have shown that acetonitrile is a solvent that greatly elutes peptides from a C18 stationary phase [38], and so acetonitrile was chosen as organic modifier in the second dimension. To obtain the best efficiency both duration and composition of the organic linear gradient were changed several times during the method optimization. Further, refocusing on the analytical column in the second dimension was achieved by using bigger particle size in the trap columns (5 μm) than the particle size in the analytical column (3.5 μm).

Due to all the parts in the system that must interact properly, the method development was a time consuming process. Samples from brain stem and cerebellum was used as test samples as it was expected to find fewer compounds linked to stress/non-stress in these brain regions compared with the other brain regions. None of the compounds in the test samples was identified, but the test samples were rather used for optimisation of the fractionation and separation of the compounds.

The total ion chromatogram (TIC) (Fig. 6a) was not used for quantification of any compounds, but it was used as a normative measurement for the system stability by ensuring that the baseline trend looked the same in every fraction in the no-injection run. By studying the base peak chromatogram (BPC) from a no-injection run (Fig. 6b) it was possible to decide which peaks originated from the system (mobile phases, stationary phase bleeding, couplings, gradient effects etc). The start of every linear gradient is marked with arrows and acquisition duration in minutes in the BPC.

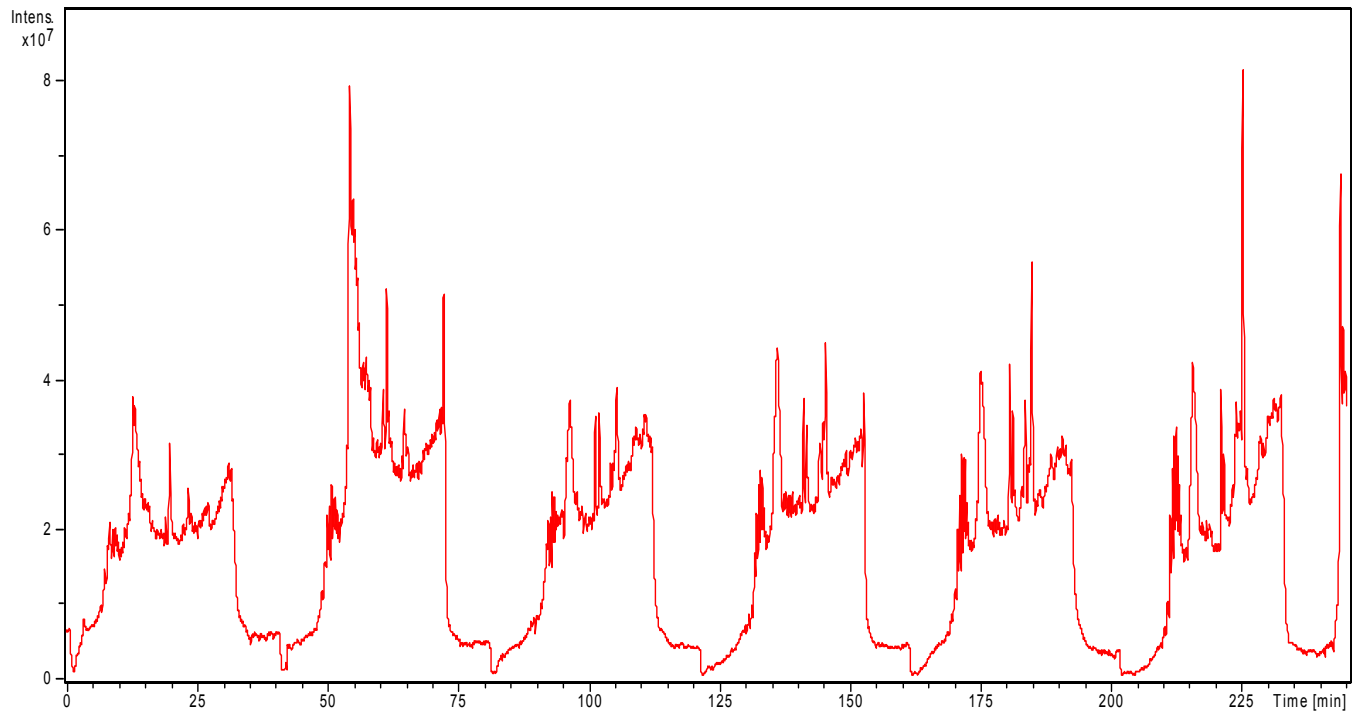

Fig. 6a Total Ion Chromatogram (TIC) from a no-injection run

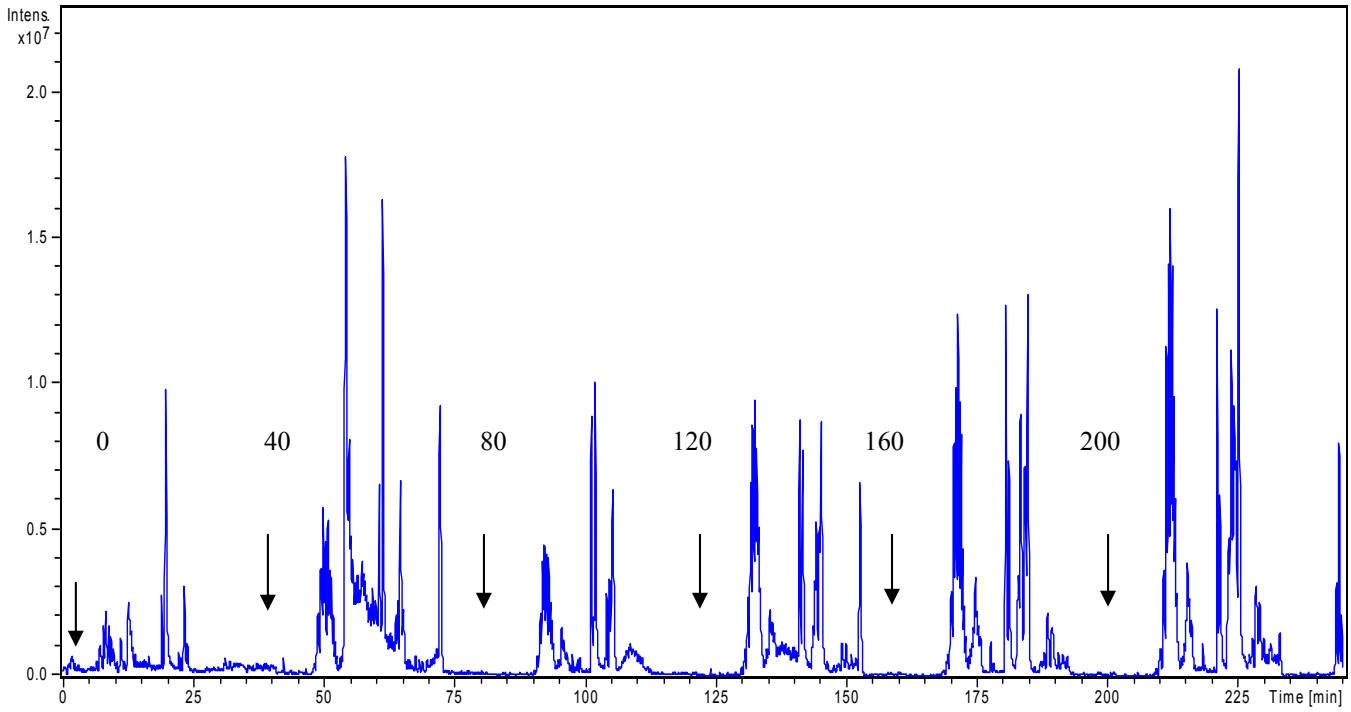

Fig. 6b Base Peak Chromatogram (BPC) from a no-injection run. Arrows mark start of every RP gradient. Numbers are runtime.

Due to polyethylene glycols (PEG's) in the mobile phase water, a very high background was observed in the first runs with the final system. By changing the mobile phase water from MilliQ- to HPLC grade- water, and adding polystyrene divinylbenzene (PS-DVB) pre columns to the dilution pump and the salt buffer gradient pump, the background was significantly reduced. Other compounds with origin in the organic mobile phase also contribute to the background, but these compounds were not identified. In the single MS run the interfering agents are negligible, but during the auto MS run they could make problems because of their high intensity. However, since the masses were known they were registered in the MS-software so even though their intensity exceeded the threshold value, MS/MS was not performed on them. The zoomed fraction in the BPC from the no-injection run (Fig.7) shows the background peaks that occur in every fraction. The first eight masses are PEG's while the last masses are unidentified.

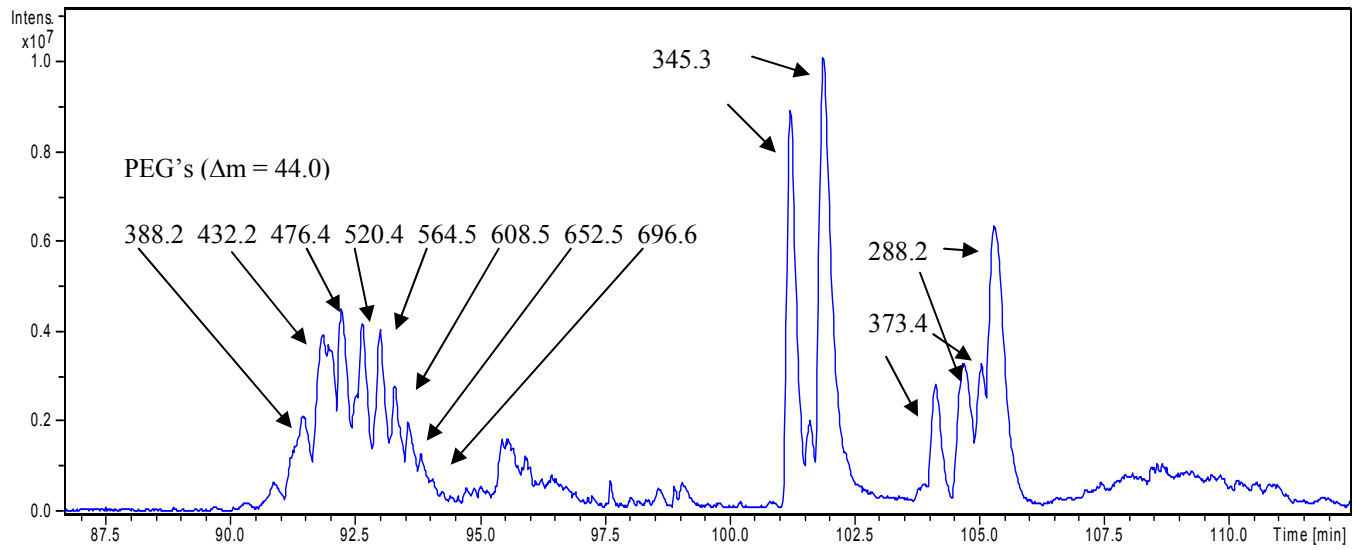

Fig. 7 Zoom of BPC from one of the no-injection fractions showing the background masses

The greatest challenge in this project was sequencing of the compounds of interest. It was not known in advance which compounds that could exist in the matrix, and the use of standard solutions to compare with was therefore excluded. Also, normally when working with biological samples containing amino acid sequences, the molecule (mostly proteins) has reacted with a known enzyme that cleaves the amino acid sequence at specific positions in the chain. After the fragmenting is done with the mass spectrometer the data analysis software is fed with what kind of enzyme the analytes was treated with. This information gives the database search algorithm a hint which limits the amount of possible structures the analyte could have. When the database search is finished, the software suggests which compound the analyte is most likely to be. The suggestions are represented as "hits", which gives information about the structure and the possibility for the respective structure.
In the current case, the hint is absent because the sample is not treated with any kind of enzyme. Consequently, the search can not be narrowed down and suggested possible structures for every compound is considerably increased.

During the MS fragmentation procedure the point of cleavage can be located in several positions in the chain, both in the backbone and the sidebone. However, it is most likely that the cleavage finds place between the C- and N- terminal of two amino acids, known as the peptide bond. The part that follows the N-terminal from the precursor ion is called the y-fragment while the other part belonging to the original C- terminal is called the b-fragment (Fig. 8). The nomenclature for naming such fragments is described in detail by Michael Kinter and Nicholas E. Sherman [40].

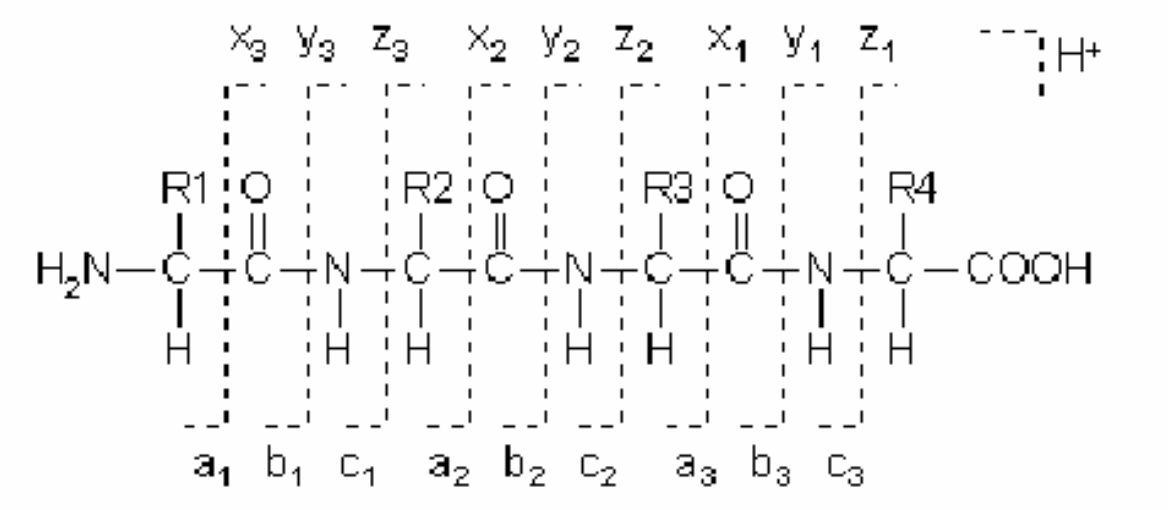

Fig. 8 Fragmentation of an amino acid sequence

To prove the reliability of the Mascot search algorithm, a complete example of how identification of a known compound (Bradykinin) was performed is shown in appendix 5.3.

3.3.1 Internal Standards

Due to different reasons two of the three internal standards added to the samples during the sample preparation could not be used in the analyses. After optimization of the instrument parameters, the scanning range was starting on *m/z* = 250 which is beyond the *m/z*-value of ibuprofen (M_w = 206.28 $gmol^{-1}$) and L-dopa-2,5,6-d_3 (M_w = 197.19 $gmol^{-1}$). In addition L-dopa-2,5,6-d_3 have no retention on the trap columns and will elute to waste. Therefore only propranolol (M_w = 259.35 $gmol^{-1}$) was useful as an internal standard. As can be seen from the extracted ion chromatogram (EIC) below (Fig. 9) propranolol appeared mainly in the first fraction, but a small signal is also visual in the second fraction.

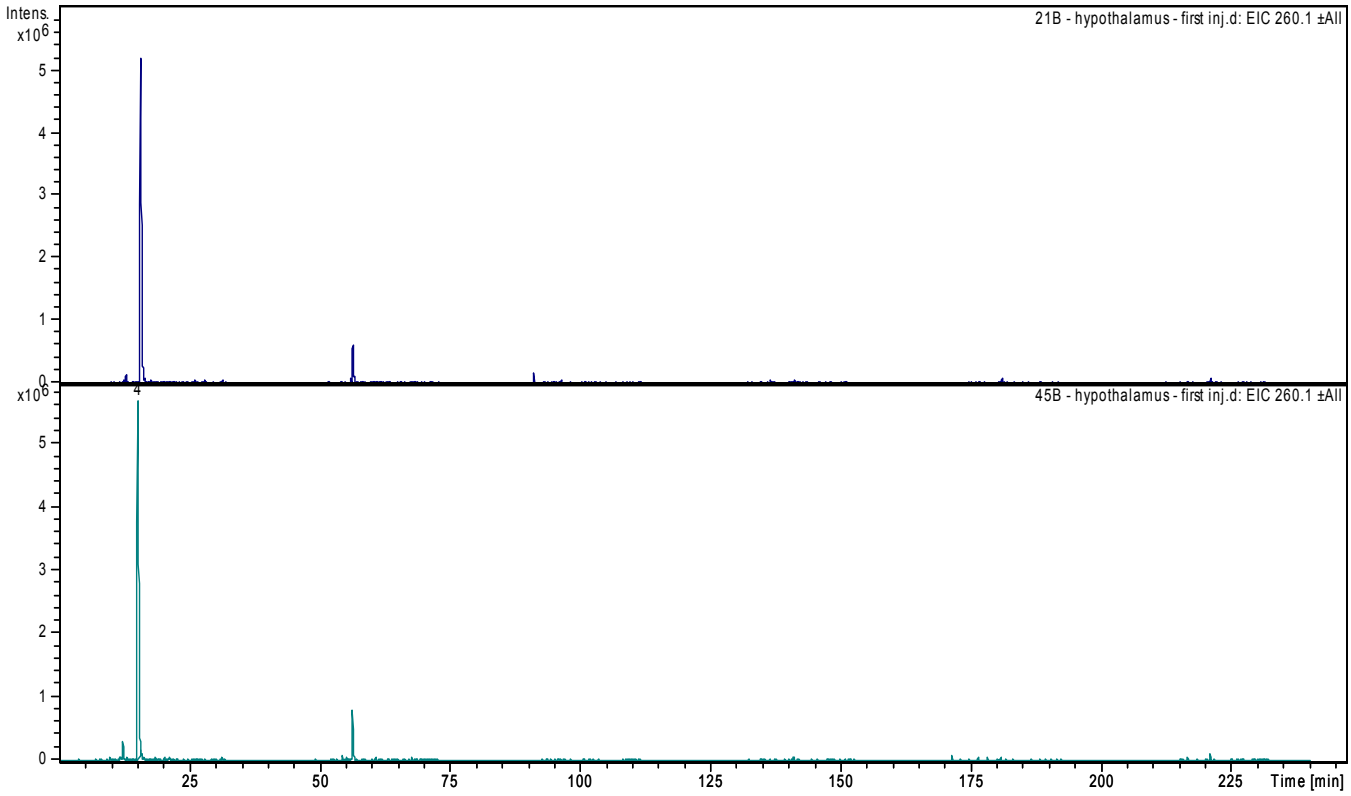

Fig. 9 The peak from the internal standard propranolol in the extracted ion chromatograms from both the stressed (upper) and non-stressed (lower) samples

3.3.2 Repeatability

To prove the systems repeatability the peptide angiotensin II was used as test substance. The repeatability testing was performed by four injections of the test sample in a concentration of 20 ng/ml (1 ng absolute mass) with the same system conditions as used with the real sample runs. The within day results from the four injections are given in the figure below (Fig. 10), while the statistical values regarding retention time and areas are represented in table 4. As can be seen from the figure and table below, intensity fluctuations occurred during the day of repeatability testing. Of unknown reasons the signal decreased even though the concentration of the test substance was the same. The in-house conclusion was that the error originated in the mass spectrometer, and this theory was strengthened by other users which also have reported high RSD values in connection with the mass spectrometer [41-42].

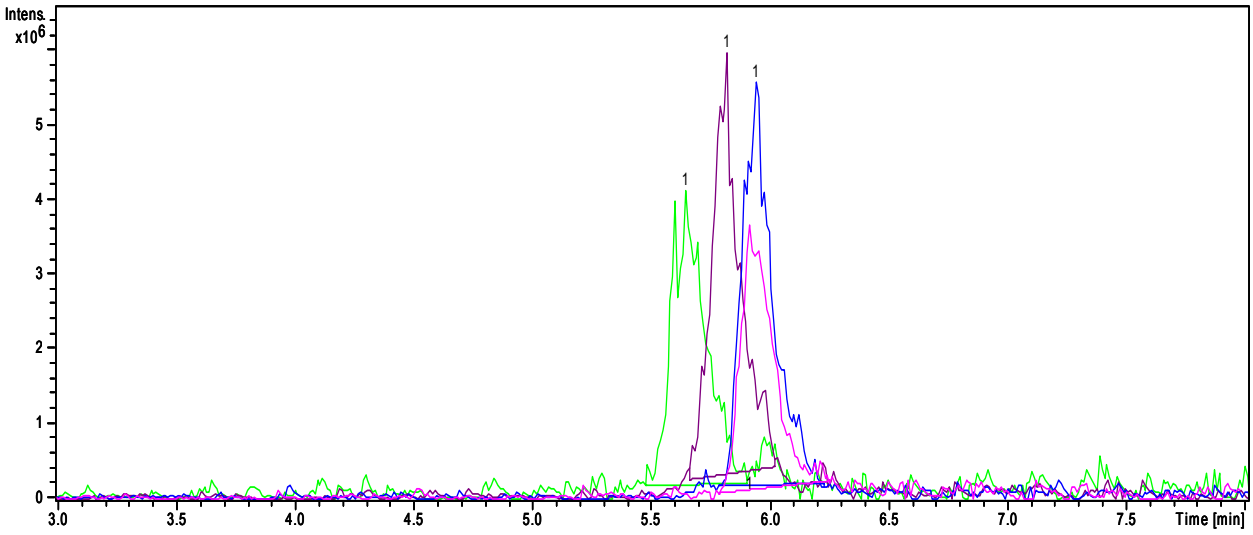

Fig. 10 Overlaid chromatogram of the four injections of Angiotensin II performed with the final 2-D LC system. The analyte was eluted in the first salt step fraction.

Table 4: Repeatability testing results

Replicate no. (x)	R_t	area
1	5.6	38540876
2	5.8	45590527
3	5.9	47072893
4	5.9	33125626
x_{ave}	**5.8**	**41082481**
σ_x	**0.0577**	**6480196**
RSD (%)	**0.995**	**16**

3.3.3 Limit of detection

The limit of detection was defined as three times the noise (S/N = 3). With a 50 µL loop the lowest solution concentration obtained, according to the definition, was **1 ng/mL** with a corresponding absolute mass of **50 pg**. The results are shown in table 5 and figure 11.

Table 5: Limit of detection

Inj nr	RT [min]	Area
1	5.3	12317682
2	4.9	9480197
3	5.1	7648713
Ave	**5.1**	**9815531**
STDEV	**0.2**	**2352478**
RSD (%)	**3.921569**	**23.9669**

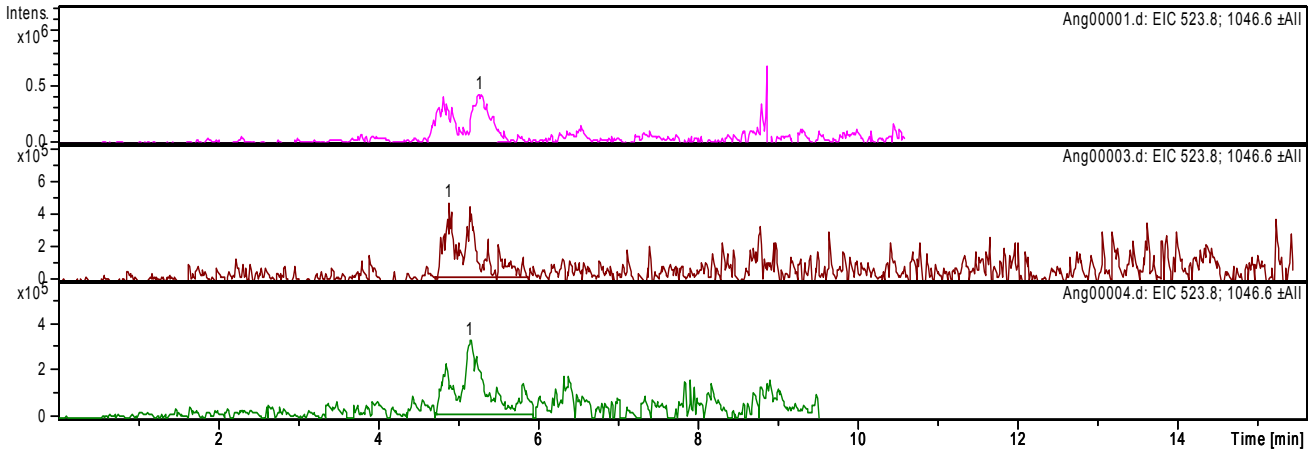

Fig. 11 Chromatograms of the three injections from the determination of limit of detection with angiotensin II

From this result it is possible to estimate the smallest amount of analyte that must be present in the rat brain tissue to be detected. The final sample volume after sample preparation was approximately 150 µL and with a concentration equal to the LOD (1 ng/mL) the absolute mass would have been 150 pg. With an average mass of the brain tissue samples of 35 mg this concentration corresponds to 4.3 ng analyte per gram brain tissue.

3.4 Analysis of rat brain tissue samples

In contrast to the TIC, the BPC was important when determining the relative abundance of analytes in the samples from the stressed and non-stressed rats. The BPC monitored the most abundant *m/z*-value at any time, and the value was used to decide which analyte that should be further investigated. Figure 12 shows an overview chromatogram from hypothalamus which represents a stressed rat (blue line) and not stressed rat (green line) overlay. The arrows points at peaks where big differences are visual.

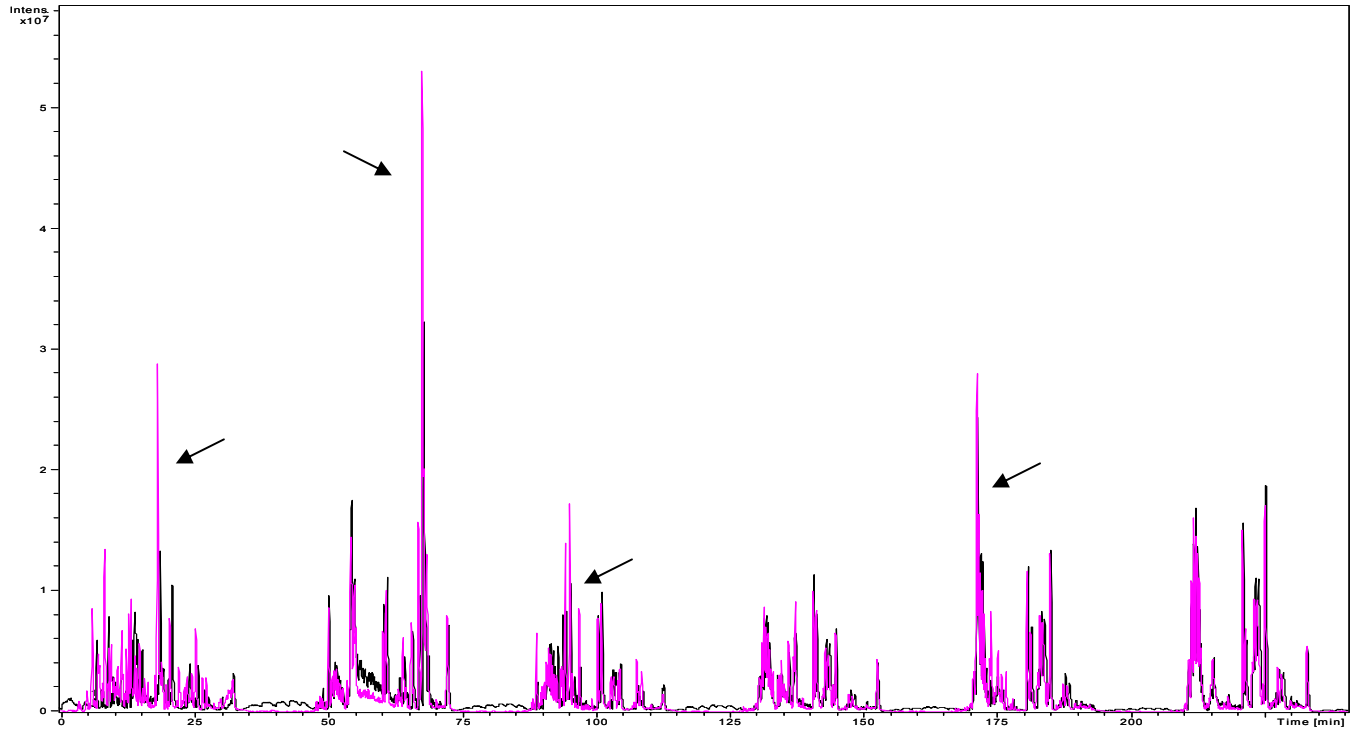

Fig. 12 Overlay of two chromatograms from rats exposed to stress (black) and not exposed to stress (pink). Arrows marks the most visual differences in this overview chromatogram.

The zoom of the 20 mM fraction from hypothalamus (Fig. 13) shows an overlay ("fingerprint") of a stressed rat (green line) and a non-stressed rat (blue line). The intensity differences are evident! There is a little time delay between the two runs and therefore two red arrows are marking the respective peak in both two fractions. The red marked peak was one of the further investigated peaks due to abundance differences in the stressed and non-stressed fraction. As an example the mass spectra of this compound are shown below, while the peaks' profile spectrum before and after fragmentation are presented in figure 14a and 14b, respectively.

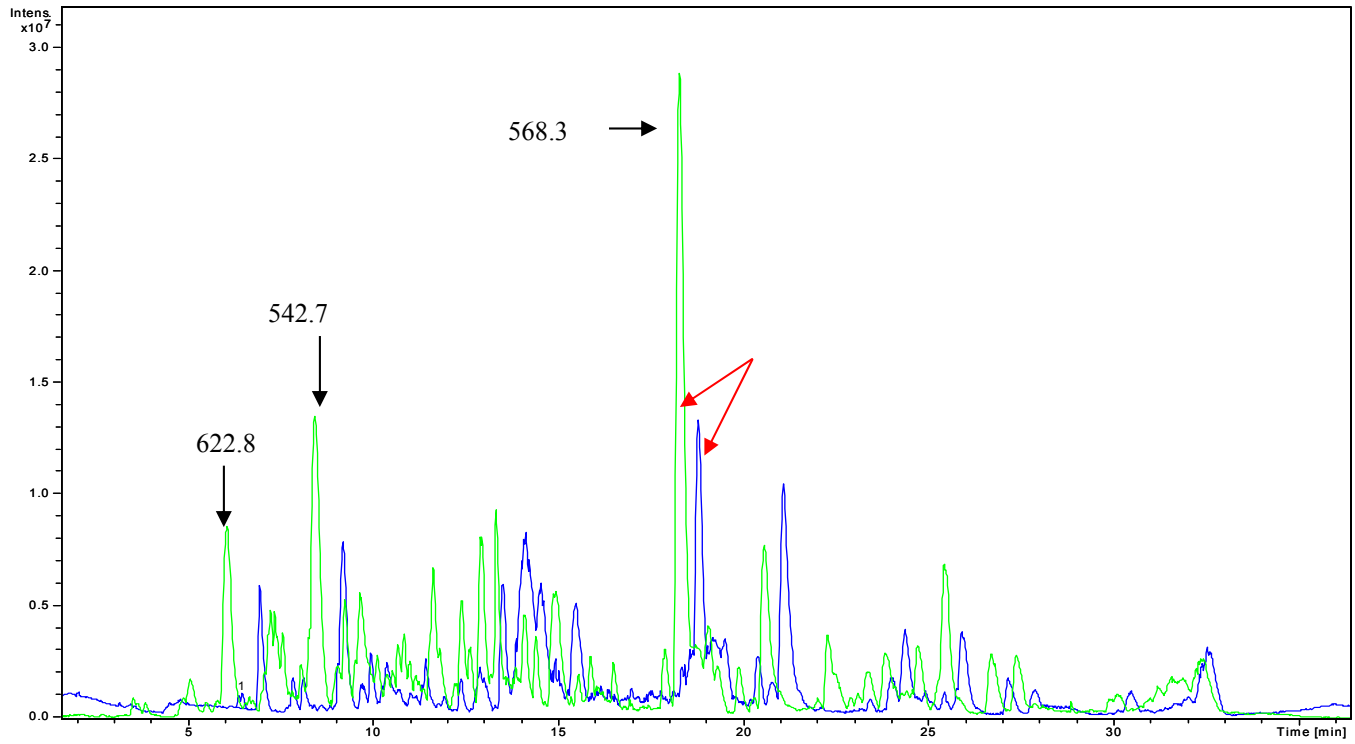

Fig. 13 Zoomed BPC overlay of the 20 mM fractions from stressed (green line) and non-stressed (blue line) rats

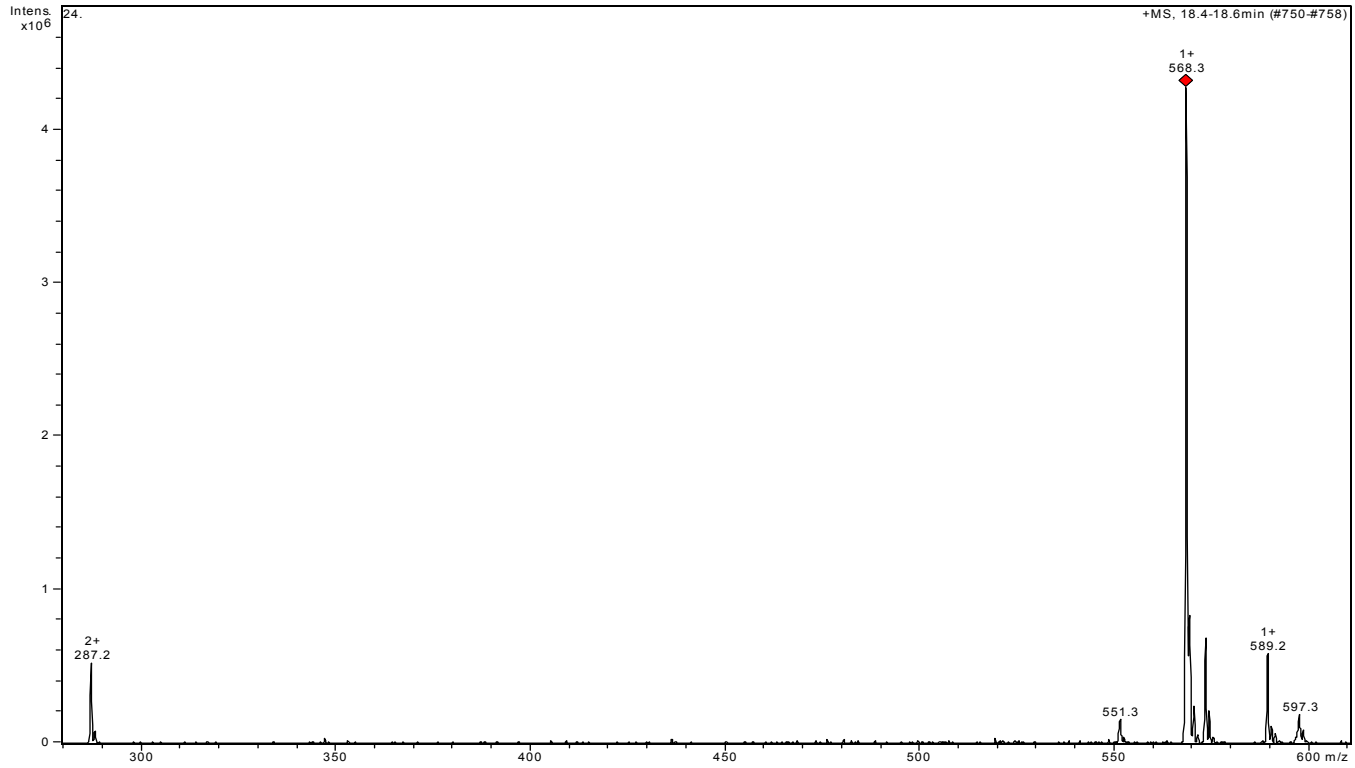

Fig. 14a Mass spectrum showing the parent ion chosen by the software (through the threshold value) to be fragmented. Red spot is ion chosen by the software to be fragmented.

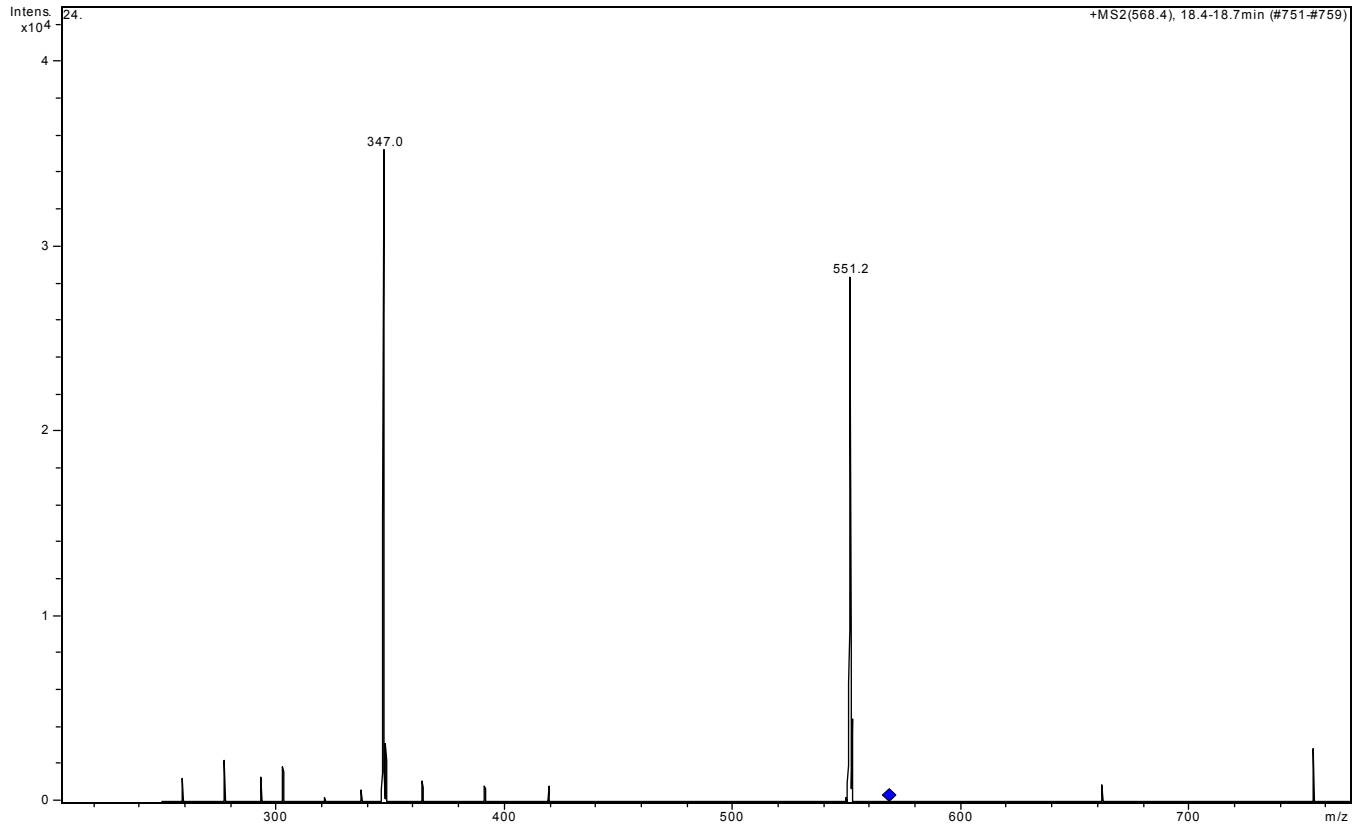

Fig. 14b Mass spectrum showing the daughter ions from the fragmented parent ion (Blue spot is where parent ion was before fragmentation)

Three compounds were tentative identified in this study. Every search in the NCBInr database returned a list of suggested compounds that could match the inquired compound. The suggestions were ranged after a score which described how much of the analyte amino acid sequence the respective suggestions covered.

The compounds with highest score for each of the three inquired compounds in the database are shown in the table 6. All the three compounds had a sequence coverage of 100%, and this is an often occurring result with small compounds as peptides. Bigger proteins seldom have a sequence coverage as good as 100 %, but rather in the range from 30-80 % [39].

Results represented in the field of proteomics by Per Andrèn and his co-workers show promising results where several peptides are found in the same kind of matrix as used here [17-18], even though they have only used one-dimensional separation techniques.

Table 6: The three tentative identified compounds

m/z-value	Charge	Retention time	Compound name	Amino Acid sequence
622.8	2+	6.9	SOMATOSTATIN-28	SANSNPAMAPRE
542.7	1+	8.9	OLIGOPEPTIDE NO.15.- vectors.	PLVTL
568.3	1+	18.3	CYTOCHROME OXIDASE II (FRAGMENT).- Pseudoregma koshunensis (Bamboo aphid).	MTWM

3.5 Future plans

Even though the method development in this project is finished and the analysing conditions are optimized, there are still several samples left to be analysed. Further work will be based on continued identification of the most abundant compounds in the samples and find additional results that describe the brain's activity during stress. The connection between age and how stress is handled is also an area of interest.

4. Conclusion

The method development was a time consuming and challenging task. After optimizing the different parts of the system one by one, the most demanding work was to make all the components in the system work properly together. Anyway, the current system has shown good functionality and high resolution power and is ready for the analyses that will be performed in the future.

As mentioned Per Andrèn and his group obtained identification of several peptides by the use of a not so complex separation technique as employed in this study. Unfortunately some work still remains in this project before comparison with others results can be done.

It would have been preferred to have a fully automized system, but due to different manufacturers the components cannot communicate. Anyway, with run times as long as in the present system (beyond four hours), an autosampler would have been a great addition to exploit the systems capacity.

5. References

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6. Appendix

6.1 The function of a neuron

A neuron activates by transmitting electrical signals along its axon (Fig. 15). When the signal reaches the end of the axon, pouches called vesicles are influenced by the electrical signal and releases neurotransmitters. Neurotransmitters bind to receptor molecules on the surface of adjacent neurons. The area of virtual contact is known as the synapse (as earlier shown in Fig. 1).

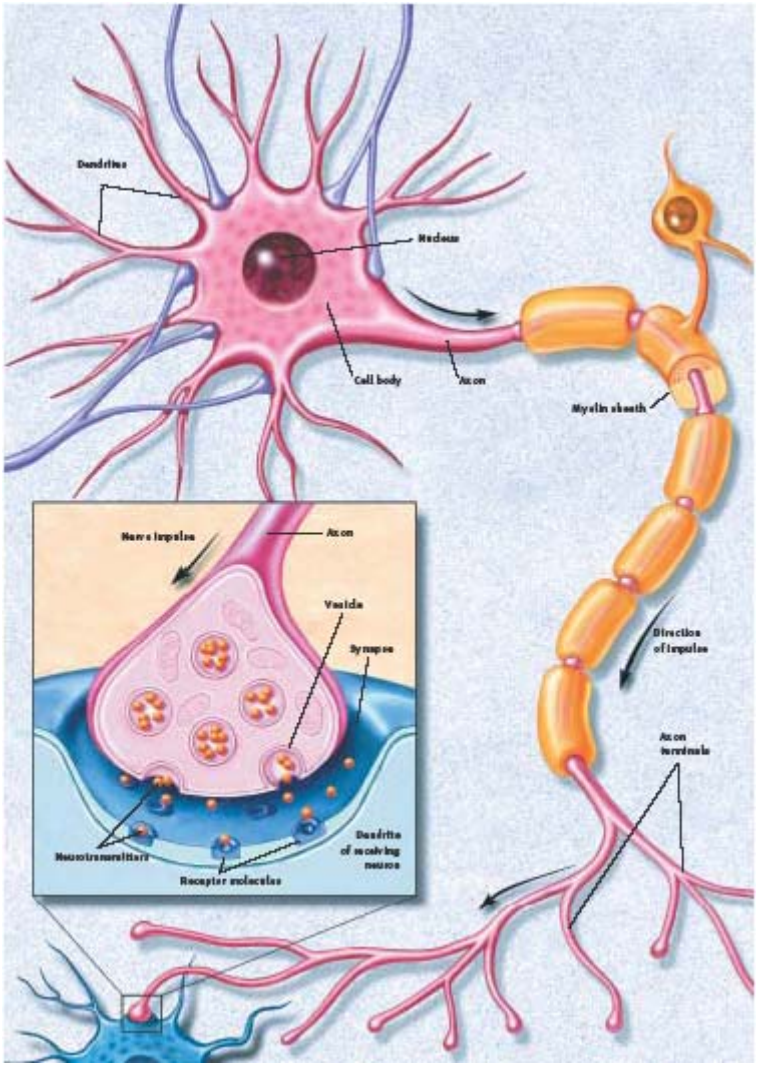

Fig. 15 The function of a neuron

6.2 Identification of bradykinin

To ensure the Mascot databases' reliability, bradykinin was analysed and identified by the use of Mascot online database. The arrowed peak below exceeded the preset threshold value and MS/MS was performed. Since bradykinin occurs with three different charges, three *m/z*-values are found in connection with this peak (Fig. 16). The mass spectra' are shown in figure 17.

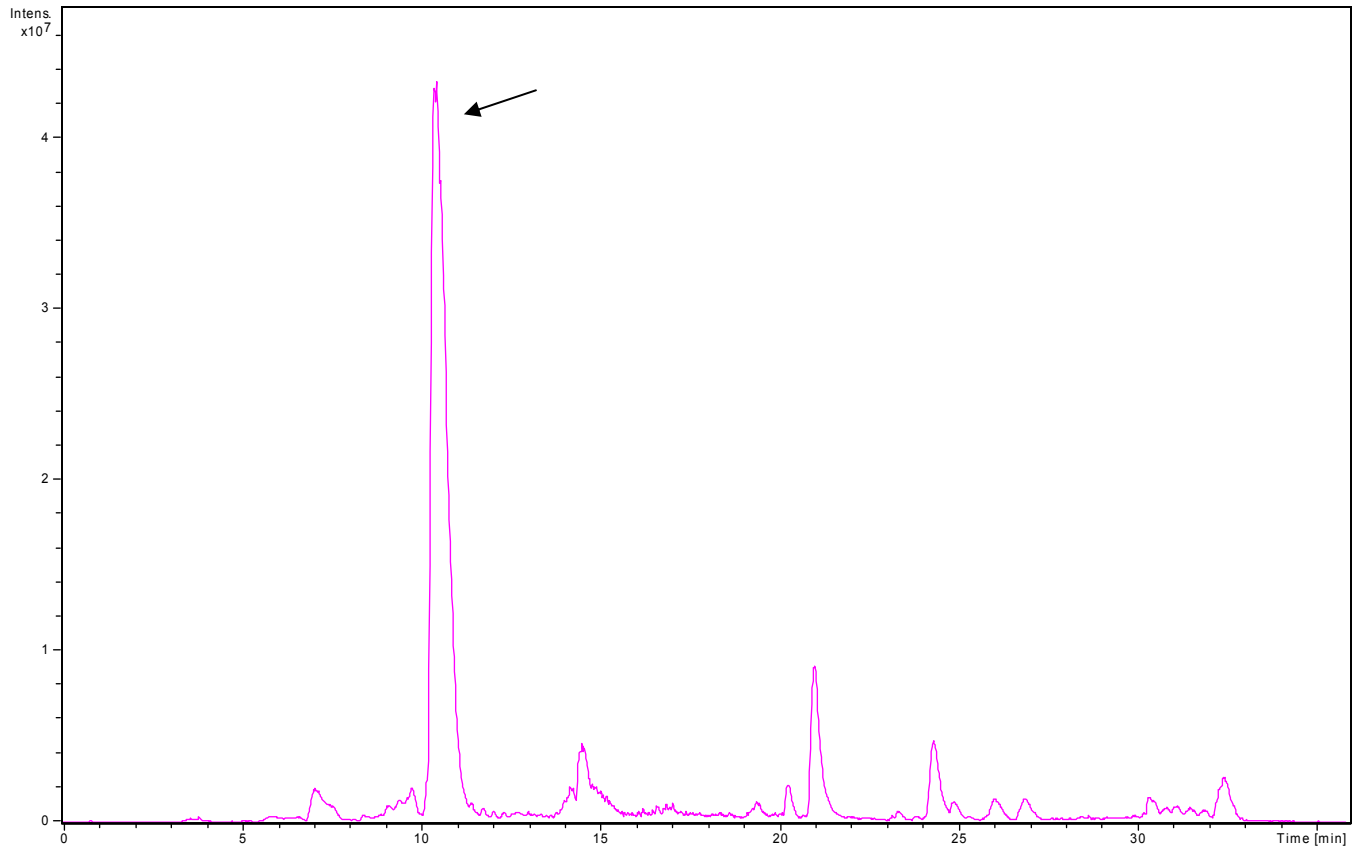

Fig. 16 BPC of bradykinin from the first salt step fraction

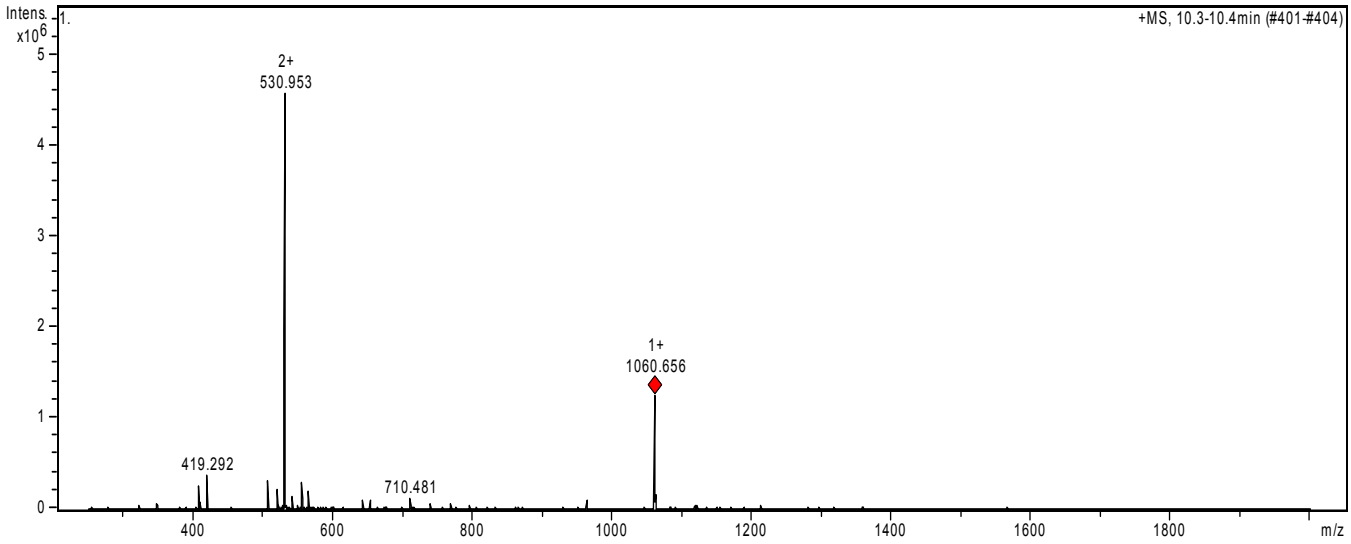

Fig. 17a MS spectrum showing the *singly* charged parent ion chosen to be fragmented

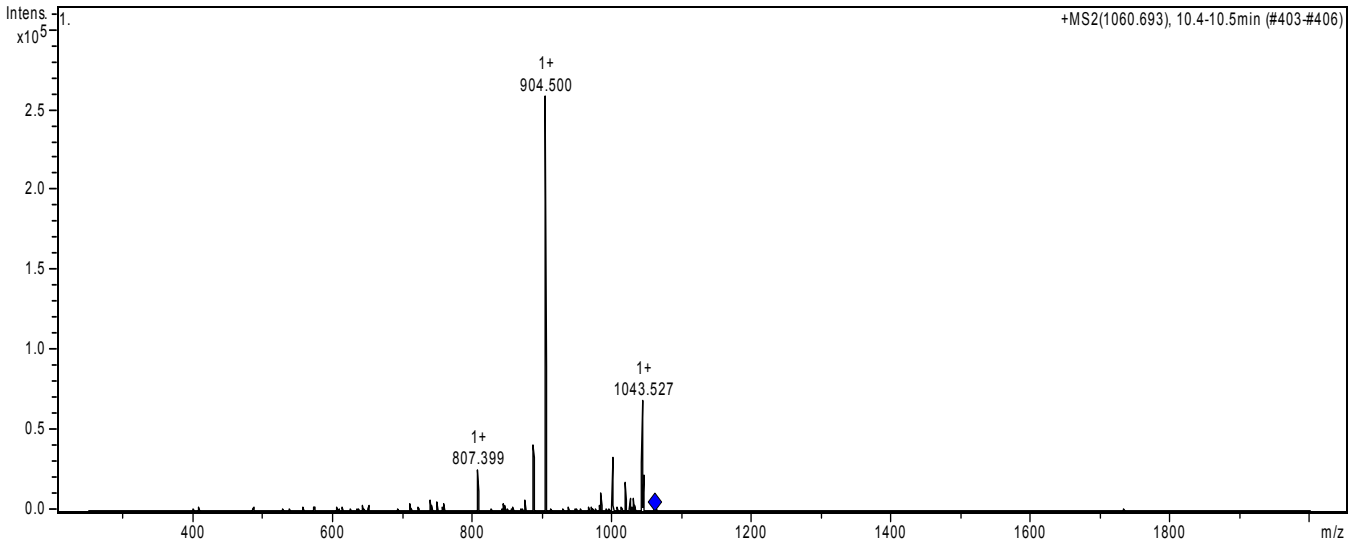

Fig. 17b MS/MS spectrum showing daughter ions from the fragmented parent ion with m/z-value 1060.656

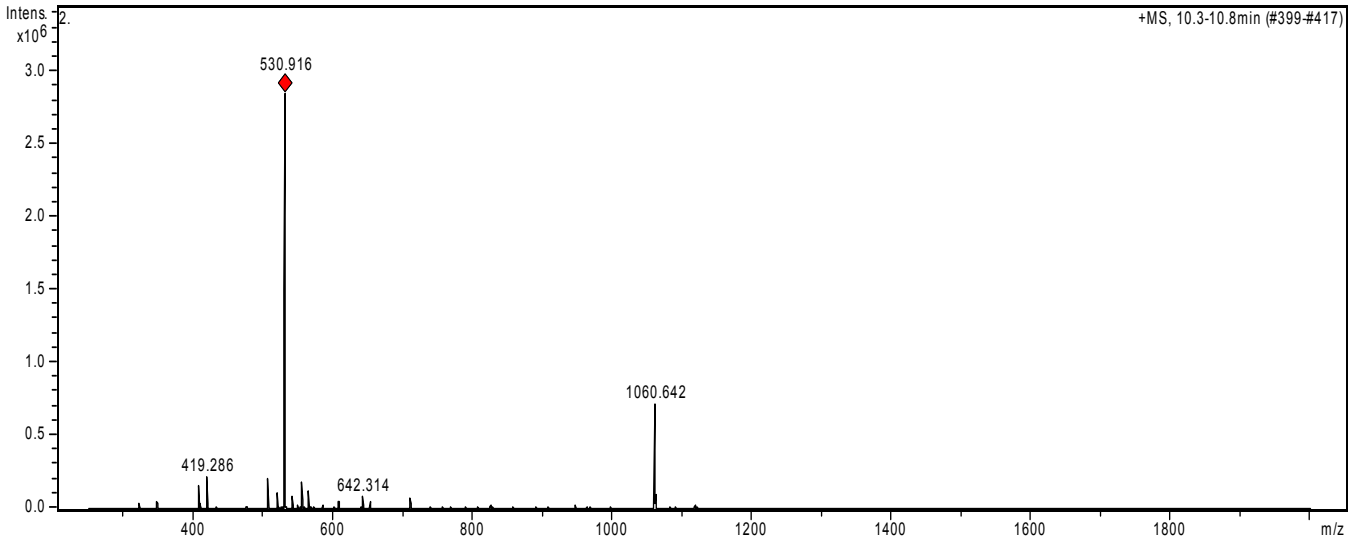

Fig. 17c MS spectrum showing the *doubly* charged parent ion chosen to be fragmented

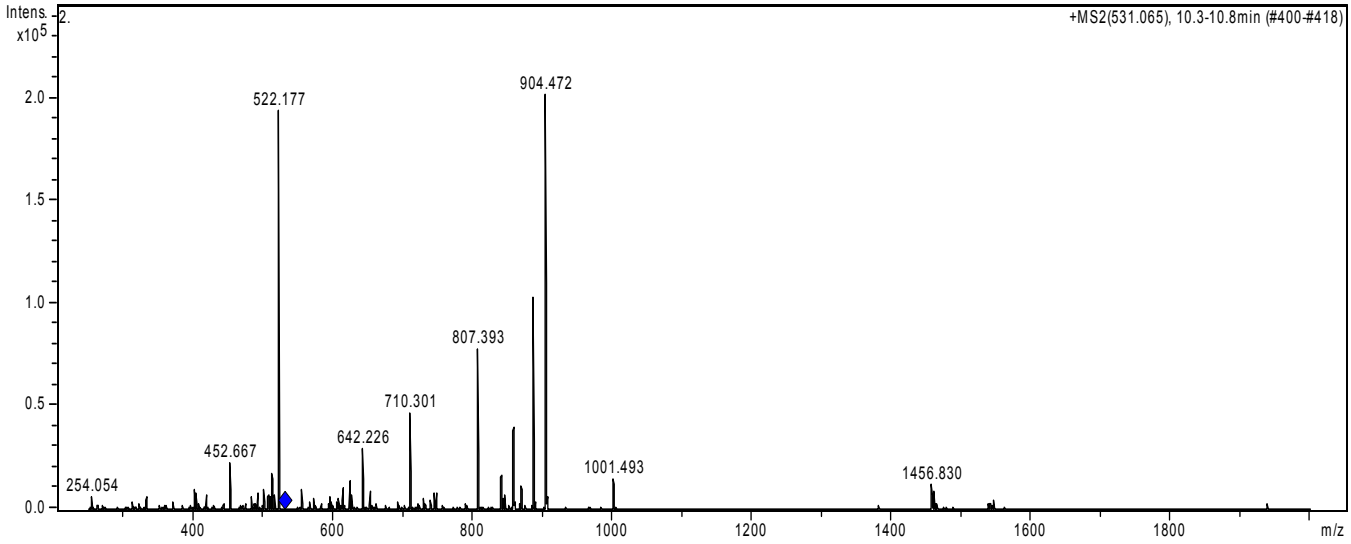

Fig. 17d MS/MS spectrum showing daughter ions from the fragmented parent ion with m/z-value 530.916

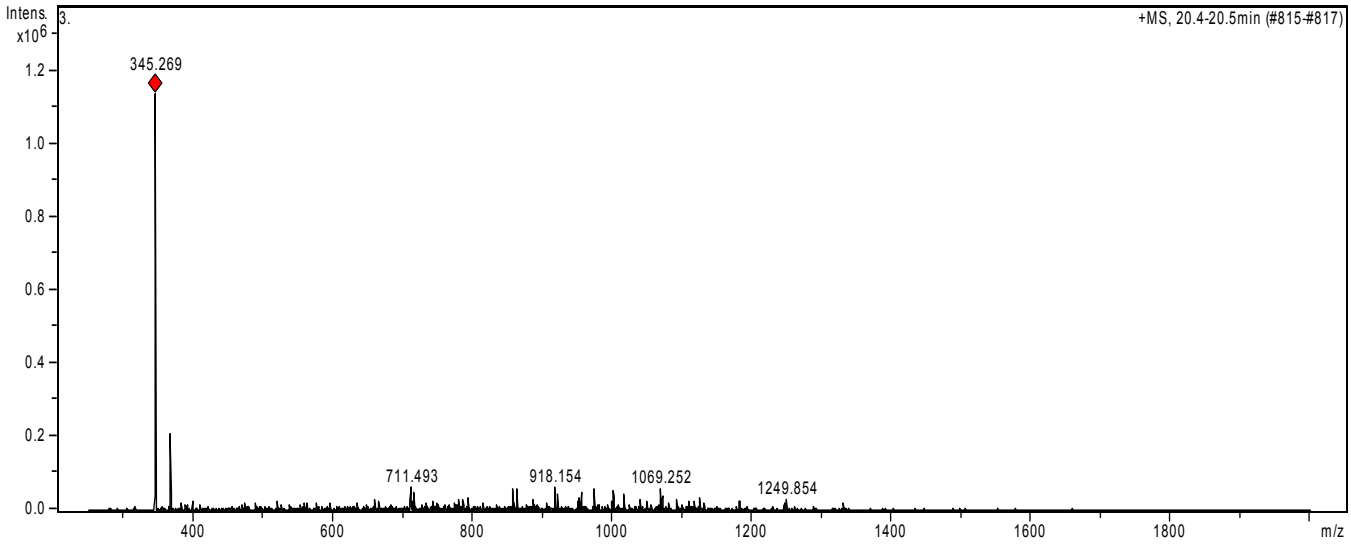

Fig. 17e MS spectrum showing the *triply* charged parent ion chosen to be fragmented

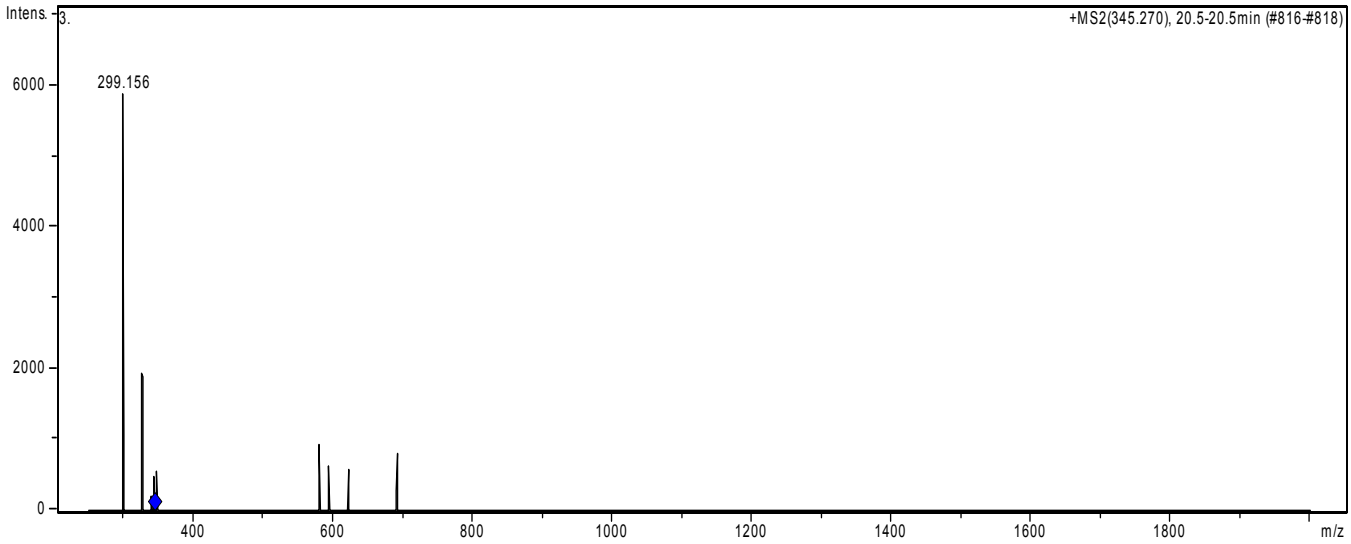

Fig. 17f MS/MS spectrum showing daughter ions from the fragmented parent ion with m/z-value 345.269

The best fragmentation pattern (with most daughter ions) was obtained with the doubly charged parent ion (Fig. 17d) and this was therefore used in the database search. The figure below (Fig. 18) shows the annotation of the profile spectra performed by the BioTools software before the database search. Here, two sequences are painted, depending on if the starting point is at the N- (y) or C- (b) terminal. The one letter code and other symbols for the amino acids are presented in appendix 6.3.

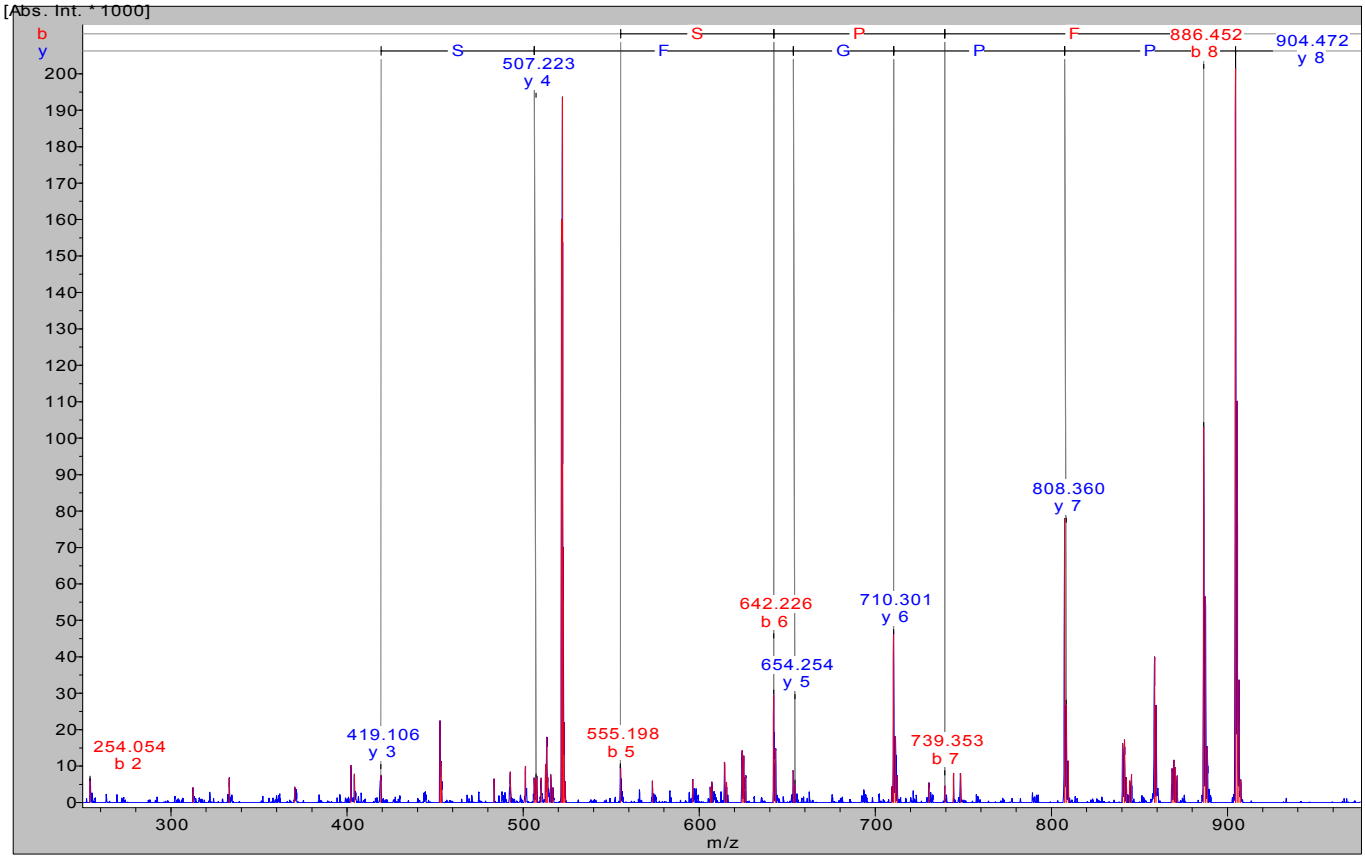

Fig. 18 Annotation of the profile spectrum from the fragmented (doubly charged) parent ion.

The return of the search is first displaying a "Probability Based Mowse Score" (Fig. 19), which is a histogram presenting the number of hits and the hits' individual score.

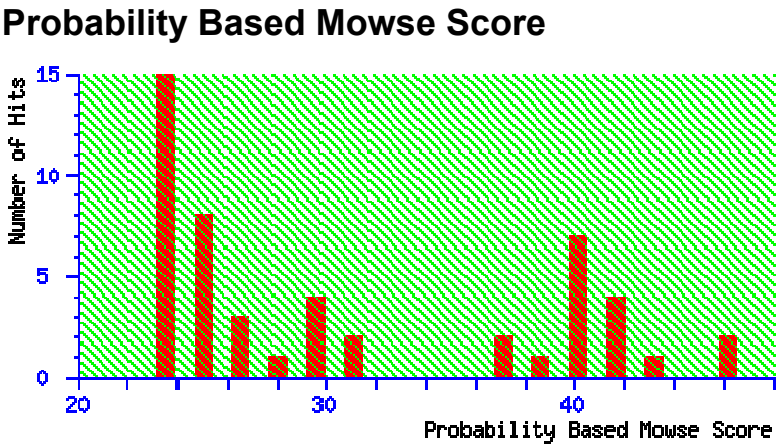

Fig. 19 Probability Based Mowse Score. Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 74 are significant (p<0.05).

Further the index list of suggested compounds is listed by decreasing score.

Table 7 Index. A list of the hits with the highest score

Index

	Accession	Mass	Score	Description
1	A61363	1060	46	bradykinin - common frog
2	S65433	1060	46	bradykinin - horn fly (fragment)
				KALLIDIN (DEKAPEPTIDE , INTERMEDIATE PEPTIDE)
3	E972904	1188	43	- unidentified.
4	E977704	1180	42	OLIGOPEPTIDE.- vectors.
5	A61365	1336	42	phyllokinin - Rohde's leaf frog
6	S13279	1260	42	Ile-Ser-bradykinin - human (fragment)
				ARTIFICIAL KALLIDIN FRAGMENT FROM PATENT
7	E972905	1299	42	EP0207402.- unidentified.
8	A61360	1345	40	vespakinin M - hornet (Vespa mandarinia)
9	A61359	1343	40	vespakinin X - hornet (Vespa xanthoptera)
				PROTEIN SEQUENCE 5 FROM PATENT NUMBER
10	E972907	1299	40	EP0207402.- unidentified.

By following the link A61363 from the top of the list information about (amino acid) sequence coverage is displayed.

Table 8 The sequence of the hit with the highest score (bradykinin – common frog) is displayed

No enzyme cleavage specificity

Sequence Coverage: **100%**

Matched peptides shown in **Bold Red**

1 **RPPGFSPFR**

Since the hit with highest score was the same as the injected compound (bradykinin), the Mascot database functions as expected.

6.3 Amino acids and their symbols

Table 9 Formulas and molecular weights

Formulas and Molecular Weights

Table A-1 Amino acid residue masses (mono-isotopic and average) together with 3- and 1-letter code and elemental composition

Name	Symbol	S.	C	H	N	O	S	Monoisotopic Mass	Averaged Mass
Alanine	Ala	A	3	5	1	1	0	71,03712	71,079
Cysteine	Cys	C	3	5	1	1	1	103,0092	103,145
Aspartic acid	Asp	D	4	5	1	3	0	115,027	115,089
Glutamic acid	Glu	E	5	7	1	3	0	129,0426	129,116
Phenylanaline	Phe	F	9	9	1	1	0	147,0684	147,177
Glycine	Gly	G	2	3	1	1	0	57,02146	57,052
Histidine	His	H	6	7	3	1	0	137,0589	137,141
Isoleucine	Ile	I	6	#	1	1	0	113,0841	113,159
Lysine	Lys	K	6	#	2	1	0	128,095	128,174
Leucine	Leu	L	6	#	1	1	0	113,0841	113,159
Methionine	Met	M	5	9	1	1	1	131,0405	131,199
Asparagine	Asn	N	4	6	2	2	0	114,0429	114,104
Proline	Pro	P	5	7	1	1	0	97,05277	97,117
Glutamine	Gln	Q	5	8	2	2	0	128,0586	128,131
Arginine	Arg	R	6	#	4	1	0	156,1011	156,188
Serine	Ser	S	3	5	1	2	0	87,03203	87,078
Threonine	Thr	T	4	7	1	2	0	101,0477	101,105
Valine	Val	V	5	9	1	1	0	99,06842	99,133
Tryptophan	Trp	W	#	#	2	1	0	186,0793	186,213
Tyrosine	Tyr	Y	9	9	1	2	0	163,0633	163,176

6.4 Instrumental settings in the mass spectrometer

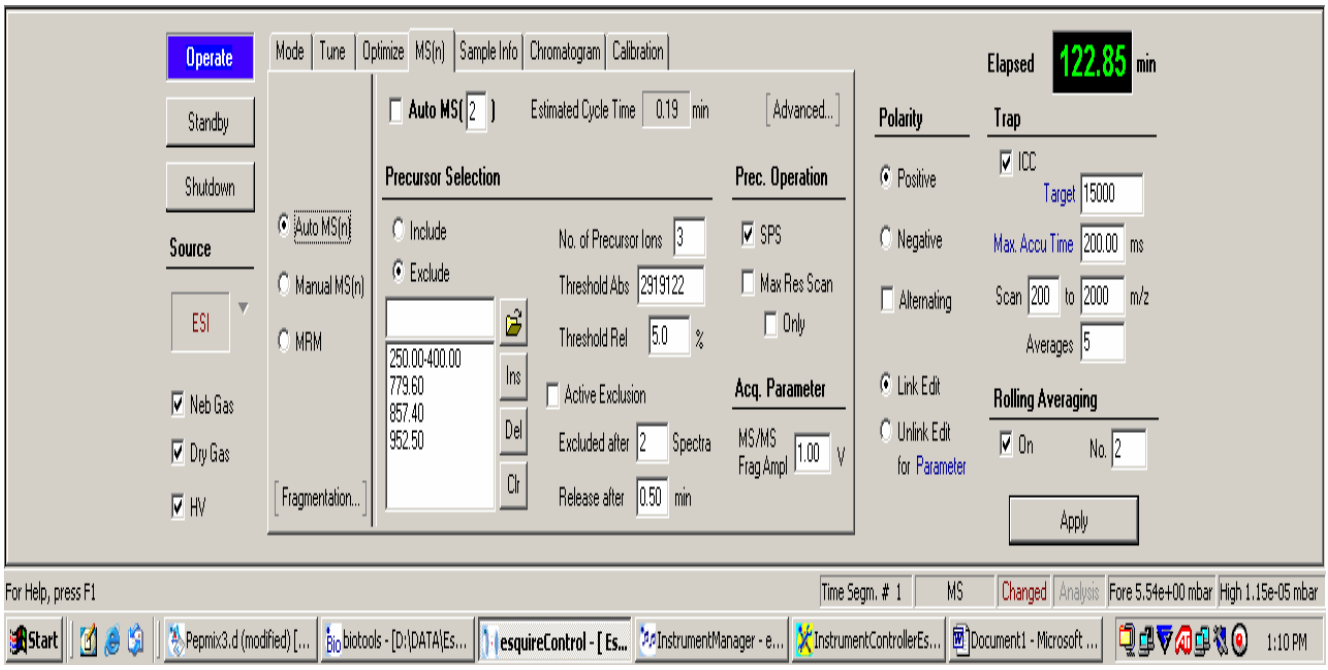

Fig. 20a: MS-conditions (MSn page)

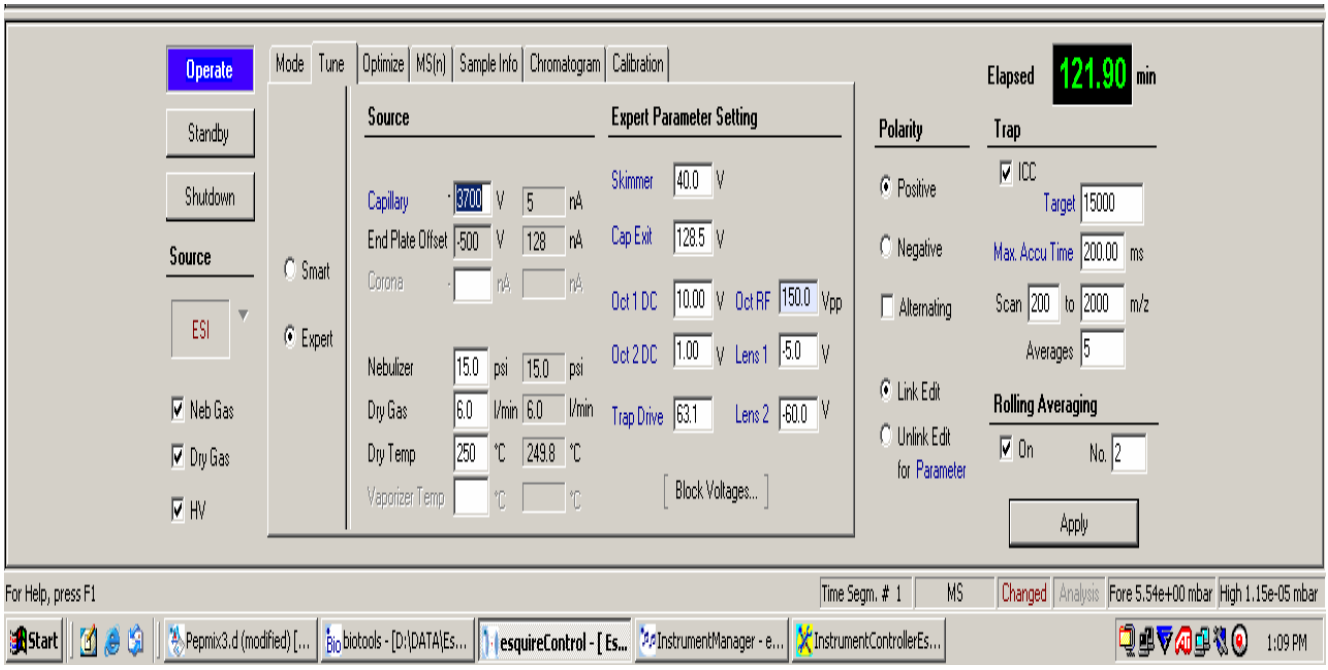

Fig. 20b: MS-conditions (Tune page)