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of Candidata Scientiarum

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Stability of angiotensin II and bradykinin solutions investigated by capillary liquid chromatography

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"Music speaks what words cannot"

Contents

Preface

This graduate study has been carried out in the Department of Chemistry at the University of Oslo from January 2003 to April 2005. My supervisors during the study were Cand.Scient. Steven R. H. Wilson, Professor Elsa Lundanes and Professor Tyge Greibrokk. Parts of the preliminary work were presented at the 16. Norwegian Symposium of Chromatography in Sandefjord in January 2004 (see the Appendix for a copy of the presentation poster). Parts of the study will be submitted for publication. Some of the theoretical courses for the degree were taken at the Universität Leipzig, Germany the summer 2003.

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I also would like to thank Hege Lynne and her staff at the analytical course lab for always being helpful in finding equipment and chemicals. I also want to thank the electronic and instrumental workshops at the Chemistry Department for help with old detectors, pumps and other equipment.

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Finally I would like to thank all my musician friends for a great time outside Blindern, and my family for all their love and support. Thank you, Espen, for being a loving and patient husband, and for sharing both the tears and the laughter.

Oslo, Norway, 6. December 2005

Aase Marit Sørum Ramton

Abstract

A robust, fast and sensitive capillary column switching reversed-phase (RP) liquid chromatography (LC) method with back flushing of the pre-column and ultraviolet (UV) detection has been developed to investigate the stability of angiotensin II and bradykinin stored in different matrices and by different temperatures. The loading mobile phase (MP) containing acetonitrile (AcN) – H_2O – formic acid (FA) (2 : 97.95 : 0.05, v/v/v) was delivered isocratically at a flow rate of 0.200 mL/min. Manual injections of 200 µL were made with an external loop, and the samples were loaded on a HotSep Tracy column (Kromasil C_{18}; 5 x 1 mm (inner diameter (ID)), 5 µm). After a loading time of 3.5 min the analytes were back flushed from the pre-column and transferred to the separation column, a HotSep Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm), using an eluent consisting of AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added, delivered at a flow rate of 0.005 mL/min. In-line UV detection was performed at 210 nm. The limits of detection (LOD) were ~10 ng/mL for bradykinin and below 1 ng/mL for angiotensin II. The robustness of the system is discussed.

An isocratic and a gradient eluting nano LC-UV system and a column switching gradient nano LC-UV method, all with a Kromasil C_{18} nanocolumn (150 x 0.1 mm (ID), 3.5 µm), were developed for bradykinin determination.

The signal responses for bradykinin dissolved in H_2O and 20 % AcN with FA added in different amounts were compared using direct infusion electrospray time-of-flight mass spectrometry (direct infusion-ESI-TOF-MS) at different capillary voltages.

The ratio of theoretical dilution of two different capillary columns was calculated and compared to the experimental dilution ratio obtained with those columns using capillary RPLC coupled to an electrospray time-of flight mass spectrometer (ESI-TOF-MS).

Abbreviations and definitions

a.a.	ammonium acetate
AcN	acetonitrile
a.f.	ammonium formate
ang II	angiotensin II
ANOVA	analysis of variance
As	asymmetry
α	selectivity
Bl.	blank
brad	bradykinin
C18, C_{18}	octadecyldimethyl-silane
cLOD	concentration limit of determination
conc.	concentration
D	theoretically dilution
df	degree of freedom
ESI	electrospray ionization
F	freezer
FA	formic acid
H	Peak height
HFBA	heptafluorobutyric acid
ID	inner diameter
Inj.	injection
Inj. seq	injection sequence
LC	liquid chromatography
K	refrigerator
m/z	mass to charge ratio
MeOH	methanol
mLOD	mass limit of detection
MP	mobile phase
MS	mass spectrometry

N/m	plate number per meter
neu	neurotensin
OD	outer diameter
OHAc	acetic acid
P1	pump 1; loading pump
P2	pump 2; analytical pump
PE	polyethylene
PP	polypropylene
R	room temperature
RP	reversed-phase
Rs	resolution
RSD	relative standard deviation
s	absolute standard deviation
S/N	signal to noise ratio
SP	stationary phase
SPE	solid-phase extraction
TFA	trifluoroacetic acid
TOF	time-of-flight
t_R	retention time
UV	ultraviolet
V1	valve 1; injection valve
V2	valve 2; switching valve
$\overline{X}$	mean, average

1. Introduction

1.1 Bradykinin and angiotensin II

Proteins and peptides are compounds that consist of long chains of 20 different amino acids connected to each other through peptide bindings. Molecules with less than 50 amino acids are often referred to as peptides, while the larger molecules are called proteins [1]. Kinins are peptides that are produced from a plasma protein (called kininogen) under influence from specific proteases called kallikreins. Among the kinins is the nonapeptide bradykinin (brad) with the following amino acid sequence: arg - pro - pro - gly - phe - ser - pro - phe – arg. The molecular structure of bradykinin is shown in Figure 1. The molecular mass of brad is 1060.2206 g/mol. Kinins have a very short half-life; they are inactivated through corresponding peptidases after a few seconds. The Angiotensin-Converting-Enzyme (ACE, also called kininase) is of significant importance in this process (Figure 2) [2].

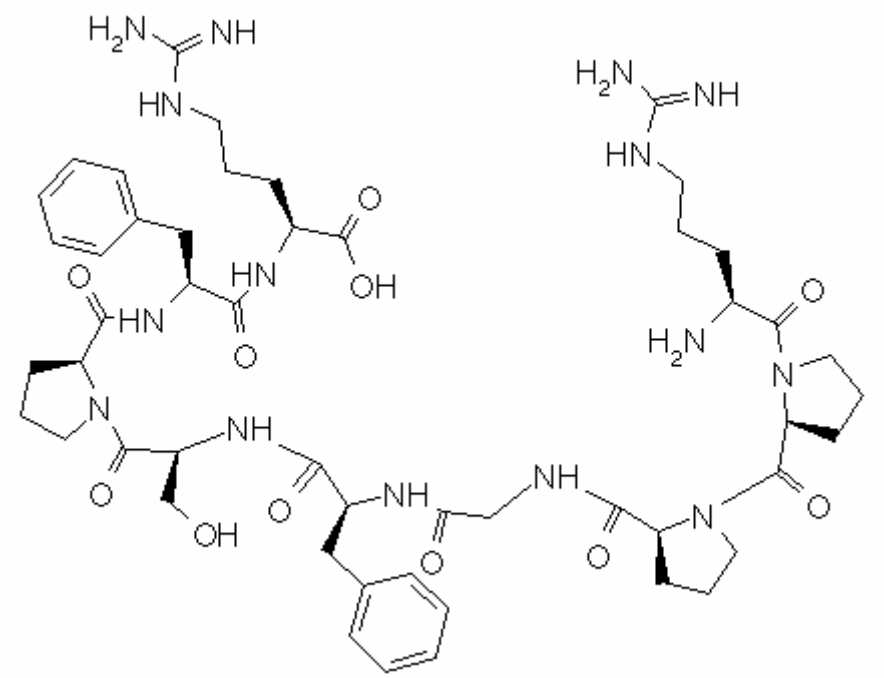

Figure 1: The bradykinin molecular structure [3].

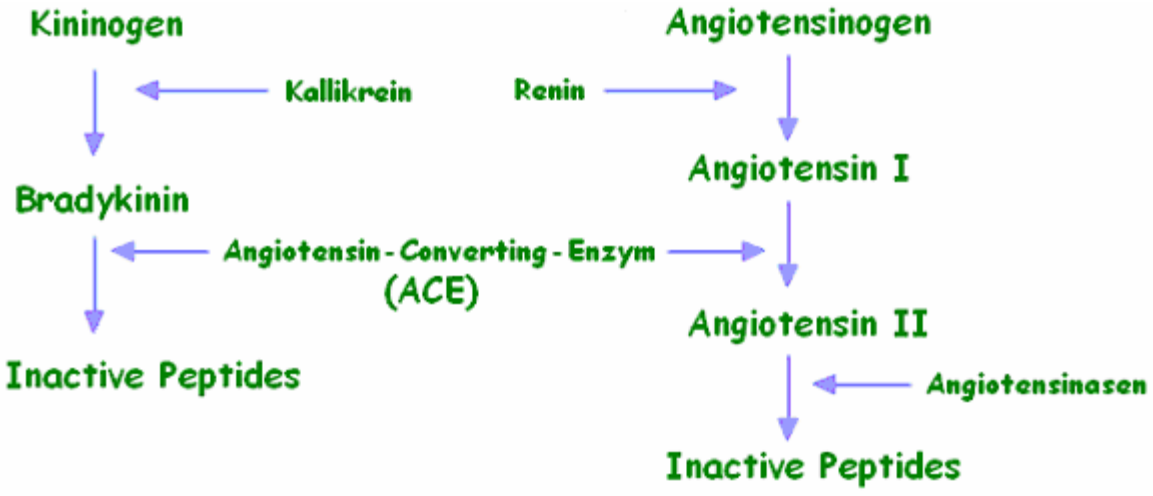

Figure 2: Biosynthesis and breakdown of bradykinin and angiotensin II [2, 4].

In humans, cats and rats contracting muscles release kinins. Bradykinin contributes, among other things, to increased blood pressure, heart rate and blood flow in the muscles. It has also been observed that bradykinin increases the glucose utilization of the working muscles [5]. In order to investigate bradykinin's possible contribution to muscle pain, it is useful to monitor the kinin level in muscle tissues. This has been done by the sampling of substances from the muscle tissue of rats using micro dialysis, with Ringer's acetate as dialysis solvent (Experimental, Table 2). Methods, including radioimmunoassay (RIA, described in Section 1.2) [6] and LC-ESI-MS [7], have been used for the quantification of the kinins in the dialysate.

Angiotensin II (ang II) is an octapeptide with the amino acid sequence: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (Figure 3), that is created when the biologically inactive angiotensin I is cleaved by the ACE, the same enzyme that catalyses the breakdown of bradykinin into inactive peptides (Figure 2). The molecular mass of ang II is 1181.359 g/mol.

Ang II is one of the most potent vasoconstrictors known, and thus important for the maintenance of blood pressure and volume. Ang II is also among the hormones that regulate water and electrolyte content and it also facilitates the release of norepinephrine [2, 8].

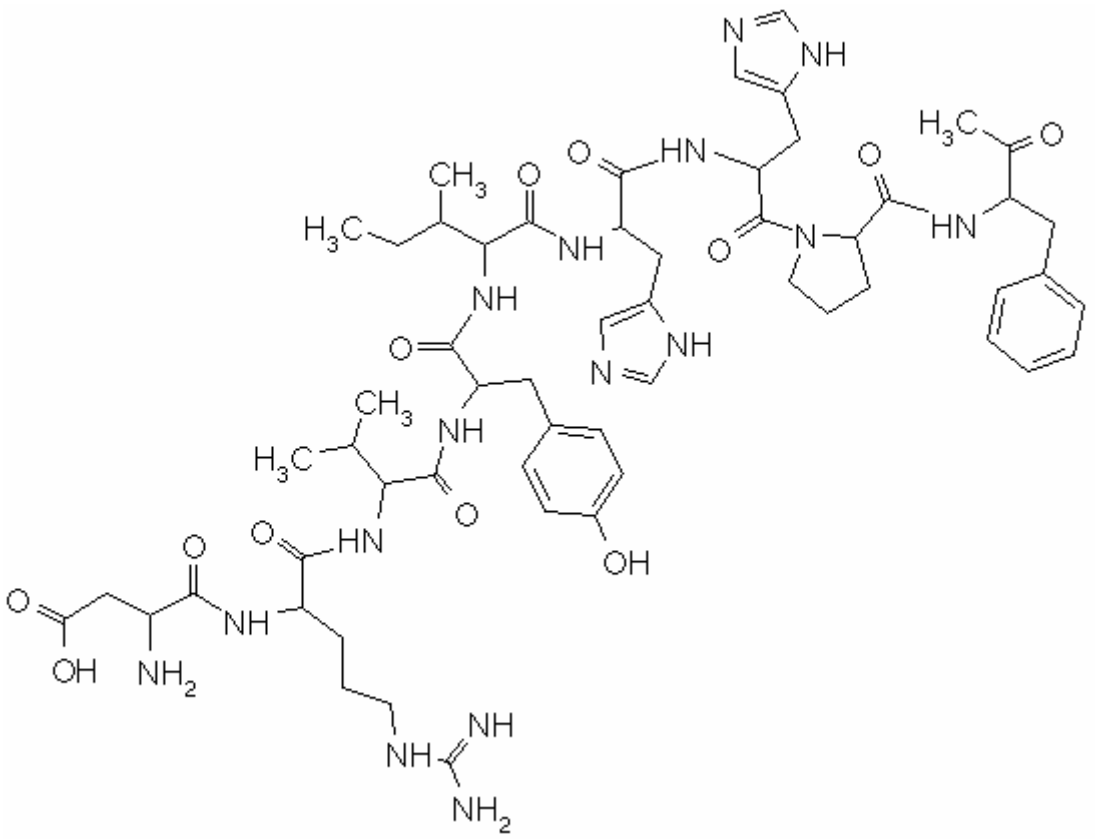

Figure 3: The angiotensin II molecular structure [3].

E. R. Vickers et al [9] investigated the stability of bradykinin in saliva. 0.5 mL saliva donated from 6 volunteers (three females and three males between the ages of 23 and 40 years) was added to 0.5 mL 0.1 M orthophosphoric acid and the pH was measured before the solution was vortex-mixed and centrifuged. Each of the 6 samples was divided into two aliquots and added bradykinin giving an absolute mass of 50 and 500 ng per HPLC injection, respectively. Then all samples were subdivided into two aliquots and stored at 20ºC for 5 days and 4ºC for 60 days, respectively. The stability was monitored during the period using a HPLC-UV system at room temperature with a mobile phase containing AcN – H_2O – trifluoroacetic acid (TFA) (19:80.9:0.1, v/v/v) delivered isocratically at a flow rate of 0.4 mL/min. The column used was C_8 (7 µm particles, 100 Å pore size, 150 x 3.9 mm (ID)). UV detection was performed at 220 nm. Bradykinin in these solutions was found to be stable for 5 days stored at 20ºC and for 60 days when stored at 4ºC.

1.2 Methods of determination

There are three techniques that are often applied for peptide and protein determination: two-dimensional (2D) gel electrophoresis, liquid chromatography, and capillary zone electrophoresis (CZE). LC and CZE are suitable for both protein and peptide separation, while 2D gel electrophoresis is only suitable for protein separation and large peptides (> 10 kDa). Capillary electrophoresis (CE) has higher separation efficiency than LC, while repeatability is better and peak capacity is higher for LC. Both CE and LC are suitable for on-line detection and automation, and both perform high protein recoveries and fast separation times [10-12]. However, CE suffers from poor concentration sensitivity, due to the limited sample volume that can be loaded. Short path lengths in the absorbance-related detectors make the method even less sensitive. To overcome these problems creating longer path lengths should be considered, for example by using Z-cells or bubble cells, or on-line preconcentration techniques [13-15]. Another disadvantage is that capillary electrophoresis, capillary zone electrophoresis and capillary electro-chromatography are not easily coupled with MS [14]. On-line preconcentration for CE of bradykinin, angiotensin and related peptides can be done when using a C_{18} concentrator cartridge with a dynamic pH junction within the capillary that focuses the analytes [15]. Matsubara et al reported separation of eleven angiotensin II analogues using capillary electrophoresis [16].

CZE has been applied for separation of kinins (bradykinin and similar substances) with laser fluorescence detection [17] and purity profiling of among others bradykinin and angiotensin I with UV detection [18].

Several LC detection methods for angiotensin II and bradykinin have been reported: for example UV detection [9, 19-21] and MS detection, including both ESI-MS [7, 9, 19, 22] and MALDI-MS (coupled off-line to microbore HPLC) [23]. Also HPLC-fluorescence methods for angiotensin II and bradykinin detection are reported [24-26] all including derivatization in order to make the peptide fluorescent. A disadvantage is that the derivatization may be rather time consuming when doing it off-line.

Radioimmunoassay (RIA) is an extremely sensitive and selective method used to test hormone levels in the blood. It involves mixing a radioactive antigen with its antibody, then adding an unlabeled (or "cold") antigen in known quantities and finally measuring the amount of labelled antigen displaced. Several methods for determination of bradykinin in blood and plasma by radioimmunoassay have been reported [27-29]. Voelker and co-workers reported a method that coupled solid-phase extraction (SPE), HPLC and radioimmunoassay (RIA) for quantifying angiotensin II in plasma [30].

The degradation of peptides and proteins can be of both physical and chemical nature. Changes of the peptides and proteins, due to physical instability, involves the unfolding of the peptides/proteins and the aggregation of the compounds (while the chemical structure remains the same); these can be caused by changes in temperature, ionic strength, pH or other factors [31]. Changes in molecular size and shape, due to the unfolding process, can be studied with size exclusion chromatography [32, 33] and electrophoresis [34, 35]. Among other methods that can be applied to monitor the unfolding process are spectroscopic methods, like fluorescence spectroscopy, since the spectroscopic properties of the proteins/peptides are changed, and Fourier transform infrared (FT-IR) [36] and ^{1}H-NMR, that can measure the changes in chemical shifts.

The chemical instability of peptides and proteins (changes of the primary structure), is caused by degradation reactions such as oxidation/reduction, deamidation, hydrolysis, racemisation, arginine conversion and β-elimination [31].

Oxidation, one of the major degradation pathways, often gives hydrophilic products; mass changes, as well as changes in spectroscopic and electrochemical properties, may also be observed [31]. In RP-HPLC-UV the oxidation products have a shorter retention time compared to the original protein/peptide, and UV detection, while relatively little specific, is widely applied [37, 38]. Fluorescence [38-40] and electrochemical detection [41] have also been reported. Protein/peptide oxidation can also be studied by single or tandem MS [42, 43], which provide information on the molecular masses and how the products fragmentize.

Reduction of proteins/peptides gives more hydrophilic products than the original compound for peptides and smaller proteins. RP-HPLC and electrophoresis are suitable for study of this process.

1.3 Micro and capillary chromatography

Horváth et al introduced microcolumn LC in 1967 [44]; the breakthrough, however, did not come before the mid 1970's [45]. Narrow column diameter makes connecting to MS without flow splitting easier. Miniaturized LC requires less solvents, reagents and packing materials [46, 47], and limited sample amounts are needed [45]. One of the main advantages of using narrower columns is enhanced detection performance with the use of concentration sensitive detection devices. This is due to reduced chromatographic dilution [47-50]. In addition increased sensitivity of electrospray ionization has been observed due to more efficient ionization at lower flow rates [14]. The decreased inner diameter of the columns also lowers the heat capacity and thus permits much easier control of column temperature and more rapid response to temperature gradients [46, 47]. Although micro and capillary LC has these advantages over conventional LC, it also has disadvantages. Extra column band broadening, mostly originating from dead volumes in the injection valve, connecting tubes and/or detection flow cell, have to be minimised to prevent loss of efficiency and resolution of the packed capillary and micro LC columns [51]. Connecting tubes with narrower ID will lead to a reduction of the dead volume [14]. Another disadvantage is that small injection volumes or masses cause decreased detection sensitivity [45]. On-column focusing techniques may eliminate this problem by using sample solvent with low eluent strength compared to the mobile phase used in order to focus the analytes in a small plug on the column entrance [51-53]. However, the most promising way to increase the sensitivity is sample enrichment using a switching valve system. This is described in Section 1.4.

To further increase the sensitivity, nano LC can be used. The first commercial nano LC system was produced by LC Packings (Sunnyvale, CA, USA) in the mid 1990's. This

technique often requires flow splitting of the mobile phase prior to injection. By UV detection, according to Beer-Lambert Law (A = εbc = abc), the absorbance is proportional to the concentration and the optical path length. If the path length is too long, diffusion of the analyte will occur at low flow rates (i.e. nano LC-UV), causing peak broadening. To avoid this problem UV-detectors with U- or Z-shaped configurations can be used to reduce the dead volumes, while maintaining a relatively long optical path length [54].

In proteomics nano LC is often connected to MS and MS/MS (tandem MS). ESI can be connected on-line to LC and is therefore traditionally the most used ionization technique for LC [45]. An electrospray interface with a narrower inner diameter of the needle tip (usually 1-3 μm) is called nanospray; a reduction of the flow rate (usually < 100 nL/min) is achieved [55]. The primary application of capillary and nanoscale LC-ESI-MS have been in protein and peptide analysis [45]. Schmelzer et al used both LC-ESI-ion trap-MS and nano ESI-qTOF-MS to characterize human skin peptides produced by proteolytic digestion [56].

Saito et al [47] classified the LC columns according to their internal diameter (Table 1). As can be seen from the table; columns with an ID of 0.01 – 0.1 mm are classified as nanocolumns.

Table 1: LC classified according to the ID of the columns [47].

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

1.4 Large volume injection

To increase the concentration sensitivity of miniaturised LC systems, large volume injections using column switching technique with pre-column and analytical column could be applied [51]. To prevent broadening of the peaks, the switching valve must provide a low dead volume flow path. The valve must also be capable of high-pressure operations without deterioration [57]. As observed by Vissers et al [51] on-column focusing (i.e. dissolving the sample in a non-eluting solvent) must be applied when injecting large sample volumes in order to avoid band broadening effects. Further the loading mobile phase must be a weak eluent with respect to the separation mechanism on the second column and it also must be miscible with the separation mobile phase [57]. This allows improvements of the limits of detection (LOD) by several orders of magnitude.

As Vissers (1999) emphasized, large volume injections are routinely applied for the analysis of protein and peptide samples [45].

1.5 Aim of study

In our research group we are working towards the determination of the concentration of neurotransmitters in rat brain tissue, bradykinin and similar compounds in muscle tissue, and other projects involving peptides. Thus, it is important to know how to store peptides solutions without decrease in concentration.

Hence the aim of this study was to develop a fast, robust, selective and sensitive chromatographic method for the determination of peptides with bradykinin and angiotensin II as model substances, and measure their concentration after storage in different water matrices at different temperatures.

The robustness of a gradient capillary LC-UV column switching system was investigated and discussed in detail. Additionally, preliminary testing of nano LC systems to be used in further proteomic studies was done. Finally MS-conditions for peptide analysis were optimized.

2. Experimental

2.1 Chemicals and reagents

HPLC grade AcN and glass distilled grade toluene was obtained from Rathburn Chemicals Ltd. (Walkerburn, UK). Grade 1 water was obtained from a Milli-Q plus 185 system (Millipore, Bedford, MA, USA). Bradykinin, angiotensin II and neurotensin were provided by Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate ($NH_4^+CH_3COO^-$, analytical grade (AG)), acetic acid glacial 100% (OHAc, analytical grade) and trifluoroacetic acid (for spectroscopy) were provided by Merck (Darmstadt, Germany). Water (HPLC grade), ammonium formate ($NH_4^+HCOO^-$, analytical grade for mass spectroscopy) and 50 % formic acid (FA, analytical grade for HPLC) were obtained from Fluka (Steinheim, Germany), uracil from Nutritional Biochemicals Corporation (Cleveland, OH, USA) and carbon tetrachloride (CCl_4) from Prolabo (Paris, France). Helium (4.6, 99.996 %) and nitrogen (4.0, 99.99 %) were provided by AGA (Oslo, Norway). Ringer's acetate was made in-house by solving the prescribed salts (Table 2) in HPLC water.

Table 2: Salt content and electrolytes in Ringer's acetate.

Salts pr 1000 mL solution:	**Mass**	**Producer**	**Electrolyte**		
Magnesium chloride hexahydrate (AG)	203 mg	Fluka	Magnesium	1	mmol/L
Calcium chloride dehydrate (AG)	294 mg	Merck	Calcium	2	mmol/L
Potassium chloride (AG)	298 mg	Merck	Potassium	4	mmol/L
Sodium acetate (AG)	4.08 g	Merck	Acetate	30	mmol/L
Sodium chloride (AG)	5.84 g	Merck	Chloride	110	mmol/L
			Sodium	130	mmol/L

2.2 Columns and column preparation

The columns that were compared in the preliminary testing are listed in Table 3.

Table 3: Testing of columns for peptide separation.

Type	Manufacture	ID [mm]	Length [cm]	Pore size [Å]	Particle size [µm]
(1) Poroshell 300 SB-C18 (short)	Agilent Technologies, CA, USA	0.3	7.5	300	5.0
(2) HotSep Kromasil C18-100	G&T Septech AS (Kolbotn, Norway)	0.3	5.0	100	3.5
(3) ProntoSIL C18-300	G&T Septech AS	0.3	5.0	300	3.0
(4) Poroshell 300SB-C18 (long)	Agilent Technologies	0.5	15.0	300	5.0
(5) ACE (packed in-house)	Advanced Separation Technologies (Whippany, NJ, USA)	0.3	10.1	100	3.0
(6) ACE (packed in-house)	Advanced Separation Technologies	0.3	9.9	100	3.0

Other analytical columns that were used during this work were Kromasil C_{18} (150 x 0.1 mm (ID), 3 µm) purchased from G&T Septech AS and Zorbax SB-C18 (150 x 0.5 mm (ID), 5 µm) purchased from Agilent Technologies. HotSep Tracy Kromasil C_{18} pre-columns (5 x 1 mm (ID), 5 µm) were purchased from G&T Septech AS.

The ACE C_{18} analytical column (Table 3), and the Kromasil C_{18} (Eka Nobel; Bohus, Sweden) pre-column used for the preliminary testing were slurry packed using AcN and grade 1 water (70:30, v/v) as packing fluid. The Kromasil C_{18} pre-columns were ~ 5.5 cm long and had a particle size of 10 µm and a pore size of 100 Å. The fused silica capillary (Polymicro Technologies Inc.; Phonex, AZ, USA) used to pack the columns had an ID of 320 µm and an outer diameter (OD) of 450 µm.

A suspension of ~30 mg of the packing material and 200 µL carbon tetrachloride was sonicated for ~10 min in an ultrasonic bath. A desired length of the capillary was cut and a nut, graphite/vespel ferrule (FS1.4), filter (2SR1) and union (1/16) obtained from Valco Instruments Co., Inc. (Houston, TX, USA) were installed at one end. The other end of the capillary was connected to a packing chamber (made in-house) through a nut and ferrule. The sonicated slurry of packing material was then transferred to the packing chamber using a syringe made in-house of a Omniflex 2 mL plastic syringe connected via an union, nut and ferrule to a ~12 cm long fused silica capillary (320 µm ID/450 µm OD). The other end of the packing chamber was connected to an ISCO Model 100 DM syringe pump with an ISCO Series D pump controller (Teledyne Isco Inc., Lincoln, NE, USA). The pump, delivering the packing fluid containing AcN - grade 1 H_2O (70:30, v/v), was preset to start at 100 bar and increase the pressure at 100 bar/min until 650 bar was reached. The vent between the pump and packing chamber was opened, and when equilibrium at 100 bar was obtained, the program was started. The pressure was held at 650 bar for ~15 min. Then the vent was closed, letting the column depressurize to atmospheric pressure (~20 min). The rest of the system was depressurized using a program with a rate of -100 bar/min. Finally, the packing chamber was disconnected from the pump, and the column was disconnected from the packing chamber, and nut, ferrule, filter and union were installed at the open end of the column. As Saito et al emphasize in their article [47] attention should be paid during the packing process in order to avoid any undesirable void volume in the column as well as obtaining a stable packing structure.

2.3 Instrumentation

2.3.1 Reversed-phase capillary LC-UV switching system

A reversed-phase capillary LC switching system with back flushing of the pre-column was used during this study (a sketch is shown in Figure 4). Capillaries used were either fused silica capillaries or steel tubing obtained from Polymicro Technologies (Phonex, AZ, USA) and Supelco (Bellefonte, PA, USA), respectively; the types and dimensions are shown in Figure 4. External injection loops with volumes of 50, 75, 100, 150, 200 and 300 µL were prepared using steel tubing. All connections were made with ZU1C unions, FS1.4 graphite/vespel ferrules and nuts purchased from Valco.

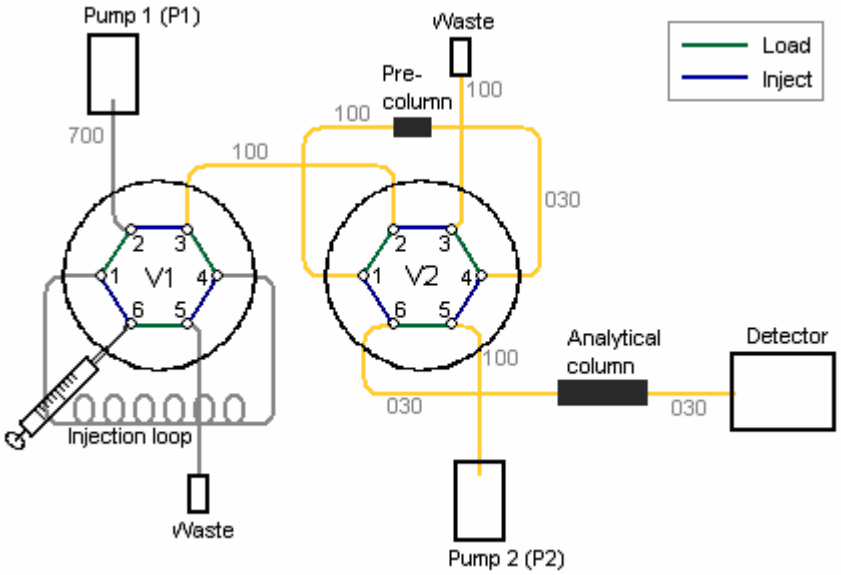

Figure 4: Schematic drawing of the capillary LC column-switching system. Yellow; fused silica capillaries and grey; steel tubing. Dimensions: 030 = 30 µm ID, 100 = 100 µm ID and 700 = 700 µm ID.

Pump 1 (P1) delivered the non-eluting loading mobile phase and pump 2 (P2) delivered the eluting mobile phase. Two 6-port valves were used as valve 1 (V1) and valve 2 (V2). With both V1 and V2 in position 1 the external injection loop was filled manually using a 1 mL sterile BD Plastipak syringe obtained from Becton Dickinson UK Ltd. (Oxford, UK). By switching V1 to position 2 the sample was loaded on the preconcentration column by the loading mobile phase at a flow rate of 0.070 mL/min when Kromasil C_{18} (55 x 0.320 mm (ID), 10 µm, packed in-house) were used as pre-column or at 0.200

mL/min when HotSep Tracy Kromasil C_{18} (5 x 1 mm (ID), 3.5 µm) were used as pre-column. After 3.5 min, V2 was also switched to position 2, and thus the analytes were back-flushed from the pre-column and transferred to the analytical column by the separation mobile phase.

The outlet of the analytical column was connected to a Linear UVIS UV-detector (Linear Instruments, Reno, NV, USA) equipped with a 100 µm ID fused capillary for in-line detection at a wavelength of 210 nm. The signals from the detector were converted by a Perkin Elmer Nelson 900 Series Model 941 interface (Perkin Elmer, Boston, MA, USA) and transferred to a PC with TotalChrom Navigator (Perkin Elmer) where the chromatogram could be further processed.

2.3.1.1 System 1 (isocratic capillary LC-UV column switching system)

System 1 is an isocratic system used for preliminary investigation of different MP (Section 3.1.3). Two isocratic Hitachi LC pumps from Merck, P1 and P2, were delivering the loading MP at a flow rate of 0.100 mL/min and the analytical MP at a flow rate of 0.005 mL/min, respectively. Two 6 port valves, a Rheodyne 7000 (Rohnert Park, CA, USA) and a ChemInert C2-1006 from Valco, operated as V1 and V2, respectively. The pre-column used was a Kromasil C_{18} column (55 x 0.320 mm (ID), 10 µm, packed in-house), and the analytical column was a HotSep Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm).

2.3.1.2 System 2 (gradient capillary LC-UV column switching system)

A HotSep Tracy Kromasil C_{18} column (5 x 1 mm (ID), 5 µm) and a HotSep Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm) were used as pre-column and analytical column, respectively. An isocratic Hitachi LC pump (Merck) delivered the non-eluting loading mobile phase. The loading MP used when testing the maximum injection volume contained AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added, delivered at a flow rate of 0.200 mL/min. For stability testing the loading MP consisted of AcN – H_2O – FA (2 : 97.95 : 0.05, v/v/v). An Agilent 1100 capillary gradient pump (Palo Alto, CA, USA;

pump 2) with an incorporated on-line vacuum degasser delivered the eluting analytical mobile phase from two chambers; A and B. The mobile phase used was as follows: In chamber A: AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+HCOO^-$ and 0.05 % formic acid added, pH = 3.1; In chamber B: AcN – H_2O (90:10, v/v) with 20 mM $NH_4^+HCOO^-$ and 0.05 % formic acid added, pH = 5.2. The gradient used was: 10-50 % B in 5 min and then held at 50 % B in 2 min at a flow rate of 0.005 mL/min. Also here a Rheodyne 7000 and a ChemInert C2-1006 were used as V1 and V2, respectively.

2.3.2 Nano LC and direct-infusion MS systems

2.3.2.1 System 3 (nano LC-UV)

Injections were performed with a ChemInert C4-0004 (Valco) injection valve equipped with a 20 nL internal loop. An Agilent 1100 capillary gradient pump delivered the eluting analytical MP. The mobile phase used was as follows: In chamber A: AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+HCOO^-$ and 0.05 % formic acid added, pH = 3.1; In chamber B: AcN – H_2O (90:10, v/v) with 20 mM $NH_4^+HCOO^-$ and 0.05 % formic acid added, pH = 5.2. For isocratic elution 16 % B with a flow rate of 0.3 µL/min was used, and for gradient elution 0-100 % B in 10 min was delivered at a flow rate of 0.6 µL/min. As separation column, a Kromasil C_{18} nanocolumn (150 x 0.1 mm (ID), 3.5 µm) from G&T Septech was used. The detection was performed at a wavelength of 210 nm using a Linear UVIS UV detector, and the data was processed by TotalChrom Navigator.

2.3.2.2 System 4 (gradient nano LC-UV column switching system)

System 4 is similar to System 2 only with a few modifications. A Kromasil C_{18} column (53 x 0.320 mm ID, 10 µm) and Kromasil C_{18} column (150 x 0.100 mm (ID), 3.5 µm) were used as pre-column and analytical column, respectively. The loading flow rate was 0.200 mL/min and the analytical flow rate was 0.6 µL/min. The gradient used was 0-100 % B in 10 min. V1 used was ChemInert C2-1036 (Valco) and V2 was still ChemInert C2-1006 (Valco).

2.3.2.3 System 5 (direct infusion-ESI-TOF-MS)

Direct infusion electrospray mass spectra were obtained using a Harvard Apparatus Pump 11 infusion pump (Holliston, MA, USA) operating at a flow rate of 10 µL/min directed into the Micromass LCT orthogonal accelerated ESI-TOF-MS (Manchester, UK). The mass to charge ratio (*m/z*) scan range was 200-1300. The MS was equipped with a Z-spray atmospheric pressure electrospray ionization source, which was modified to handle µL/min flow rates. The ionization was performed in the positive mode and bradykinin was observed as $[M+H]^{2+}$ with a *m/z* of 530.8. The voltages used are listed in Table 4.

Table 4: Voltage settings in the MS.

Capillary voltage	3.5 kV
Extraction cone	3 V
Sample cone	20 V

Some fragmentation of the analyte was observed between the capillary and the MS due to the relatively high sample cone voltage. The nebulizer gas and desolvation gas (nitrogen 2.0 (99.99 %)) were delivered at rates of 50 and 250 L/hour, respectively. This ensured a stable spray and supported solvent vaporization. The instrument was controlled and the data processed by Mass Lynx version 4.0 software (Micromass).

2.3.2.4 System 6 (gradient capillary LC-ESI-TOF-MS)

System 6 is similar to the gradient nano LC-UV System (System 3) described in Section 2.3.2.1. As analytical columns two different capillary columns were used; Kromasil C_{18} (50 x 0.3 mm (ID), 3.5 µm) and Zorbax SB-C18 (150 x 0.5 mm (ID), 5 µm) with flow rates of 5 and 10 µL/min, respectively. The UV-detector was substituted by the same ESI-TOF-MS described in System 5 (Section 2.3.2.3) and the same MS settings were used.

2.3.3 Other instruments

2.3.3.1 Molecular Absorption Spectrometry

To measure the absorbance of different solutions a Hitachi Model U-2000 double-beam spectrophotometer with screen and printer was used. The quartz cuvettes used had a light path of 1 cm.

2.3.3.2 pH-meter

All pH-measurements were done with a pH-electrode from Hanna Instruments (Bedfordshire, UK) after adding all ingredients, including eventual organic solvents. Generally, pH is defined as the negative logarithm of the hydrogen ion activity in an aqueous solution. In this work however, the term pH refers to the apparent pH (the value measured with a pH-meter).

2.4 Preparation of solutions

Salts and other solids were weighed using an analytical balance with 4 decimals. Volumes below 10 mL were measured using microliter pipettes, volumes below 100 mL were measured using volumetric pipettes and volumes above 100 mL were measured with glass volumetric flasks. HPLC grade water obtained from Fluka was used to make all the peptide stock solutions, diluted peptide solutions, Ringer's acetate and also the $NH_4^+HCOO^-$ solution and MPs used during the stability testing.

2.4.1 Mobile phases

The mobile phases were degassed with 4.6 helium in 10-15 min prior to use. The MP used in the stability testing was also filtered through a Millipore type HVLP 0.45 μm filter (Billerica, MA, USA). MP delivered with the Agilent gradient pump was not degassed prior to use, since it had an on-line incorporated vacuum degasser.

2.4.2 Ringer's acetate

The Ringer's acetate solution was made by solving the salts listed in Table 2 in water. It was then sonicated and filtered through a 0.45 μm Minisart-plus filter with CA-membrane and GF-prefilter (Sartorius AG, Göttingen, Germany) prior to use.

2.4.3 Stock solutions

1 mg/mL stock solutions of peptides were made by adding 5 mL of H_2O to 5 mg of peptide stored in the original container and then transferred to 1.5 mL polypropylene (PP) Plastibrand Transparent Microsentrifuge tubes (Brand GMBH, Wertheim, Germany).

2.4.4 Peptide solutions used during the column testing

Working solutions of ~0.1 mg/mL of bradykinin, angiotensin II and neurotensin used during the column testing were made by appropriate dilution of the peptide stock solutions.

2.4.5 Peptide solutions for stability testing

6 different peptide samples (Table 5) were prepared for stability testing. The peptide concentration was 1 μg/mL in all solutions.

Table 5: Test solutions of 1 µg/mL ang II and brad for stability testing.

No	Peptide	Solvent	R[a]	K[b]	F[c]
1	Ang II	H_2O	1 PE vial[d]	25 PP vials[e]	25 PP vials
2	Ang II	27.7 mM Ringer's acetate	3 PE vials	30 PP vials	--
3	Ang II	20 mM $NH_4^+HCOO^-$ solution, pH = 3.0 (adjusted with formic acid before adding ang II)	3 PE vials	30 PP vials	--
4	Brad	H_2O	1 PE vial	25 PP vials	25 PP vials
5	Brad	27.7 mM Ringer's acetate	3 PE vials	30 PP vials	--
6	Brad	20 mM $NH_4^+HCOO^-$ solution, pH = 3.0 (adjusted with formic acid before adding brad)	3 PE vials	30 PP vials	--

[a] room temperature
[b] refrigerator
[c] freezer
[d] 20 mL polyethylene vials for scintillation counting
[e] Plastibrand 1.5 mL polypropylene vials

Solution no 1 and no 4 were prepared by appropriate dilution of the peptide stock solutions in water. Solution no 2 and no 3 were made by appropriate dilution of peptide stock solution and 277 mM Ringer's acetate in water. A 20 mM $NH_4^+HCOO^-$ solution was adjusted to pH = 3.0 using FA. To make solution no 3 and no 6 the peptide stock solutions were diluted to the desired concentration using the ammonium acetate solution.

Each peptide sample was divided into several aliquots in Plastibrand 1.5 mL PP vials (Brand GMBH) and 20 mL polyethylene (PE) vials for scintillation counting (Zinsser Analytic GMBH, Frankfurt, Germany) and stored in room temperature (R, ~20ºC), refrigerator (K, ~11ºC) and freezer (F, ~ -23ºC) (Table 5). All solutions were made the same day as the stability testing was started; defined as day 0.

2.4.6 Bradykinin solutions for MS

Three bradykinin solutions with a concentration (conc.) of 10 µg/mL dissolved in water, 20 % AcN and different amounts of formic acid (Table 6) were prepared for the preliminary testing of MS conditions.

Table 6: Bradykinin solutions for MS. (The % is given in volume-%.)

No	**Brad conc. (µg/mL)**	**% formic acid**	**% AcN**	**% H_2O**
1.	10	0.05	20	79.95
2.	10	0.5	20	79.5
3.	10	1.5	20	78.5

3. Results and discussion

Bradykinin and angiotensin II are often represented at low concentrations in biological samples, for instance in muscle tissue. Determination of bradykinin and angiotensin II is included in several finished and future projects in our research group. Therefore it is useful to know by which temperature and in which matrices low concentrations of those peptides can and cannot be stored when using them for practical work over time. Sensitivity is achieved by using a reversed-phase capillary LC column switching system with back flushing of the preconcentration column and UV detection.

3.1 Method development

3.1.1 Stationary phases

Octadecyldimethyl-silane (C_{18}) is a well-known packing material in reversed-phase HPLC. It is often used for peptide analysis [58]. In this work 5 different C_{18} materials were tested for their ability to separate peptides in preliminary experiments (Appendix, Section 6.1.2). A Kromasil column (50 x 0.3 mm (ID), 3.5 μm) was found to be the best choice with regard to plate number per meter, selectivity and asymmetry. In addition our group has had good results with Kromasil as stationary phase (SP) in both pre-column and analytical column from previous work with peptides and other analytes [19, 59-69].

In a column switching system the stationary phase in the pre-column should preconcentrate the analytes in a small band at the column entrance. The stationary phase in the separation column should give slightly higher relative retention than the SP in the pre-column in order to provide refocusing of the analytes. Since the particle size of the separation column was 3.5 μm, an in-house made Kromasil C_{18} column with particle size of 10 μm was chosen as pre-column in order to ensure refocusing of the analytes. Larger particle size also lowers the column pressure enabling higher flow rates. This is favourable when injecting large sample volumes in order to complete the sample loading in a short period of time. The choice of SP is in agreement with the conclusions that

Thomas Bjellaas obtained during work in his thesis [70]. The commercial Kromasil pre-column used in parts of the study had a particle size of 5 μm (also larger than the particles in the analytical column). This pre-column was shorter than the in-house made one; the length influenced the back-pressure, thus allowing higher flow rates even though the particle size was smaller.

3.1.2 Mobile phases

Separation of peptides using reversed-phase liquid chromatography is performed with a mobile phase that contains water, organic modifiers and ion-pairing agents or buffers. The organic solvent ensure suitable elution strength to desorb the peptide from the stationary phase, while the buffer or ion-pairing agent adjusts the pH and interacts with the peptide to ensure separation with sufficient resolution. As an organic modifier for peptide analysis, AcN is widely used due to both its low viscosity that limits the back-pressure and its extremely low UV absorbance even at low wavelengths [58]. Issaq et al. [71] compared two organic modifiers, methanol (MeOH) and AcN with respect to the chromatographic separation, and found that AcN generally provided better resolution, selectivity, symmetry and efficiency than MeOH.

Peptides are large molecules, thus having a large surface. This enables interactions between the peptides and C_{18} chain at several places. In addition peptides often have several basic amino acid groups that can attach to the deprotonated residual silanol groups of the packing material, called secondary interactions, when a neutral or basic mobile phase are used. Since all these interactions have to be broken before the peptides elute from a column, tailing and peak broadening are often observed. At lower mobile phase pH however, both the peptides and the silanol groups are protonated, thus decreasing the secondary interaction and giving less tailing.

Adding trifluroacetic acid to the mobile phase has three main advantages; it decreases the pH value of the mobile phase, acts as an ion-pairing agent and, in addition, has little UV-absorption at low wavelengths. However, the main disadvantage is that TFA causes

decreased sensitivity in ESI-MS detection, due to ion suppression of the analytes [58, 72, 73].

0.1 % TFA was added to the mobile phases used during the column testing (Appendix, Section 6.1.2). Since the chromatographic method developed in this thesis was meant to be connected to an ESI-TOF-MS, a MP with no TFA added was desired, thus other ion-pairing agents were investigated. In order to avoid the ion suppression caused by TFA, formic acid and acetic acid are often used as ion-pairing agent for peptide separations. A good resolution is unfortunately not always obtained using these acids [58]. McCalley [74] reported recently a study of the chromatographic separation of basic peptides obtained by using different mobile phase additives; TFA, FA, ammonium formate and phosphate buffer, at constant pH (2.3-3.2). Since mobile phases that contained FA produced poor separation, inorganic salts are non-volatile and thus not favourable by MS detection and TFA gives rise to ion suppression of the analytes, McCalley concluded that ammonium formate was the best choice among the studied additives.

3.1.3 Preliminary mobile phase testing

The next step was to set up an isocratic reversed-phase capillary LC-UV switching system with back-flushing of the pre-column (System 1, Section 2.3.1.1) and test different mobile phases to find the mobile phases best suited for the determination of angiotensin II and bradykinin. The mobile phases that were tested are listed in Table 7. Different combinations of loading and separation MP were compared; all containing H_2O and AcN with an additive like ammonium formate (a.f.), ammonium acetate (a.a.) or formic acid (FA) added.

Table 7: Overview of the mobile phases tested.

No	Loading mobile phase				Analytical mobile phase			
	% AcN	% H_2O	Additive	pH-adjustment	% AcN	% H_2O	Additive	pH-adjustment
1	2	98	20 mM a.a.	--	16	84	20 mM a.f.	--
2	2	98	20 mM a.a.	--	16	84	20 mM a.f.	3.15
3	2	98	20 mM a.a.	--	16	84	20 mM a.f.	3.37
4	2	98	20 mM a.a.	--	16	84	20 mM a.a.	--
5	2	98	20 mM a.a.	3.15	16	84	20 mM a.f.	3.15
6	1	99	20 mM a.f.	--	16	84	20 mM a.f.	3.15
7	2	98	20 mM a.f.	--	16	84	20 mM a.f.	3.15
8	2	98	20 mM a.f.	--	16	84	20 mM a.a.	--
9	2	97.95	0.05 % FA	--	16	83.95	0.05 % FA	--
10	2	97.95	0.05 % FA	--	16	84	20 mM a.f.	3.15
11	1	99	20 mM a.f.	--	16	84	20 mM a.f.	3.15
12	2	98	20 mM a.f.	--	16	84	20 mM a.f.	3.15
13	2	97.95	0.05 % FA	--	14	85.95	0.05 % FA	--

When comparing the chromatograms for the different MPs, some observations were easily made. MPs no 9 and 13 (both with FA as additive in both loading MP and analytical MP) are not suitable for peptide separations, due to bad repeatability and too short retention times, thus they are excluded from the calculations below (Figure 5-7). Also MP no 4, 8 and to a lesser extent no 1 gave relatively bad repeatability. None of the MPs showed good results with respect to peak tailing, but some are worse than others. MP no 6, 7 and 8 gave very asymmetric peaks and MP no 2 also gave relatively asymmetric peaks (Figure 5). The plate number per meter (N/m) was also calculated, showing that MP no 4, 6 and 12 gave the lowest value, and MP no 10 showed the best results (Figure 6). Figure 7 shows the k-values obtained with the different mobile phases. As can be seen MPs no 1, 4 and 8 gave too high k-values, while the other MPs provided relatively ideal k-values (~ 2). From the evaluation above, MP no 3, 5, 10 and 11 (all having AcN – H_2O (16:84, v/v) with 20 mM ammonium formate added pH-adjusted to

either 3.15 or 3.37 as analytical mobile phase) showed the best performance with respect to asymmetry and plate number per meter. Among those four mobile phases no 3 and 5 showed the largest peak height (no 3 was slightly better than 5). However, low pH for both loading MP and separation MP was chosen in order to avoid secondary interactions. Therefore the following solvent mixtures (MP no 5) were chosen as loading MP and separation MP, respectively: AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added and AcN – H_2O (16:84, v/v) with 20 mM $NH_4^+CH_3COO^-$ added, both adjusted to pH = 3.15 using formic acid. Bacterial growth was observed in the loading mobile phase, therefore AcN – H_2O – FA (2 : 97.95 : 0.05, v/v/v) were used as loading MP during the stability testing (MP no 10), because it showed similar asymmetry (As) and better N/m than MP no 3.

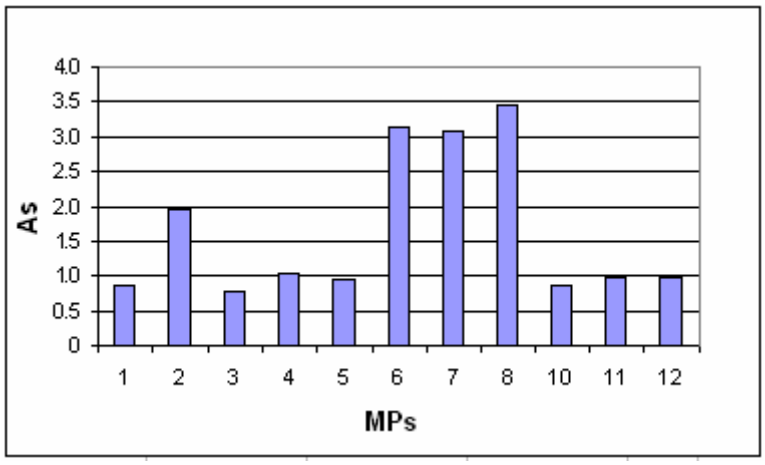

Figure 5: Asymmetries of the bradykinin peaks using different MPs.
(The numbers at the y-axis refers to the MPs listed in Table 7)

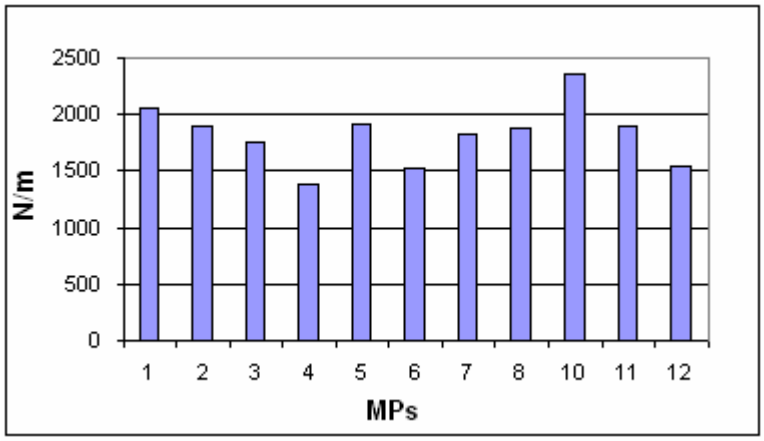

Figure 6: The calculated plate number per meter (bradykinin) using different MPs.
(The numbers at the y-axis refers to the MPs listed in Table 7)

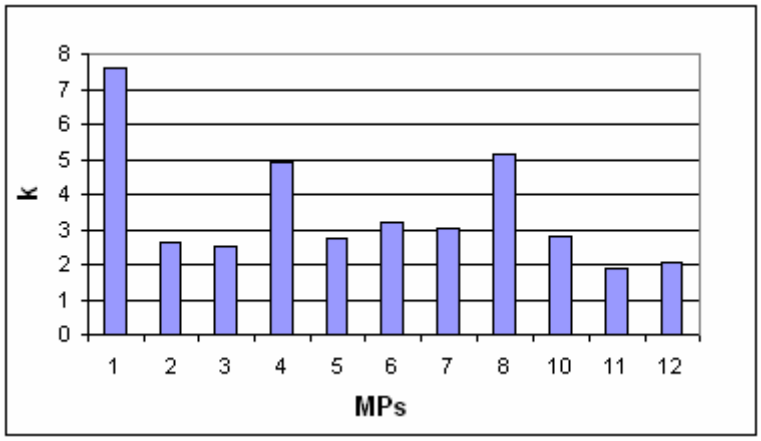

Figure 7: The k-values for bradykinin obtained with different MPs.
(The numbers at the y-axis refers to the MPs listed in Table 7)

Since the chosen MP still showed some tailing, gradient elution was used for the rest of the study. The analytical pump had two chambers delivering the MP as follows. In chamber A: AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+HCOO^-$ and 0.05 % formic acid added; In chamber B: AcN – H_2O (90:10, v/v) with 20 mM $NH_4^+HCOO^-$ and 0.05 % formic acid added. The gradient used was: 10-50 % B in 5 min and then held at 50 % B in 2 min at a flow rate of 0.005 mL/min.

When injecting large volumes it is essential that the stationary phase of the pre-column and the loading mobile phase used provide sufficient focusing of the analytes. Therefore the breakthrough of the analytes on the chosen pre-columns, the in-house made Kromasil C_{18} precolumn (53 x 0.320 mm (ID), 10 μm) and the commercial Kromasil C_{18} pre-column (5 x 1 mm (ID), 5 μm) were tested with the chosen loading mobile phases. Both loading MPs used during this work were tested: one containing AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added and the other containing AcN – H_2O – FA (2 : 97.95 : 0.05, v/v/v). By substituting the column in System P (described in App. 6.1.1) with the pre-columns (one at a time), and using a flow rate of 0.200 mL/min, no signals were found 50 min after injecting the analyte (one injection with angiotensin II and one injection with bradykinin), indicating no breakthrough on either columns.

Phase collapse may occur in reversed-phase LC using alkyl bonded phases (like C_8 or C_{18}) as stationary phase and mobile phase containing less than 5 % organic solvent. This

phenomenon may cause peak tailing, gradient regeneration delays and decreased, and sometimes also non-repeatable, retention times. Washing the column with a solution containing 50 % (or more) organic solvent may regenerate the column [75]. The loading mobile phases used in this project contained less than 5 % organic modifier, but since the separation mobile phase used in the capillary LC part of the study was delivered with a gradient of 10-50 % B (containing up to 46 % AcN), no phase collapse was observed. For the nano LC even more AcN was delivered at the end of the separation MP gradient.

Investigation of the influence of Ringer's acetate on the peak shape and retention time of ang II is described in the Appendix, Section 6.1.3.

3.2 Detection

T. Bjellaas tested the ultraviolet light absorption of different peptides including ang II and brad at 210 and 280 nm, and found that the absorption at 280 nm was very weak or if observable at all [70]. At wavelengths higher than 210 nm the absorbance of MP impurities, additives and salts are smaller, thus the signal to noise ratio (S/N) may increase when the wavelength is increased from 210 nm provided that the absorbance of the analyte itself is not decreased. Therefore, in this project, the ang II signal at 210 and 230 nm were compared using System P (described in Appendix, Section 6.1.1), with a MP consisting of AcN – H_2O – OHAc (22:77.9:0.1, v/v). It was observed that 210 nm provided a higher signal and higher S/N, and thus higher sensitivity, for ang II than 230 nm. Therefore a wavelength of 210 nm was chosen for the peptides in this thesis, as in Bjellaas' thesis [70].

3.3 Injection volume

The aim of the study was to investigate the stability of low concentrations of ang II and brad stored in different water matrices and at different temperatures because these peptides are present at low concentrations in biological samples. Therefore a sensitive method was required. Large injection volumes can be performed to enhance the

sensitivity of a capillary LC method. Too high injection volumes however, are time consuming and may cause breakthrough and overloading of the column. The maximum binding capacity of polypeptides on a reversed-phase column depends on the column volume and on the size of the hydrophobic part of the molecule versus the total molecular weight [58]. Therefore investigation of the linearity of the injection volumes versus peak areas is important.

5 µg/mL of bradykinin and angiotensin II were injected at volumes of 50.6, 75.3, 100.8, 156.0 and 198.3 µL (300.0 µL of ang II was also injected) and the linearities of the injected volumes versus peak heights were then monitored. The injection volumes are later in this thesis referred to as 50, 75, 100, 150, 200 and 300 µL. A gradient capillary LC-UV column switching system (System 2; Section 2.3.1.2) was used during the investigation. The raw data can be seen in Appendix, Section 6.2.1.

The relationship between the average peak areas (of 2-5 replicates) of ang II and the injection volumes are plotted in Figure 8. The linear regression lines (drawn in red) have r-values of 0.991 (for injection volumes of 50, 75, 100, 150 and 200 µL) and 0.989 (for injection volumes of 50, 75, 100, 150, 200 and 300 µL). Figure 9 shows the relationship between the peak areas and the injection volumes (50, 75, 100, 150 and 200 µL) of bradykinin. The linear regression line (also here drawn in red) has an r-value of 0.984.

As can be seen from both Figure 8 and 9, the average peak areas obtained with an injection volume of 150 µL, are slightly smaller than expected, due to the fact that the values lay below the regression lines). This may indicate that the injection loop used for 150 µL injections may give a smaller volume than expected. The difference in r-values between the plots of peak areas of ang II versus injection volumes including and excluding 300 µL ($r = 0.991$ and 0.989, respectively), shows that the regression line for the plot excluding 300 µL fits the data better. This may indicate that 300 µL gives a slight breakthrough, but this cannot be stated for sure. Another fact that supports this theory is that the regression lines for ang II and brad should intersect the y-axis slightly above origo, since the volume inside the injection valve is neglected in the volume calculations.

The regression lines for ang II and brad with injection volumes of 50-200 µL nearly intersect origo, while the regression line for ang II, including the injection volume of 300 µL, intersects the y-axis at a higher y-value.

200 µL was chosen as the injection volume, just to be sure that no breakthrough would occur while also saving loading time.

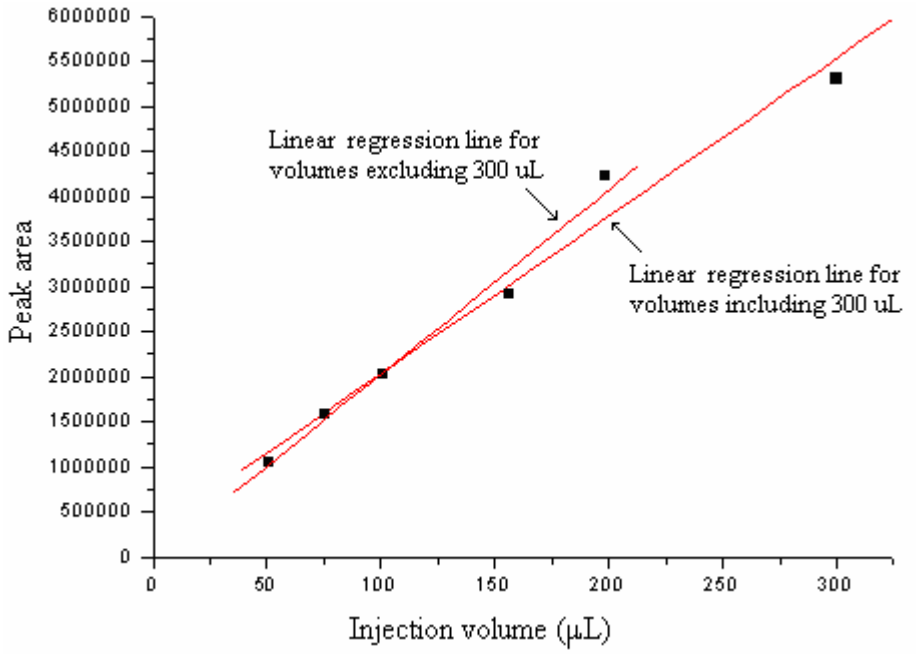

Figure 8: The relationship between the average peak areas of ang II and the injection volumes (50, 75, 100, 150, 200 and 300 µL). The linear regression lines are drawn red; r = 0.991 (for volumes excluding 300 µL) and r = 0.989 (for volumes including 300 µL).

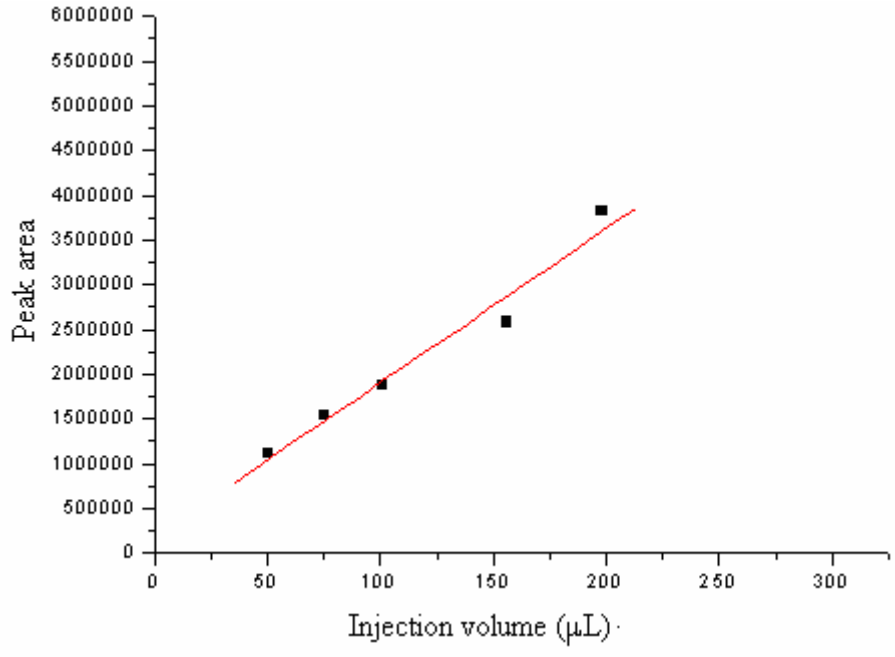

Figure 9: The relationship between the peak areas of bradykinin and the injection volumes (50, 75, 100, 150 and 200 µL). The linear regression line is drawn red, r = 0.984.

3.4 Limit of detection

The limit of detection of an analyte is most commonly defined as the concentration giving a signal that is 3 times the background noise.

Since peptides like angiotensin II and bradykinin most often are represented in biological samples at low concentrations, it is important to obtain a method with as low detection limits as possible. The detection limits for angiotensin II and bradykinin were tested using System 2 (Section 2.3.1.2) with an injection volume of 200 µL. The concentration limit of detection (cLOD) of brad were found to be approximately 10 ng/mL (Figure 10), thus giving a mass limit of detection (mLOD) of ~ 2 ng. The cLOD of angiotensin II was below 1 ng/mL giving a mLOD below 0.2 ng (Figure 11).

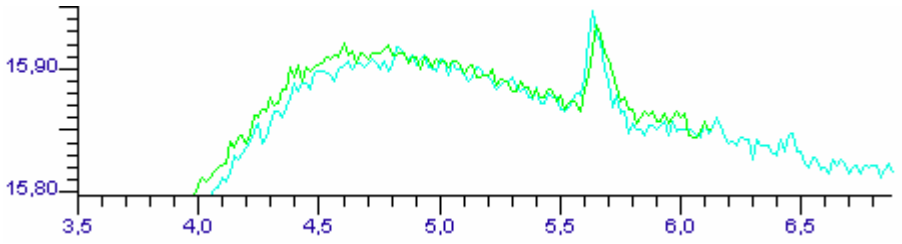

Figure 10: An overlay of two chromatograms showing 200 µL of 10 ng/mL bradykinin injected into a gradient capillary LC-UV switching system with detection wavelength of 210 nm. The columns used were a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm) and a Kromasil C_{18} pre-column (5 x 1 mm (ID), 5 µm). The loading MP (AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added) and the analytical MP (AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added) were delivered at a flow rate of 0.200 and 0.005 mL/min, respectively.

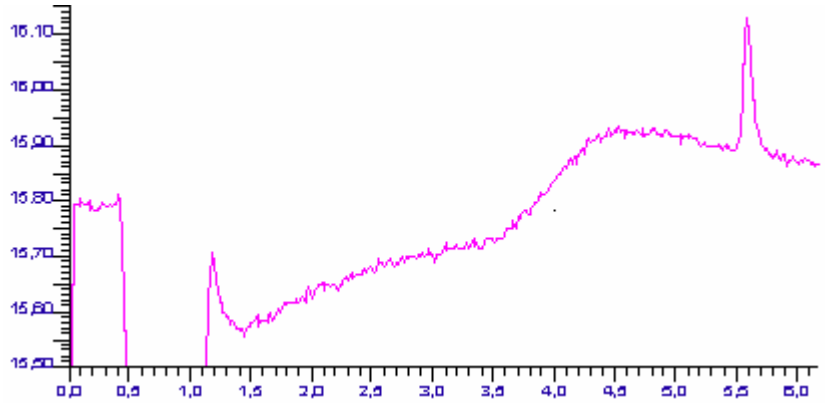

Figure 11: A chromatogram of angiotensin II at a concentration of 1 ng/mL. 200 µL ang II was injected into a capillary LC-UV switching system. The columns used were a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm) and a Kromasil C_{18} pre-column (5 x 1 mm (ID), 5 µm). The loading MP (AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added) and the analytical MP (AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added) were delivered at a flow rate of 0.200 and 0.005 mL/min, respectively. UV detection was performed at 210 nm.

3.5 Stability testing of bradykinin and angiotensin II

Investigation of the stability of 1 µg/mL angiotensin II and bradykinin stored in three different matrices and by different temperatures (Table 5) was carried out using System 2 (Section 2.3.1.2) with a injection volume of 200 µL. The total analysis time was: 3.5 min loading, 6.5 min analytical separation and 5 min reconditioning of the columns.

All peptide solutions were made the same day and stored in polypropylene or polyethylene vials in room temperature (~20ºC), refrigerator (~11ºC) or freezer (~-23ºC).

Each day 2 vials with each of the analyte solutions were taken out of the refrigerator and the freezer app. 1 hour prior to analysis.

Measurements of the peak areas for ang II were done on days 0, 1, 2, 3, 4, 5, 7, 14, 19 and 24. Brad was measured on days 0, 1, 2, 3, 4, 5, 8, 15, 21 and 24. (Day 0 is defined as the day that the solutions were made). Ang II in H_2O (in room temperature) was not analyzed on day 19 and brad in H_2O (in room temperature) was not analyzed on day 21.

Chromatograms of bradykinin dissolved in $NH_4^+HCOO^-$ (pH = 3.0) and angiotensin II dissolved in H_2O, both stored in the refrigerator and measured the 3rd day, are shown in Figure 12 a and b, respectively.

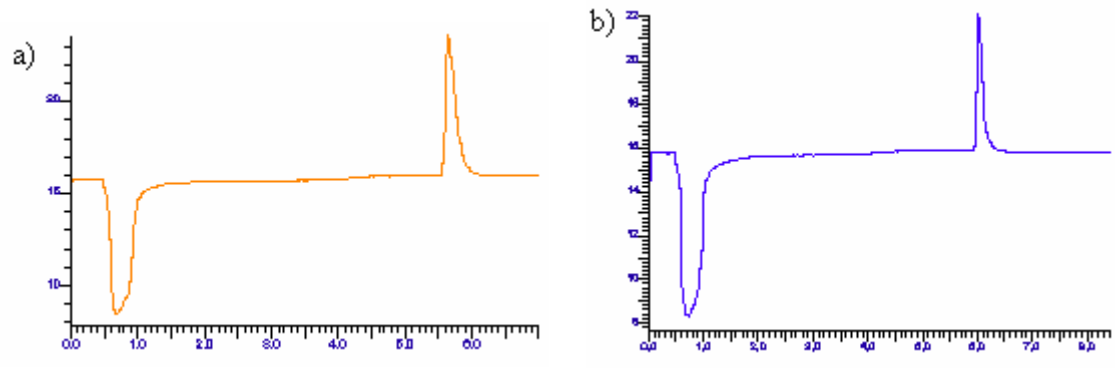

Figure 12: a) A chromatogram of bradykinin dissolved in $NH_4^+HCOO^-$ (pH = 3.0) and stored in the refrigerator for 3 days and **b)** a chromatogram of angiotensin II dissolved in H_2O and stored in the refrigerator for 3 days. 200 µL of 1 µg/mL peptides was injected in a gradient capillary LC-UV switching system with detection wavelength of 210 nm. The columns used were a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm) and a Kromasil C_{18} pre-column (5 x 1 mm (ID), 5 µm). The loading MP (AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added) and the analytical MP (AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added) were delivered at a flow rate of 0.200 and 0.005 mL/min, respectively.

Histograms showing the measured peak areas of the peptides each day are shown in Figure 13-26 (R = room temperature, K = refrigerator, F = freezer). The purple columns show the average peak areas. In those cases where three replicates were made, the values for the absolute standard deviation, s, are calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column illustrates the lowest peak area and the yellow column the largest peak area. In those cases where only one replicate was made, the peak area is shown with

a purple column. The raw data (both for peak areas and retention times) from this part of the project are shown in Table A7-A10 (Appendix, Section 6.2.2). The total averages of the peak areas for the peptides in each solution for the whole period are given in Table 8. A high value of relative standard deviation (RSD) indicates a great change in peak area during the period.

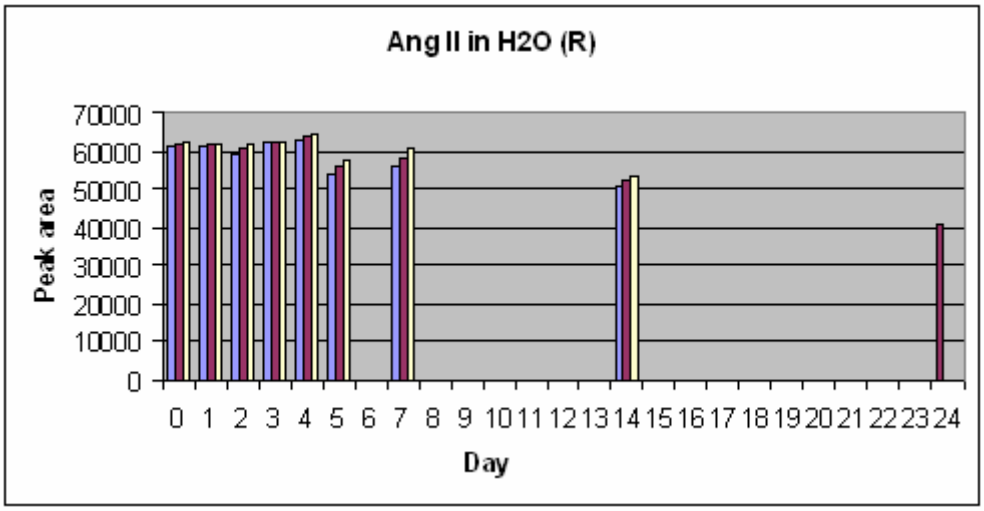

Figure 13: Peak areas obtained for ang II dissolved in H_2O and stored at room temperature. When two replicates were made; the purple column shows the average peak area, while the blue column illustrates the lowest peak area and the yellow column the largest peak area. In those cases where only one replicate was made, the peak area is shown with a purple column.

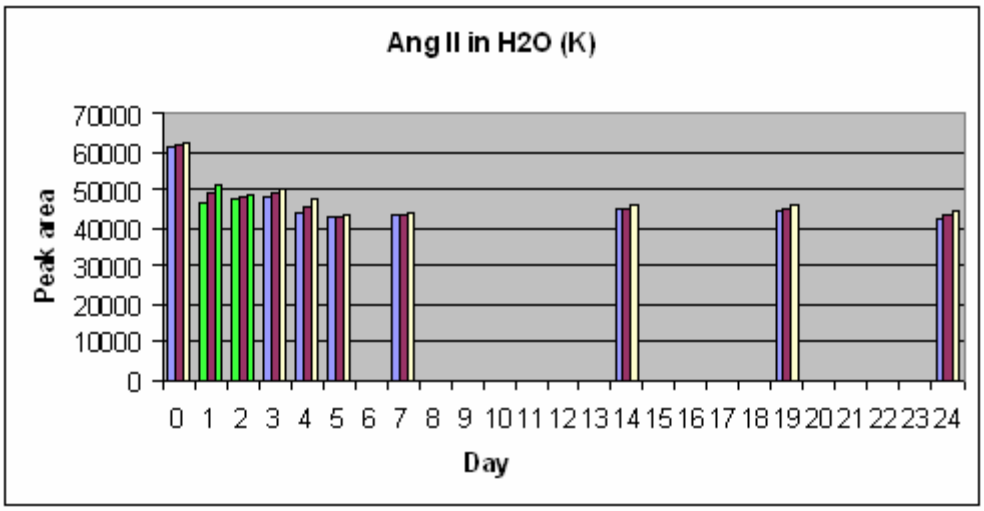

Figure 14: Peak areas obtained for ang II dissolved in H_2O and stored in the refrigerator. The purple columns show the average peak areas. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column shows the lowest peak area and the yellow column the largest peak area.

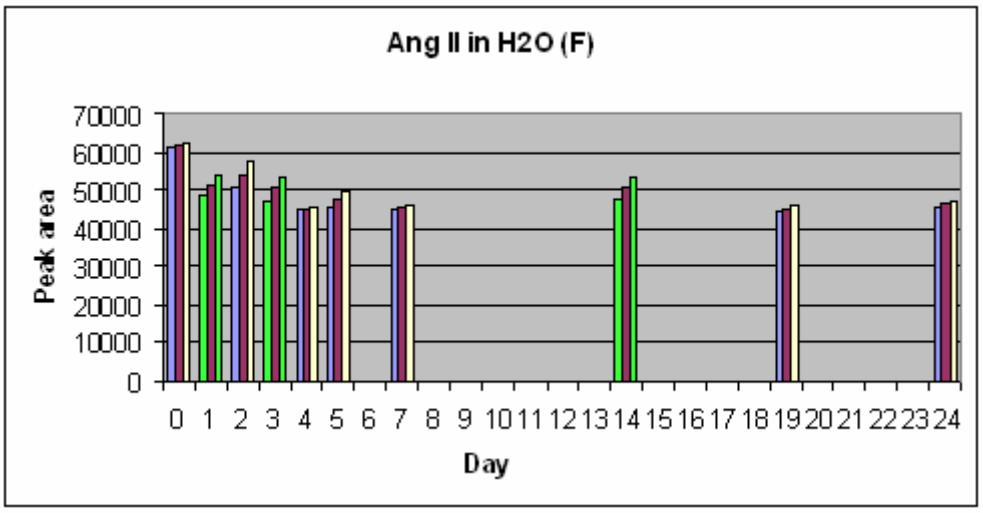

Figure 15: Peak areas obtained for ang II dissolved in H_2O and stored in the freezer. The purple columns show the average peak areas. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column shows the lowest peak area and the yellow column the largest peak area.

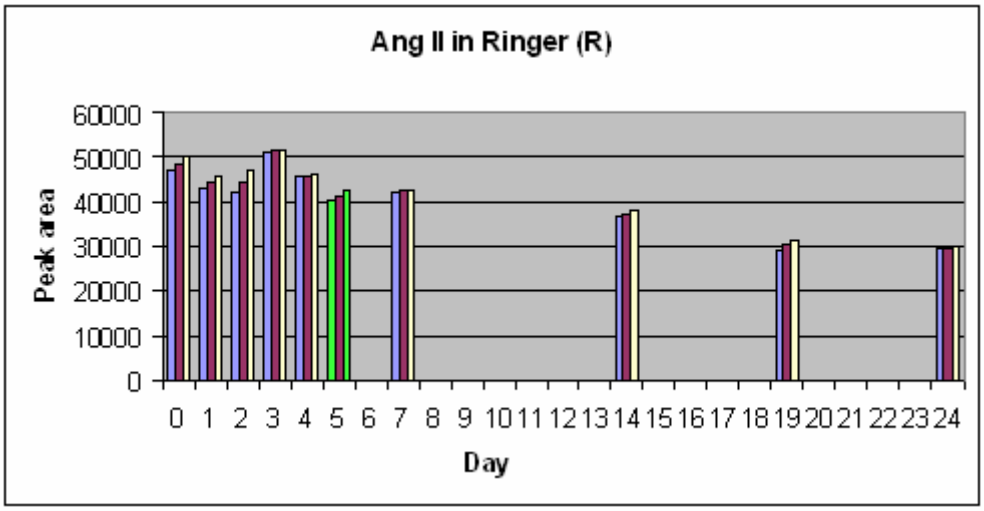

Figure 16: Peak areas obtained for ang II dissolved in Ringer's acetate and stored at room temperature. The purple columns show the average peak areas. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column shows the lowest peak area and the yellow column the largest peak area.

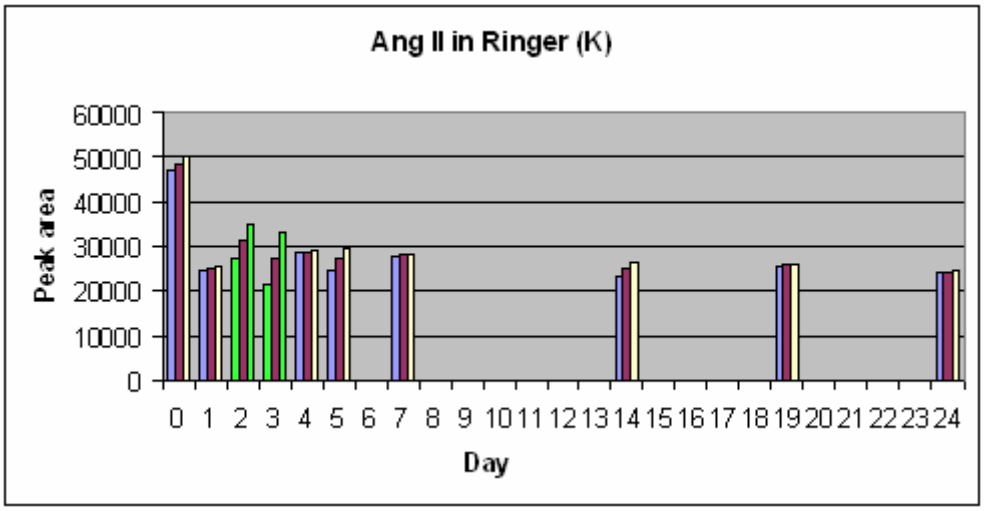

Figure 17: Peak areas obtained for ang II dissolved in Ringer's acetate and stored in the refrigerator. The purple columns show the average peak area. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column shows the lowest peak area and the yellow column the largest peak area.

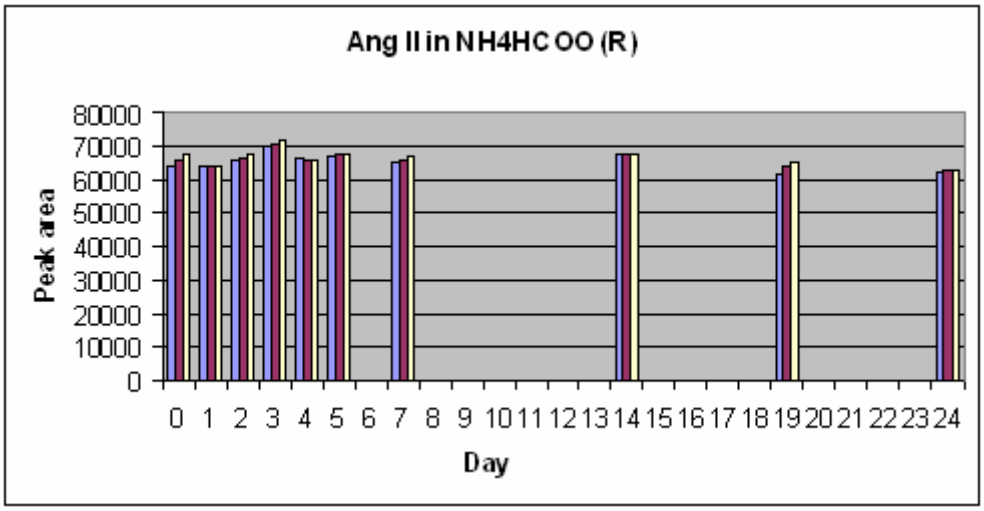

Figure 18: Peak areas obtained for ang II dissolved in $NH_4^+HCOO^-$ at pH = 3.0 and stored at room temperature. The purple columns show the average peak areas, while the blue columns show the lowest peak areas and the yellow columns the largest peak areas.

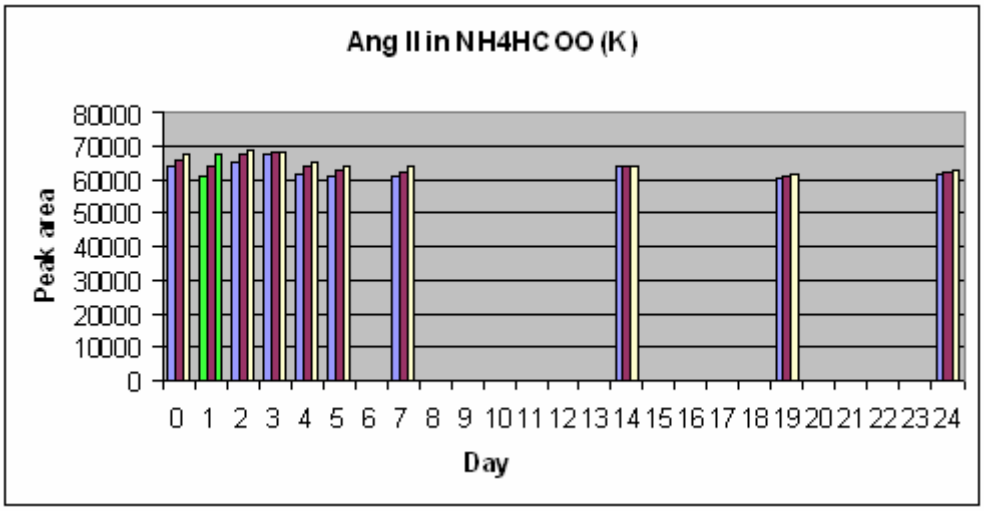

Figure 19: Peak areas obtained for ang II dissolved in $NH_4^+HCOO^-$ at pH = 3.0 and stored in the refrigerator. The purple columns show the average peak areas. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column shows the lowest peak area and the yellow column the largest peak area.

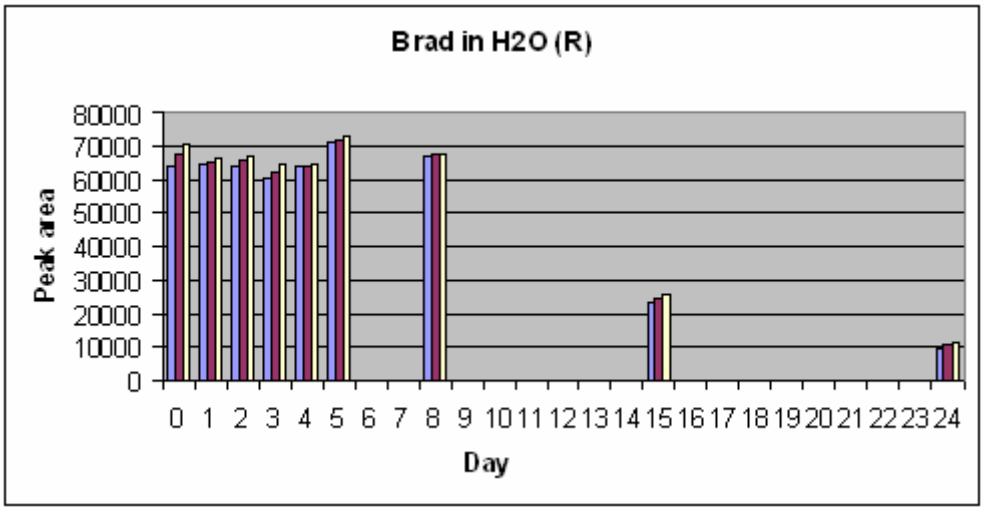

Figure 20: Peak areas obtained for brad dissolved in H_2O and stored at room temperature. The purple columns show the average peak areas, while the blue columns show the lowest peak areas and the yellow columns the largest peak areas.

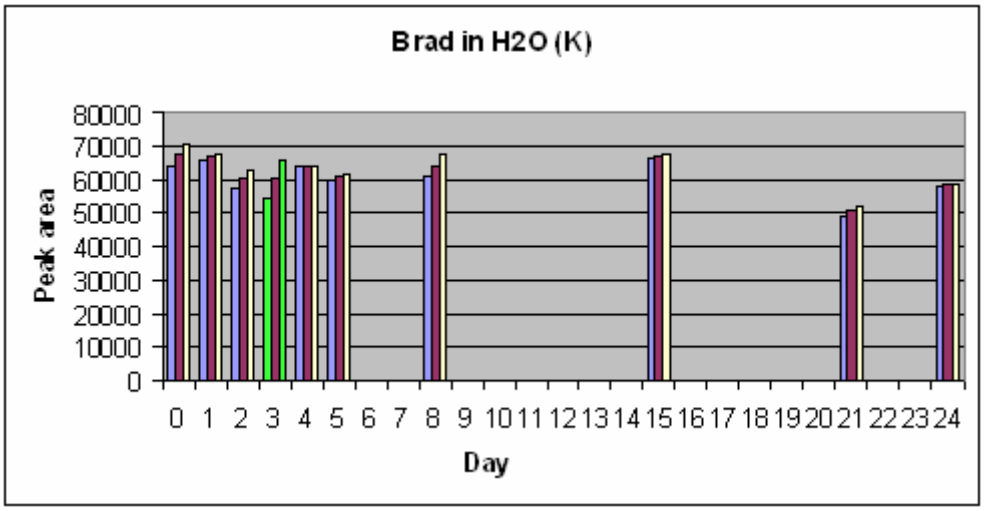

Figure 21: Peak areas obtained for brad dissolved in H_2O and stored in the refrigerator. The purple columns show the average peak areas. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column shows the lowest peak area and the yellow column the largest peak area.

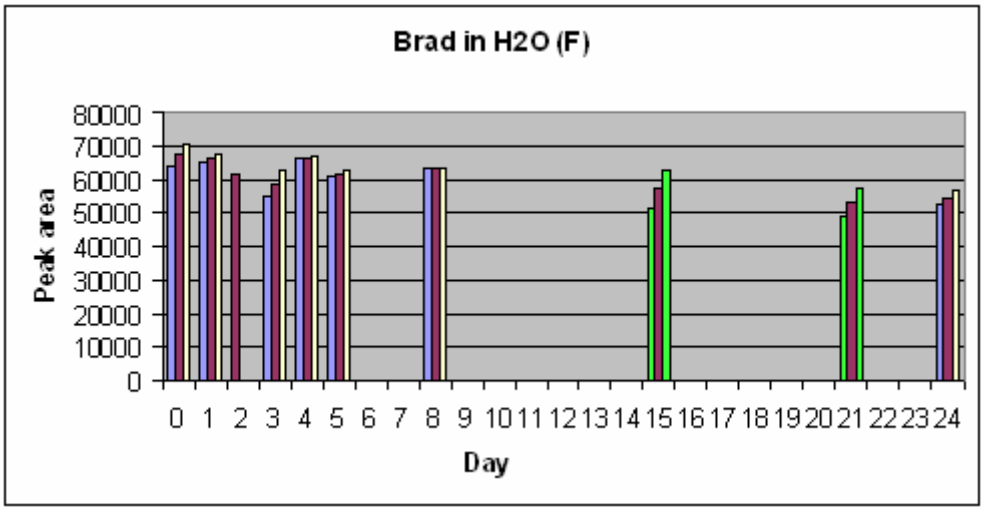

Figure 22: Peak areas obtained for brad dissolved in H_2O and stored in the freezer. The purple columns show the average peak areas. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column illustrates the lowest peak area and the yellow column the largest peak area. In those cases where only one replicate was made, the peak area is shown with a purple column.

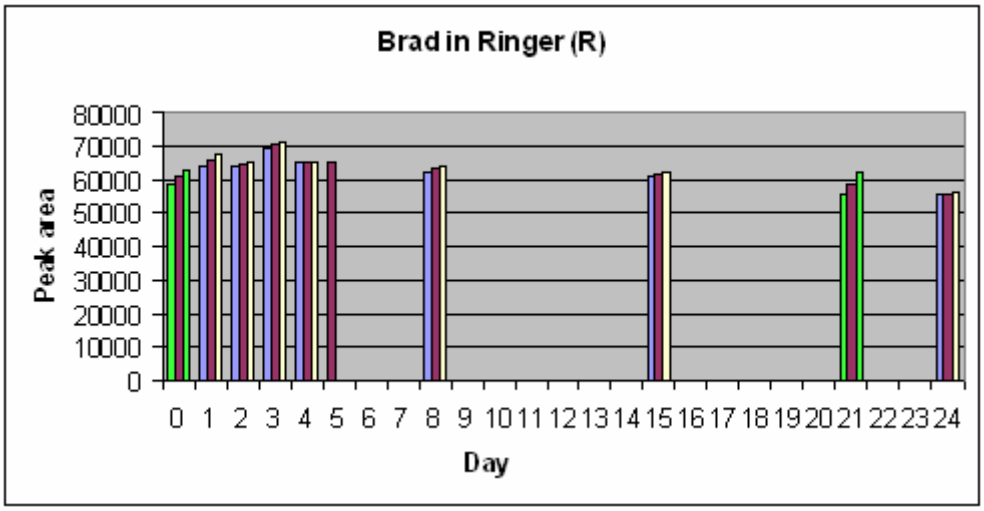

Figure 23: Peak areas obtained for brad dissolved in Ringer's acetate and stored at room temperature. The purple columns show the average peak areas. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column shows the lowest peak area and the yellow column the largest peak area. In those cases where only one replicate was made, the peak area is shown with a purple column.

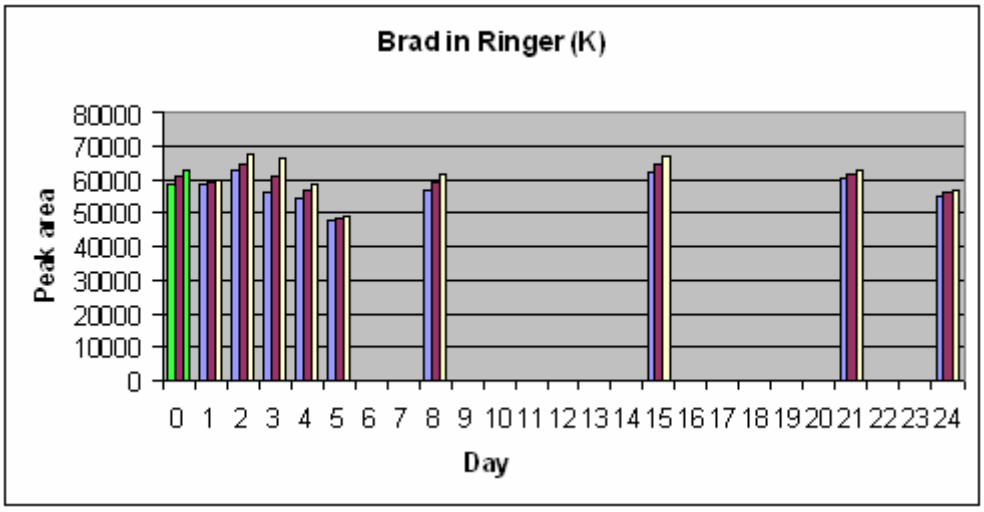

Figure 24: Peak areas obtained for brad dissolved in Ringer's acetate and stored in the refrigerator. The purple columns show the average peak areas. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column shows the lowest peak area and the yellow column the largest peak area.

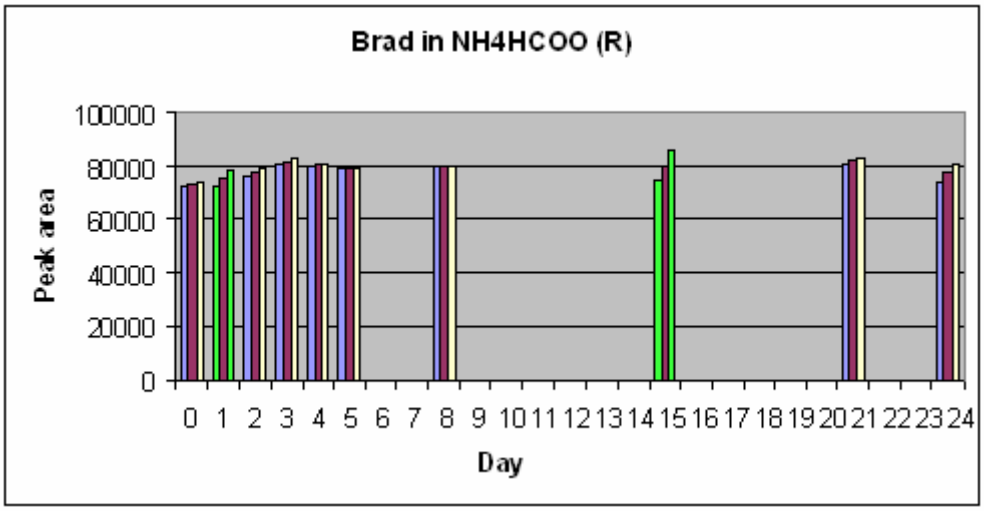

Figure 25: Peak areas obtained for brad dissolved in $NH_4^+HCOO^-$ at pH=3.0 and stored at room temperature. The purple columns show the average peak areas. In those cases where three replicates were made, the value of the absolute standard deviation, s, is calculated and the green columns illustrate the average peak area plus or minus the s value ($\overline{X} \pm s$). When only two replicates were made; the blue column shows the lowest peak area and the yellow column the largest peak area.

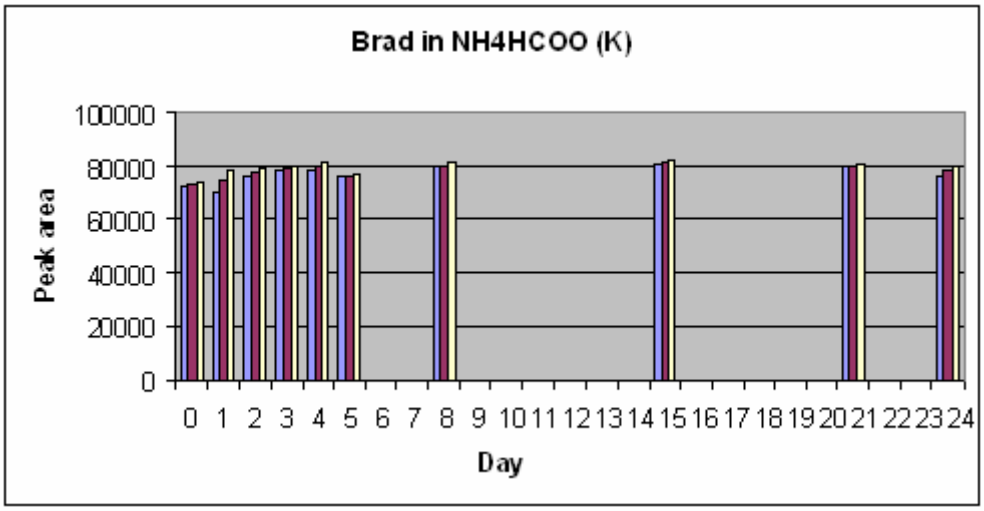

Figure 26: Peak areas obtained for brad dissolved in $NH_4^+HCOO^-$ at pH=3.0 and stored at room temperature. The purple columns show the average peak areas, while the blue columns show the lowest peak areas and the yellow columns the largest peak areas.

Table 8: The total average of the peak areas for each peptide solution. The average is calculated from the average peak area obtained each day.

Peptide solution	$\overline{X}$	S	RSD [%]
Ang II in H_2O R	$5.74 \cdot 10^4$	$7 \cdot 10^3$	13
Ang II in H_2O K	$4.75 \cdot 10^3$	$6 \cdot 10^3$	12
Ang II in H_2O F	$4.98 \cdot 10^3$	$5 \cdot 10^3$	10
Ang II in Ringer's acetate R	$4.16 \cdot 10^3$	$7 \cdot 10^3$	17
Ang II in Ringer's acetate K	$2.91 \cdot 10^3$	$7 \cdot 10^3$	25
Ang II in $NH_4^+HCOO^-$ R	$6.61 \cdot 10^3$	$2.3 \cdot 10^3$	4
Ang II in $NH_4^+HCOO^-$ K	$6.42 \cdot 10^3$	$2.3 \cdot 10^3$	4
Brad in H_2O R	$5.6 \cdot 10^3$	$2.2 \cdot 10^4$	40
Brad in H_2O K	$6.20 \cdot 10^3$	$5 \cdot 10^3$	8
Brad in H_2O F	$6.12 \cdot 10^3$	$5 \cdot 10^3$	8
Brad in Ringer's acetate R	$6.33 \cdot 10^3$	$4 \cdot 10^3$	6
Brad in Ringer's acetate K	$5.93 \cdot 10^3$	$5 \cdot 10^3$	8
Brad in $NH_4^+HCOO^-$ R	$7.88 \cdot 10^3$	$2.8 \cdot 10^3$	4
Brad in $NH_4^+HCOO^-$ K	$7.82 \cdot 10^3$	$2.7 \cdot 10^3$	4

As can be seen from the histograms in Figure 13-26 and Table 8 the peptides are most stable when dissolved in $NH_4^+HCOO^-$ (pH = 3) and stored at room temperature or in the refrigerator. Brad is also quite stable when dissolved in Ringer's acetate (especially at room temperature), and to some extent in water stored either in the refrigerator or in the freezer. The most significant loss of peptide concentration is observed in the solution of brad dissolved in water stored at room temperature. Also ang II dissolved in Ringer's acetate, especially when stored in the refrigerator experienced a significant loss of concentration. A special phenomenon is observed for ang II dissolved in Ringer's acetate and stored in the refrigerator; the concentration decreased quite significantly the first 24 hours, the same is not observed for the same solution stored at room temperature. Ang II dissolved in water and stored in the refrigerator or in the freezer also experienced a significant loss of peptide concentration the first 24 hours. The same effect is not observed in any of the bradykinin solutions.

All chemical processes (chemical reactions) are accelerated when the temperature is increased. Since the observed concentration loss the first 24 hours for angiotensin II was observed after storage at lower temperatures, this may indicate that the loss was not due

to chemical reactions. The concentration changes are more likely related to physical processes, which might include adsorption of the analyte to the walls of the vials or precipitation of the analyte. Many compounds display polymorphism, i.e. can crystallise with different crystal structures. The different crystal forms of a compound have the same solubility, but the dissolution kinetics may be different; in other words some crystal forms dissolve at different rates. This may be the reason why some angiotensin II seems to have been lost after cooling and subsequent thawing. The peptide solutions were taken out of the refrigerator and freezer ~1 hour prior to analysis, and this may have been an insufficient period of time in order to dissolve the analytes again. The same kind of argument applies to adsorption. If the angiotensin II was adsorbed to the walls of the vials, 1 hour in room temperature prior to analysis may have been insufficient time in order to dissolve all the analyte, due to the possible presence of other crystal forms of the analyte than prior to the first dissolution.

The concentration loss for angiotensin II the first 24 hours was not observed in the acidified (pH = 3) $NH_4^+HCOO^-$ solution, while it was observed in the Ringer's acetate and H_2O solutions, stored at lower temperatures, which had a pH close to 7. Following the argument above this must has been due to a differential solubility of ang II in acidic and neutral/mildly basic solutions. At pH = 7 ang II is protonated on the arginine, partially protonated on the histidine, deprotonated at the C-terminus, while the N-terminus remains neutral (to a good approximation). This gives a total of ~2.5 (positive and negative) charges. At pH = 3, the arginine is still protonated, the histidine is completely protonated and the N-terminus is protonated, while the C-terminus is neutral (again to a good approximation). This gives a total of 3 (positive and negative) charges. Generally, an increased number of charges gives rise to increased solubility in water. The lower number of charges at pH = 7 may be the reason why angiotensin II experienced a concentration loss in Ringer's acetate and H_2O after cooling but not in the acidified NH^+HCOO^- solution, since angiotensin II should have a lower solubility at pH = 7 than pH = 3.

For bradykinin the two arginines are protonated at both pH = 3 and pH = 7, the amidine functional group is much more basic than a simple amino group, and the C-terminus is deprotonated at pH = 7 and neutral at pH = 3, while the N-terminus is neutral at pH = 7 and protonated at pH = 3, thus giving a total of 3 (positive and negative) charges in both neutral and acidic solutions. This is the same number of charges that angiotensin II has in acidic solution. At pH = 7 brad has a higher number of charges than ang II, indicating that brad could be more hydrophilic than ang II at neutral pH. This is supported by the elution order of the two peptides: brad having a shorter retention time on the hydrophobic column than ang II. This may be the reason why no significant concentration loss was observed in the bradykinin solutions during the first 24 hours. Since bradykinin has the same number of charges at both pH = 3 and pH = 7, it is reasonable to assume that the solubility of bradykinin is approximately the same in both neutral and acidic solutions.

When looking exclusively at the peak shapes of angiotensin II and bradykinin during the stability testing period, it is observed that the peak shape of bradykinin only remains the same throughout the testing period when dissolved in $NH_4^+HCOO^-$ and stored in the refrigerator, the same solution is also quite stable at room temperature, only observing a slight peak tailing on day no 24. Brad dissolved in Ringer's acetate stored in the refrigerator showed some tailing with traces of peak(s) in the tailing from the 15th day (Figure 27), while stored in room temperature this phenomenon appeared from the 3rd day. Brad dissolved in water also showed some tailing with traces of peak(s) in the tailing similar to the peak shape shown in Figure 27; the phenomenon appeared at room temperature already after 5 days, and after 8 days when stored in the refrigerator or in the freezer. In both cases this traces of peak(s) in the tailing is most likely breakdown products, since there are no other substances present in the solutions than the peptide (ang II or brad) and the water matrix.

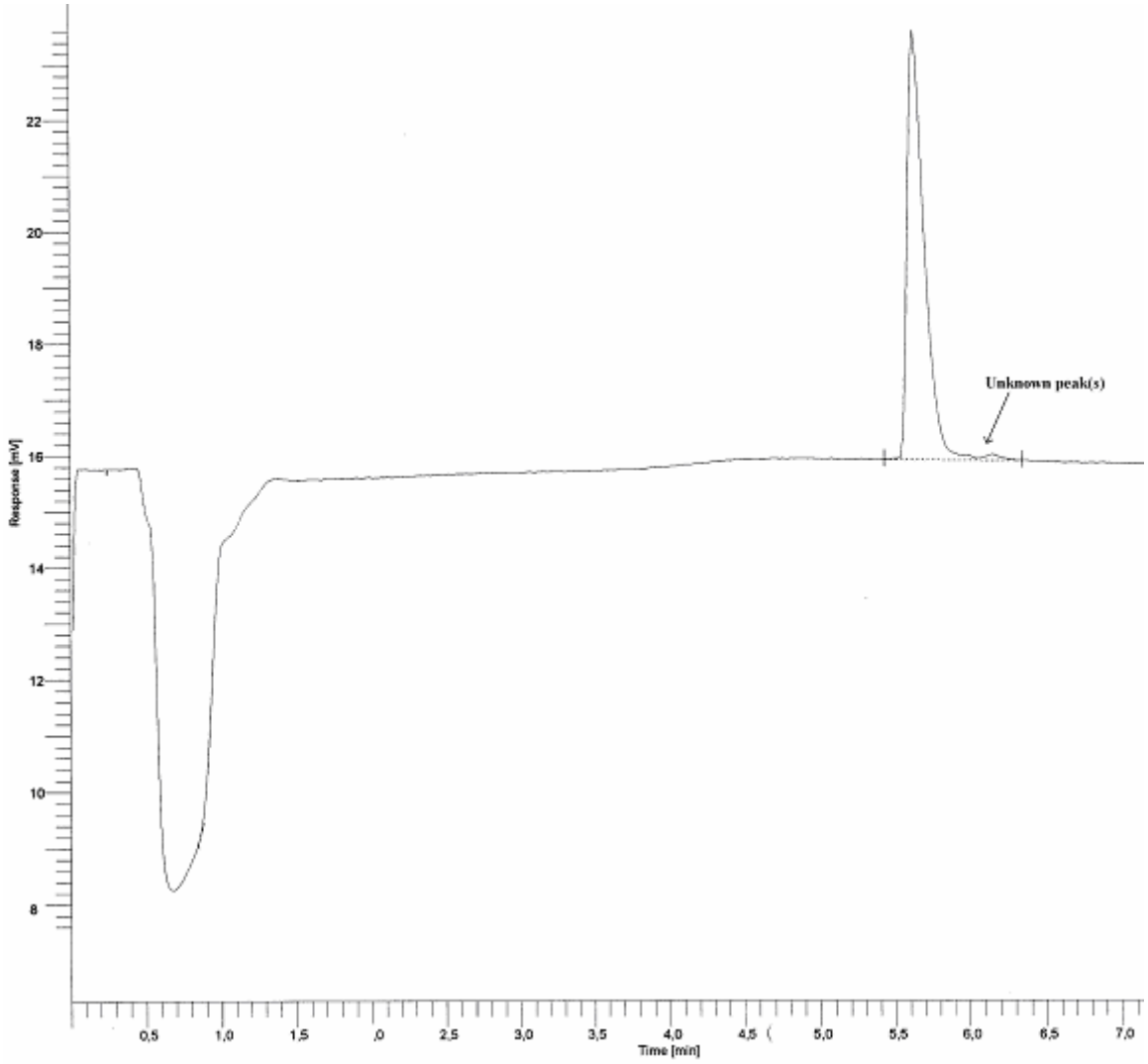

Figure 27: A chromatogram of bradykinin dissolved in Ringer's acetate and stored in the refrigerator for 15 days. 200 µL of 1 µg/mL bradykinin was injected in a gradient capillary LC-UV switching system with a detection wavelength of 210 nm. The columns used were a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm) and a Kromasil C_{18} pre-column (5 x 1 mm (ID), 5 µm). The loading MP (AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added) and the analytical MP (AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added) were delivered at a flow rate of 0.200 and 0.005 mL/min, respectively.

Angiotensin II dissolved in water and stored at room temperature, in the refrigerator or in the freezer and ang II dissolved in $NH_4^+HCOO^-$ (pH = 3.0) stored in the refrigerator showed similar results; after 7 days one tiny peak that co-eluted with the analyte was observed with a retention time that was slightly longer than ang II (an example is shown in Figure 28). This small peak had a quite stable area for the rest of the testing period. The same was observed for ang II dissolved in Ringer's acetate and stored at room temperature or in the refrigerator, but here the co-eluting peak was already observed at

day no 4, and stored at room temperature it was slightly larger at the end of the project. The worst ang II peak shape was observed when it was dissolved in acidic (pH = 3.09) $NH_4^+HCOO^-$ and stored at room temperature. Here the small co-eluting peak was observed from day no 7. From day 14 the peak had (several) tiny peak(s) in the tailing.

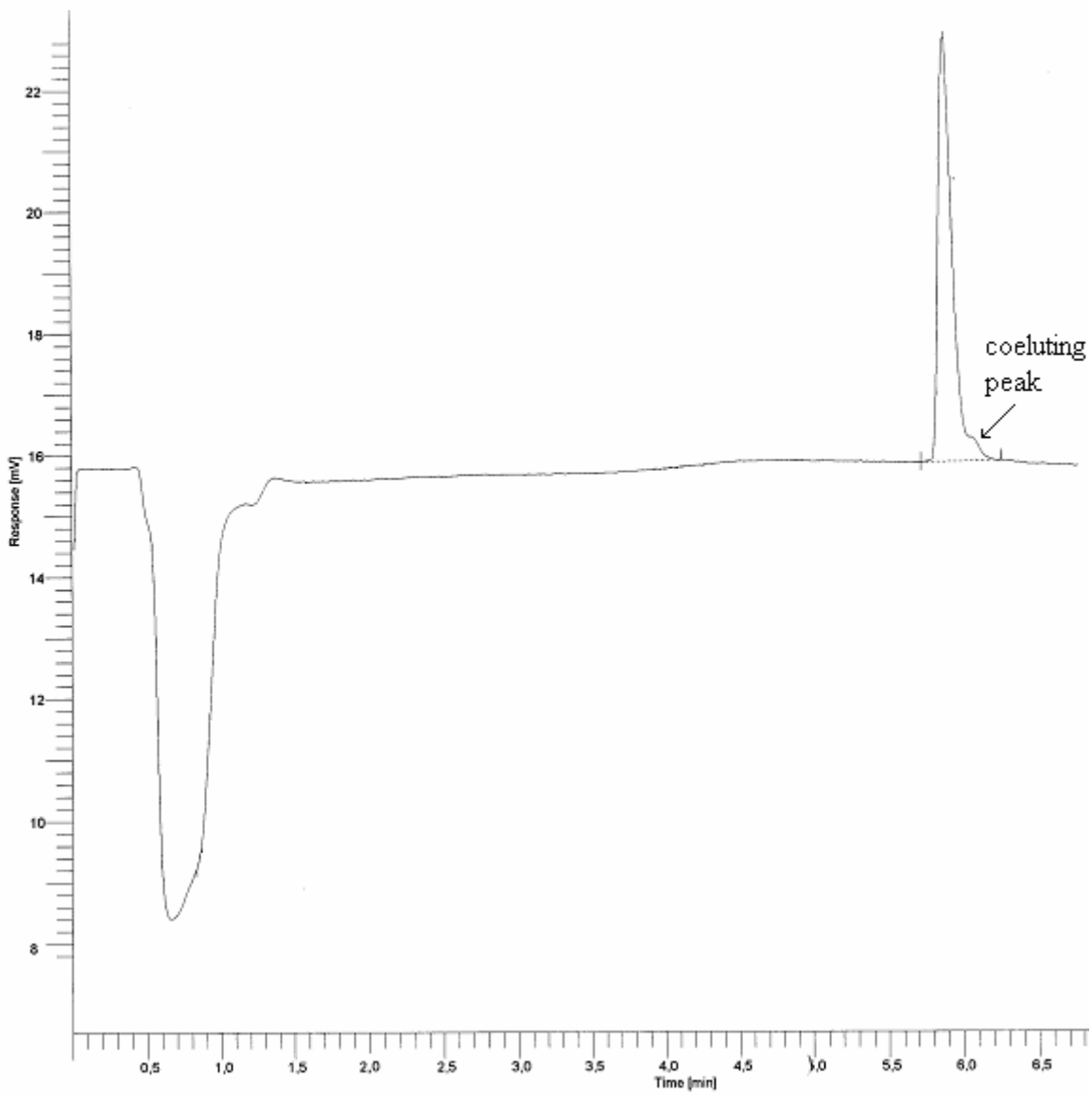

Figure 28: A chromatogram of ang II dissolved in H_2O and stored in the refrigerator for 14 days. 200 µL of 1 µg/mL ang II was injected in a gradient capillary LC-UV switching system with UV detection at 210 nm. The columns used were a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm) and a Kromasil C_{18} pre-column (5 x 1 mm (ID), 5 µm). The loading MP (AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added) and the analytical MP (AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added) were delivered at a flow rate of 0.200 and 0.005 mL/min, respectively.

In conclusion, based on both peak areas and peak shape, both angiotensin II and bradykinin are most stable when dissolved in acidic (pH = 3) $NH_4^+HCOO^-$ and stored in the refrigerator.

3.6 Carryover study

The classical carryover is caused by a cavity somewhere in the system where the analyte collects and then elutes gradually as several blanks are injected which gives a peak that is smaller for each injection. J. W. Dolan has described a procedure for solving carryover problems in systems that include an autosampler [76]. The eventual carryover in System 2 (Section 2.3.1.2) was investigated using 1 μg/mL bradykinin as test substance (solution no 1 in Table 6, Section 2.4.6, stored in the freezer). One analysis of bradykinin test solution and then four analysis with HPLC grade water (Fluka) were performed (overlay of the chromatograms; see Figure 29). A carryover of 9.2 % was observed in the first blank injection, and in blank injection no 4 there was still a carryover of 2.4 %. Since carryover problems had been experienced using Rheodyne valves at earlier occasions, this injection valve was suspected to be the origin of the carryover problems, and the Rheodyne valve was therefore rinsed with fresh AcN and investigated again following the same procedure and similar results were observed (overlay of the chromatograms is not showed).

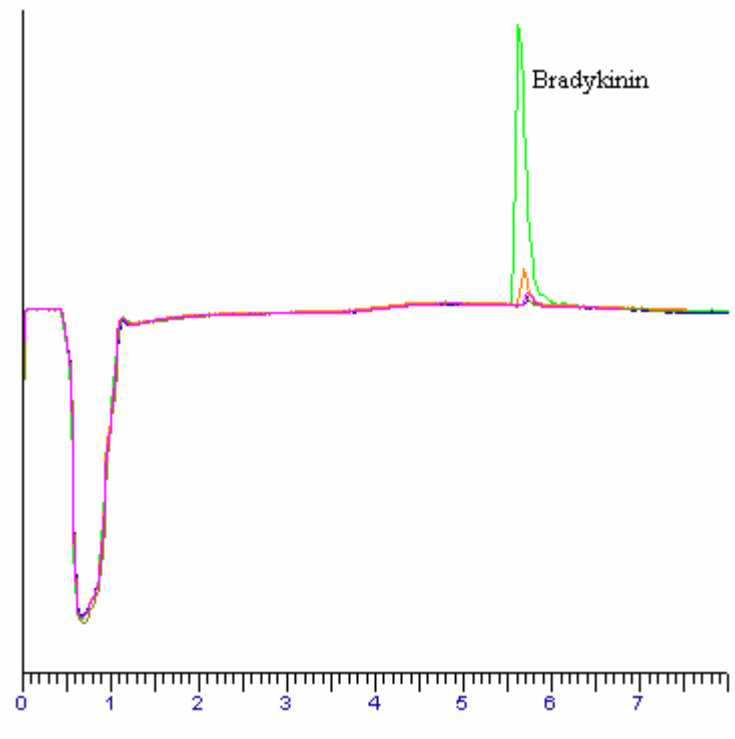

Figure 29: An overlay of the chromatograms for 1 µg/mL bradykinin and then four analysis with HPLC water injected into a capillary LC-UV switching system using a Rheodyne injection valve with a 200 µL external loop. The columns used were a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm) and a Kromasil C_{18} pre-column (5 x 1 mm (ID), 5 µm). The loading MP (AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added) and the analytical MP (a gradient of AcN – H_2O with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added) were delivered at a flow rate of 0.200 and 0.005 mL/min, respectively. UV detection was performed at 210 nm.

Then the Rheodyne valve was replaced with a Valco valve (rinsed with fresh AcN prior to use). Another bradykinin injection followed by two injections of HPLC grade water were performed (overlay of the chromatograms; see Figure 30). The results are given in Table 9 (the raw data are given in Table A11 in Appendix, Section 6.2.3).

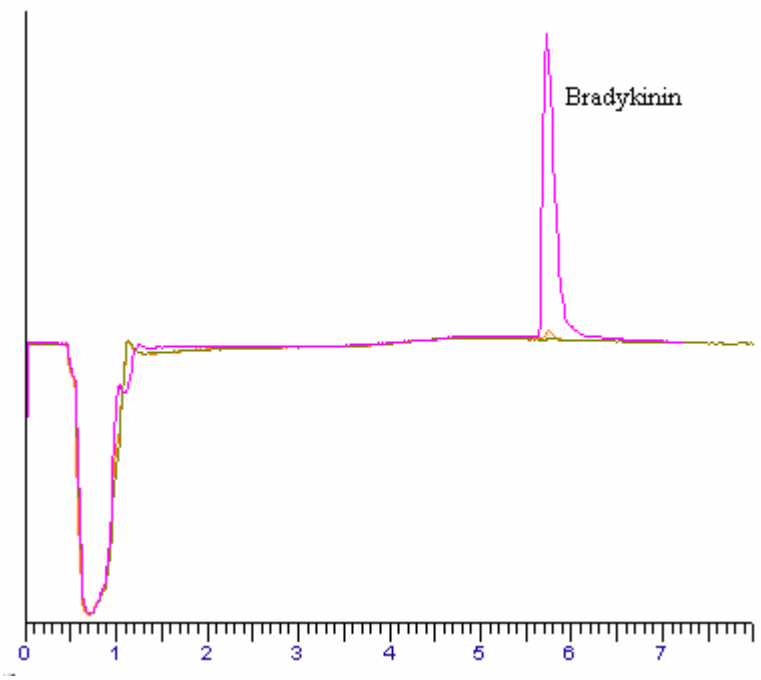

Figure 30: An overlay of the chromatograms for 1 µg/mL bradykinin and then two analysis with HPLC water injected in a capillary LC-UV switching system using a Valco injection valve (rinsed) with a 200 µL external loop. The columns used were a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm) and a Kromasil C_{18} pre-column (5 x 1 mm (ID), 5 µm). The loading MP (AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added) and the analytical MP (AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added) were delivered at a flow rate of 0.200 and 0.005 mL/min, respectively. UV detection was performed at 210 nm.

As can be seen from Table 9 app. 10 % carryover in the first blank injection, and still 2-3 % carryover in the 4th blank injection were observed using the Rheodyne valve both before and after rinsing (the carryover after rinsing of the valve was even slightly higher than before, but this may be due to random uncertainty). The Valco valve showed much better results. The carryover in the first blank injection after the bradykinin injection was just 2.0 %. That is significantly lower than the carryover observed when using the Rheodyne valve. In the second blank injection the carryover was minimal.

Table 9: Carryover for Rheodyne and Valco injection valves. The results are given in peak area relative to the peak area when bradykinin was injected. (The overlays of the chromatograms are showed in Figure 29 and 30)

Injection sequence	Rheodyne valve	Rheodyne valve (rinsed)	Valco valve (rinsed)
Brad	100 %	100 %	100 %
Blank 1	9.2 %	10.4 %	2.0 %
Blank 2	3.4 %	6.0 %	0.60 %
Blank 3	3.2 %	4.1 %	--
Blank 4	2.4 %	3.2 %	--

The carryover effect has probably not influenced the results of the stability testing since the analyte concentration was similar in every injection. The first injection in the morning may have given a slightly smaller effect because of dissolution and subsequent washing prior to the first injection. However, since the first injection often gave very bad peak shape, this analysis was not included in the stability testing.

3.7 Robustness

The robustness of a HPLC method describes the capacity of the system to remain unaffected by variations in method parameters. When working with analytical chemistry a sensitive, robust and fast system is desirable. In a reversed-phase capillary LC-UV column-switching system like System 2 (Section 2.3.1.2) several parameters influence the speed and sensitivity of the system. Several parameters have been tested in this work to obtain an optimized method for peptide determination: loading flow rate, loading time of the sample on the pre-column and reconditioning time of the pre-column with the loading MP. Consideration was also given to whether or not the peak area or shape changed when the pre-column was replaced, as well as to how the retention time was influenced by the varying age of the MP. 1 μg/mL bradykinin was used as test solution.

3.7.1 Loading flow rate and sample loading time

When injecting large sample volumes it is important to ensure a complete transfer within a reasonable period of time of the analytes from the injection loop to the pre-column. Increased flow rates will decrease the loading time, but increase the pressure in the system. Excessive pressure will shorten the lifetime of the column and other parts of the chromatographic system.

As mentioned earlier, packing materials with 10 and 5 μm particles were chosen for the in-house made and commercial pre-columns, respectively, in order to ensure refocusing of the analyte on the analytical column, which contains smaller particles. The large particles in the pre-column also create lower background pressure, and thus enable higher flow rates. In addition, a short pre-column lowers the pressure. Since leakage was observed in the valves at pressures above 200-250 bar, a flow rate that did not give more than 200 bar was chosen. For the in-house made Kromasil pre-columns that means a flow rate of 0.070 mL/min. Since the commercial Kromasil pre-columns were shorter (only 5 mm compared to 53 mm for the in-house made pre-columns) a flow rate of 0.200 mL/min could be used for those columns. No significant change in sensitivity was observed when the loading flow rate was changed in the range 0.070 – 0.200 mL/min (chromatogram not shown), and the system was therefore found to be robust with respect to such changes.

The time needed to transfer the analytes from the injection loop (through the capillaries and switching valve) to the pre-column at a flow rate of 0.200 mL/min is calculated to be approximately 1.5 minutes. To be sure that complete transfer of the analyte to the pre-column occurred, the peak areas, shapes and retention times obtained using loading times of 3.5 and 5.0 min were compared. The peak area, peak shape and retention times of the analyte were similar for both loading times, thus 3.5 minutes is sufficient time (chromatograms not shown).

3.7.2 Recondition time of the pre-column

When both valves are in position 2 the analytical mobile phase is going through the pre-column. Since the analytical MP at the end of the gradient is a stronger eluent than the

loading MP, it is essential to have a sufficient reconditioning time of the pre-column before injecting a new sample, in order to avoid breakthrough of the analytes in the pre-column. On the other hand a speedy method is desired, so the reconditioning time should not be too long. The retention times, peak shapes and areas of bradykinin obtained when using a reconditioning time of 5 min were reproducible and also similar to what was observed when using longer reconditioning times, thus 5 minutes of reconditioning was chosen.

3.7.3 Are the peak areas and shapes changing when the pre-column is replaced?

Since the pre-column has to be replaced from time to time, it is good to know how robust the system is with respect to such replacements. The peak areas of brad obtained with an old pre-column (Hot Sep Tracy, Kromasil C_{18}, 5 x 1 mm (ID), 5 μm) and a new pre-column of the same type were compared. The peak areas are given in Appendix, Table A13.

Using the t-test, the average peak areas before and after replacement of the pre-column were found to be significantly different at the 5 % level, but at the 1 % level the average were not significantly different (Appendix, Section 6.3.1). This means that the system is relatively robust, with regard to replacement of the pre-column.

3.7.4 Does the age of the MP influence the retention time?

It is time consuming to make new pH-adjusted mobile phases containing water, organic solvent and MP additives or buffers. Therefore it is convenient to make a big reservoir of MP. However, when doing qualitative analysis the age of the MP must be considered because an old MP may give longer retention times than a new MP due to AcN (or other organic solvents and modifiers used) which are more volatile than water, and will thus evaporate faster. If the retention time is significantly influenced by the age of the MP, then making MP more often (in some cases every day) should be considered. In this work the mobile phases were replaced when the reservoir was empty, but MP older than 5 days

was not used. It was investigated whether or not the retention time changed after making new MP.

The retention times for bradykinin were measured using analytical mobile phases that were several days old. Then the retention time was measured after replacing the MP in chamber A, and measured again after replacing the MP in chamber B. The retention times are given in Table A14 in Appendix. An analysis of the variance (ANOVA) was performed in order to investigate whether the sample mean values differed significantly (Appendix, Section 6.3.2). According to the ANOVA the sample mean did not differ significantly when the MP was replaced.

All retention times for bradykinin and angiotensin II obtained during the stability testing are listed in Appendix, Table A7-A10. The average of the retention time for each peptide stored under different conditions was calculated and the RSD values found for the two peptides varied between 1.1 and 2.7 %. Thus the system was found to be quite robust with regard to the age of the MP.

3.8 Nano LC-UV

3.8.1 Nano LC-UV

Nano LC gives lower radial dilution than capillary LC, and thus higher peaks. In peptide analysis the highest sensitivity possible is desirable, due to limited concentrations in biological samples. In order to obtain more experience with nano LC, some preliminary testing has been done in this project.

An isocratic nano LC-UV system (System 3, described in Section 2.3.2.1) was built, and 20 nL of 1 mg/mL bradykinin was injected into the system. A chromatogram is shown in Figure 31.

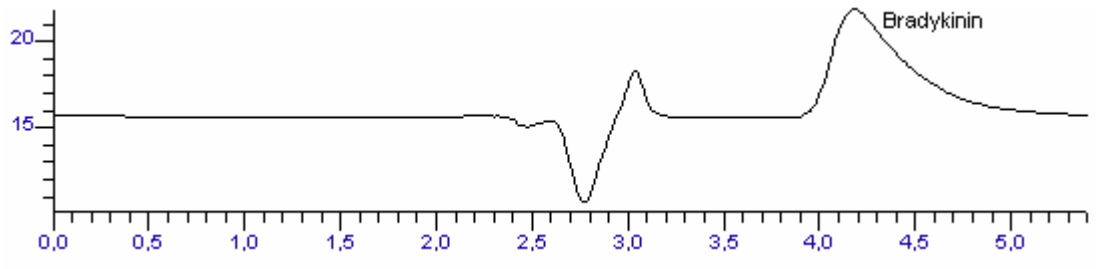

Figure 31: Chromatogram of 20 nL of 1 mg/mL bradykinin injected in an isocratic nano LC-UV system with Kromasil C_{18} nanocolumn (150 x 0.1 mm (ID), 3 µm) as separation column. The MP, consisting of AcN – H_2O (16.08 : 83.92, v/v) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added, was delivered at a flow rate of 0.3 µL/min

Since the MP gradient used in the stability testing functioned well, a similar gradient was used in this part of the work; 0-100 % B in 10 minutes (See Section 2.3.2.1, System 3 for more details). A chromatogram is shown in Figure 32.

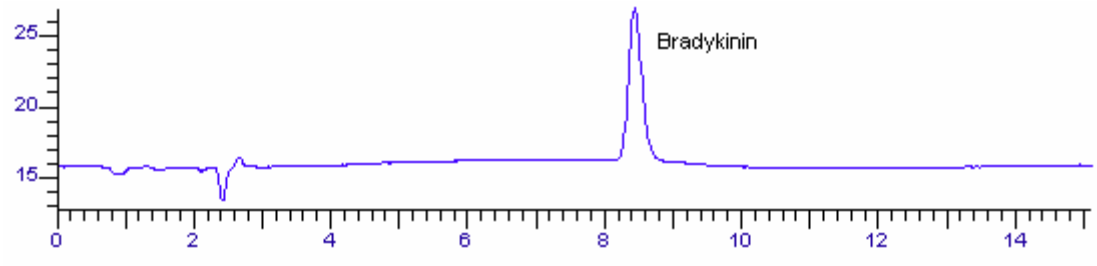

Figure 32: Chromatogram of 20 nL of 1 mg/mL bradykinin injected in a gradient nano LC-UV system with a Kromasil C_{18} nanocolumn (150 x 0.1 mm (ID), 3 µm) as analytical column. Gradient elution were performed using a MP consisting of AcN – H_2O with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added that was delivered at a flow rate of 0.6 µL/min. UV detection was performed at 210 nm.

3.8.2 Column switching gradient nano LC-UV

In this part of the work System 4 (Section 2.3.2.2) was used. 200 µL of 5 ng/mL of bradykinin was injected (chromatogram; see Figure 33).

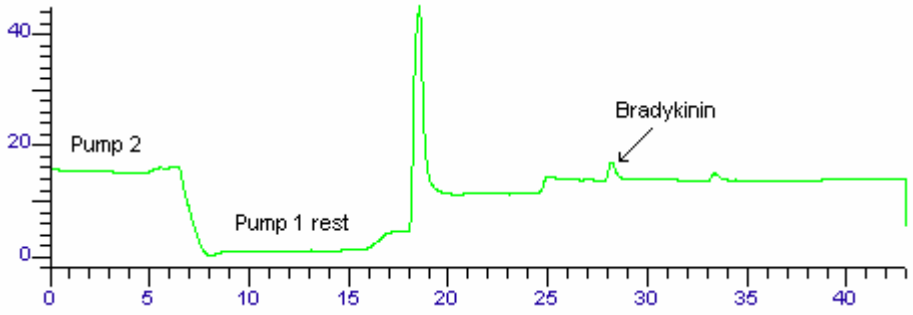

Figure 33: Chromatogram of 200 µL 5 ng/mL bradykinin injected in a gradient nano LC-UV switching system with back flushing of the pre-column. The bradykinin is eluted after ~28.5 min. The columns used were a Kromasil C_{18} nanocolumn (150 x 0.1 mm (ID), 3 µm) and a Kromasil C_{18} pre-column (53 mm x 0.320 mm (ID), 5 µm). The loading MP (AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added) and the analytical MP (AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added) were delivered at a flow rate of 200 and 0.6 µL/min, respectively. UV detection was performed at a wavelength of 210 nm.

Since the inner diameter of the analytical Kromasil nanocolumn (0.1 mm) is lower than the ID of the analytical Kromasil capillary column (0.3 mm), a lower flow rate has to be used in order to avoid excessive back pressure. The dimensions of the pre-column are (53 mm x 0.320 mm (ID)) giving a volume of 4.26 µL. This volume is relatively large compared to the flow rate used (0.6 µL/min), so the expected time used to transfer the analytes from the pre-column to the analytical column is app. (4.26 / 0.6 min) ≈ 7 min. The large negative peak in the chromatogram (Figure 33) is caused by the long time, app. 10 min, that it takes for pump 2 to flush through the pre-column, and then through the analytical column.

This experiment shows that it is important to use a small pre-column when using a slow flow rate, in order to avoid too long void times. E.g. a commercial pre-column with the dimensions: 0.320 mm x 5 mm gives a volume of 0.402 µl, thus giving a void time that is app. 10 times less than the time observed in this experiment. The disadvantages of small pre-columns are lower analyte capacity and relatively low robustness.

3.9 Mass spectrometry

3.9.1 Direct infusion-ESI-TOF-MS

When LC is connected to ESI-MS it is important to find a mobile phase that is compatible with both LC and ESI-MS; the mobile phase should generally consist of an organic modifier (AcN or MeOH) and a volatile buffer. Excessive buffer concentrations may cause ion suppression of the analyte, while insufficient concentrations may give peak tailing and poor efficiency [73]. As mentioned before, TFA gives good resolution in RP-LC, but unfortunately causes ion suppression of the analyte in ESI-MS [58, 72, 73]. Investigations of other additives, and also methods to remove the TFA post-column have been reported [73]. Methods where the mobile phase contains a mixture of different ion-pairing agents have also been reported [77, 78]. Temesi and Law [79] reported a study of the influence of TFA, formic acid and ammonium acetate in respect to analyte response obtained with the MS. They found that MPs containing formic acid gave the highest analyte signal, while TFA gave the lowest. Issaq and co-workers [71] compared the influence of different ion-pairing agents, including TFA, heptafluorobutyric acid (HFBA), acetic acid and formic acid, on the MS response and chromatographic separation of different peptides and proteins, using RPLC-ESI-MS, for the determination of peptides and proteins. TFA provided the best resolution, as expected, and the worst resolution was obtained with HFBA, while formic acid gave the highest MS response. They concluded that formic acid and acetic acid provided the best balance between chromatographic resolution and MS response.

In this study the purpose was to find the most appropriate concentration of formic acid, to obtain the highest analyte signal as possible. The results from these preliminary tests will be used in further studies in our group. To find the highest analyte signal, three different solutions containing 10 µg/mL bradykinin dissolved in 20 % AcN and different amounts of FA (0.05, 0.5 and 1.5 %) and water (Table 6) were injected directly into a Micromass LCT orthogonal accelerated ESI-TOF-MS (System 5, Section 2.3.2.3). The ESI was carried out with a Z-spray atmospheric pressure ionization source, which was modified with a spray capillary of 25 µm ID to handle flow rates in the low µL/min range. The

ionization was performed in the positive mode. The capillary voltage was changed during the experiment (Figure 34) and the MS signal intensities were obtained in a total ion chromatogram (TIC) (Figure 34). As the term TIC indicates, the total ion current is plotted against the time (or scan number), thus the MS is used as a general detector since there is no selectivity in the data. Some fragmentation of the analyte was observed between the capillary and the MS due to the relatively high sample cone voltage. The TIC (Figure 34) shows that the highest signal intensity is obtained when 10 μg/mL bradykinin is dissolved in a matrix containing AcN – FA – H_2O (20 : 0.05 : 79.95, v/v/v) and the MS is operated with a capillary voltage of 3500 V. Acids or salts in the matrix help the ionization process, but excessive concentrations of acids or salts compete for ionization with the analyte (suppression of the analyte). García et al. [80] observed an initial increase in the MS response when low concentrations of formic acid, acetic acid, TFA or ammonium formate were used as the ion-pairing agent; but at higher concentrations the signal decreased. From the TIC shown in Figure 34 the same effect can be seen at a capillary voltage of 3000 V, where 0.5 % FA gave better response, than 0.05 % FA, while 1.5 % FA gave the lowest signal. Higher capillary voltage often gives better sensitivity, but with excessive voltage electron discharge may occur, making the instrument less sensitive and shortening its lifetime. As mentioned above 3500 V gave the highest MS signal, when using 0.05 or 1.5 % FA, while 0.5 % FA gave the highest signal at 3000 V (Figure 34). A test voltage of 4000 V was also planned, but when this was tried, the ESI tip began to glow bluish indicating excessive electron discharge, so the experiment was stopped immediately.

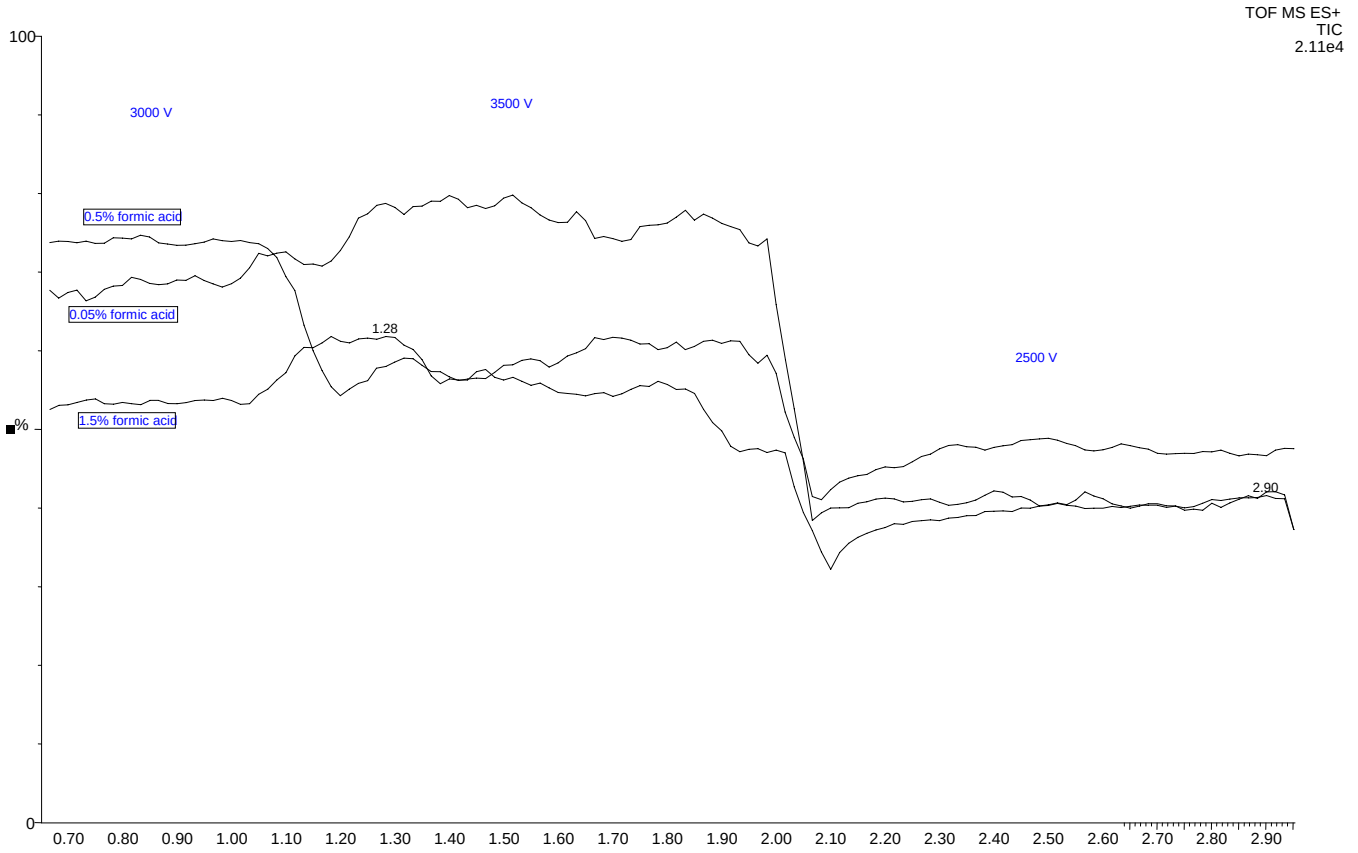

Figure 34: TIC (*m/z* = 200 – 1300) of direct infusion of 10 µg/mL bradykinin dissolved in matrices containing 20 % AcN and different amounts of FA (0.05, 0.5 and 1.5 %) and H_2O.

3.9.2 Capillary LC-ESI-TOF-MS

The theoretical and experimental ratio of analyte dilution for two different capillary separation columns were compared. 20 nL 10 µg /mL bradykinin dissolved in AcN – FA – H_2O (20 : 0.05 : 79.05, v/v) (solution no 1, Table 6) was injected into a gradient capillary LC-ESI-TOF-MS (System 6; Section 2.3.2.4). During the first three injections a Kromasil C_{18} capillary column (50 x 0.3 mm (ID), 3.5 µm) was used, and then a Zorbax SB-C_{18} capillary column (150 x 0.5 mm (ID), 5 µm) was used during the next three injections. The flow rates were 5 and 10 µL/min, respectively, which are the ideal flow rates for the columns. The results are shown in Table A12 (Appendix, Section 6.2.4). The average retention time obtained with the Kromasil column was 5.79 min (RSD = 0.29 %), while the Zorbax column's average was 7.91 min (RSD = 2.9 %). The difference in t_R is related to the different flow rates, column volumes, particle size and carbon load. The

average peak areas for bradykinin were 31.7 (RSD = 3.7 %) and 20.7 (RSD = 2.9 %) obtained with the Kromasil and Zorbax column, respectively. The RSD values show that the system gives quite repeatable retention times and peak areas. Figure 35 shows an overlay of the chromatograms of two randomly chosen analyses, one obtained with the Kromasil column and one with the Zorbax column to show the difference in peak height.

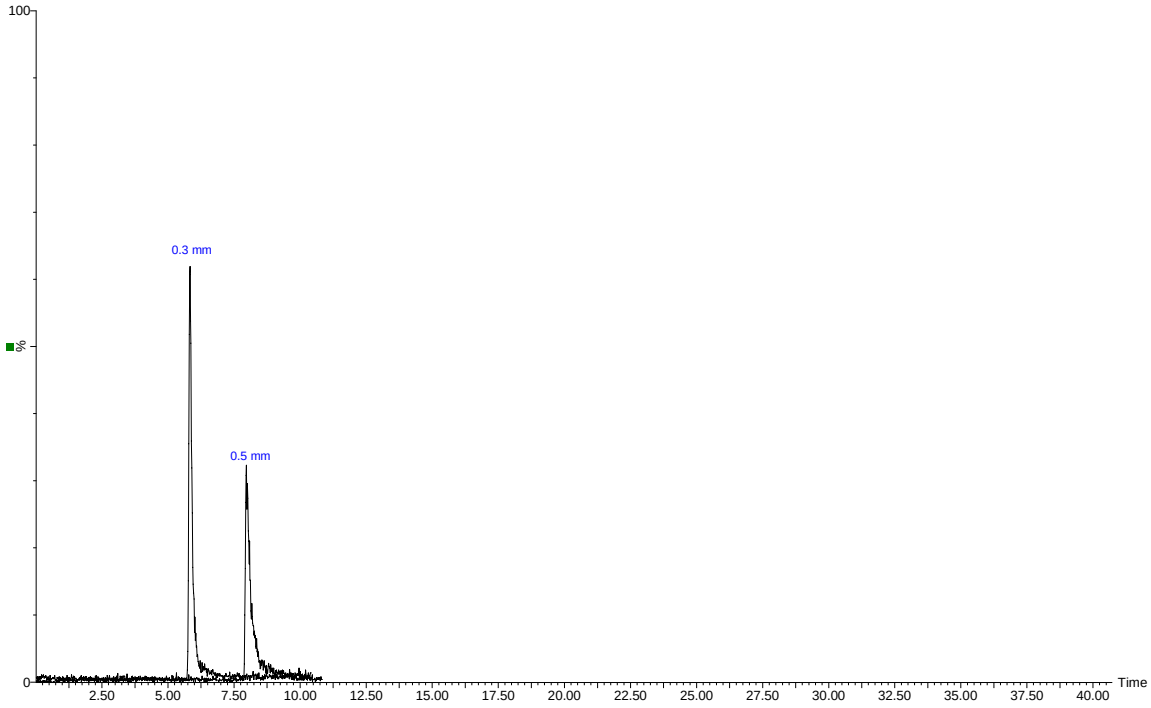

Figure 35: Overlay of two chromatograms (EIC; *m/z* = 530.8) obtained when injecting 20 nL 10 µg/mL bradykinin dissolved in a matrix containing AcN – FA – H_2O (20 : 0.05 : 79.05, v/v/v) into a capillary LC-ESI-TOF-MS first with a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm) and then using a Zorbax SB-C18 column (150 x 0.5 mm (ID), 5 µm) at flow rates of 5 and 10 µL/min, respectively. The MP used was AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.5 % FA added.

The theoretical dilution, D, ratio of the two columns is:

$$\frac{D_{Zorbax}}{D_{Kromasil}} = \frac{0.5^2}{0.3^2} = \underline{\underline{2.78}}$$

Since the Zorbax column gives rise to a 2.78 times higher dilution of the analyte than the Kromasil column the peak height (H) will be app 2.78 times higher for the Kromasil column, however the measured peak height ratio is only:

$$\frac{H_{Kromasil}}{H_{Zorbax}} = \frac{5.5}{2.7} = \underline{\underline{2.04}}$$

The difference is contributed to experimental conditions; it may be that the MS settings used are more favorable for a flow rate of 10 µL/min compared to 5 µL/min. Since Kromasil provides significantly higher peak heights, that are also slightly sharper, it can be assumed that this column also will give a better LOD.

4. Conclusion

This study has specified a robust, sensitive and fast gradient capillary LC column switching system with on-column UV detection for peptide determination. Preliminary studies of a nano LC-UV column switching system established that a low volume pre-column should be used in order to decrease the time required to transfer the analytes from the pre-column to the analytical column. By using direct infusion-ESI-TOF-MS the MS-conditions were optimized with respect to capillary voltage and the concentration of formic acid used as mobile phase additive. The theoretical dilution ratio of two different capillary columns was calculated and compared to the experimental value obtained using a capillary LC-ESI-TOF-MS system. Investigation of the stability of angiotensin II and bradykinin in different aqueous matrices demonstrated that both peptides are most stable when they are dissolved in acidic $NH_4^+HCOO^-$ and stored in the refrigerator.

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Appendix

6.1 Preliminary testing

6.1.1 Preliminary testing system (System P)

For column testing (Section 6.1.2) and preliminary mobile phase testing (Section 6.1.3) a capillary reversed-phase LC-system (called System P) was used.

During the column testing a 25 µL laboratory syringe from SGE (Ringwood, Victoria, Australia) was used to inject the sample into a Valco ChemiInert C4 injection valve equipped with a 50 nL internal loop. The different columns were kept in a Mistral oven (Spark Holland, Emmen, The Netherlands) to ensure a temperature of 25°C. An isocratic Hitachi L-7110 (Merck) pump delivered the mobile phase consisting of different ratios of AcN/H_2O with 0.1 % TFA added at a flow rate of 0.005 mL/min. UV detection was performed at a wavelength of 210 nm using a Spectra 200 UV-Vis detector obtained from Spectra-Physics (San José, CA, USA) equipped with an on-column optical cell made in-house. A Schimadzu C-R6A Chromatopac integrator processed the data.

To test different MPs a modified System P was used. The injections were performed through a Valco ChemInert C4-0004 injection valve with a 20 nL internal loop. The column was kept at room temperature and UV detection at 210 nm was performed using a Linear UVIS UV-detector (Linear Instruments) equipped with 100 µm ID fused silica capillary for on-column detection. The detector signals were converted by a Perkin Elmer Nelson 900 Series Model 941 interface and transferred to a PC with TotalChrom Navigator where the chromatogram could be read or further processed.

6.1.2 Column testing

To find suitable pre-columns and analytical columns for peptide analysis, five different columns (Table 3, Section 2.2) were compared using System P (described in Section 6.1.1). The columns were first tested using different amounts of AcN in the MP; 15, 20, 23 and 25 % AcN in H_2O with 0.1 % TFA added (column no 1, Poroshell (short) and no 4, Poroshell (long) (Table 3) were also tested with 18 % AcN). The test solution contained a mixture of 0.1 mg/mL bradykinin, angiotensin II and neurotensin (neu) dissolved in water, and the retention order was brad, ang II and neu. Two chromatograms are shown in Figure A1. k-values, plate number per meter, selectivity (α), asymmetry and peak resolution (Rs) for the different columns and mobile phases are calculated and shown in the histograms in Figure A2-A6.

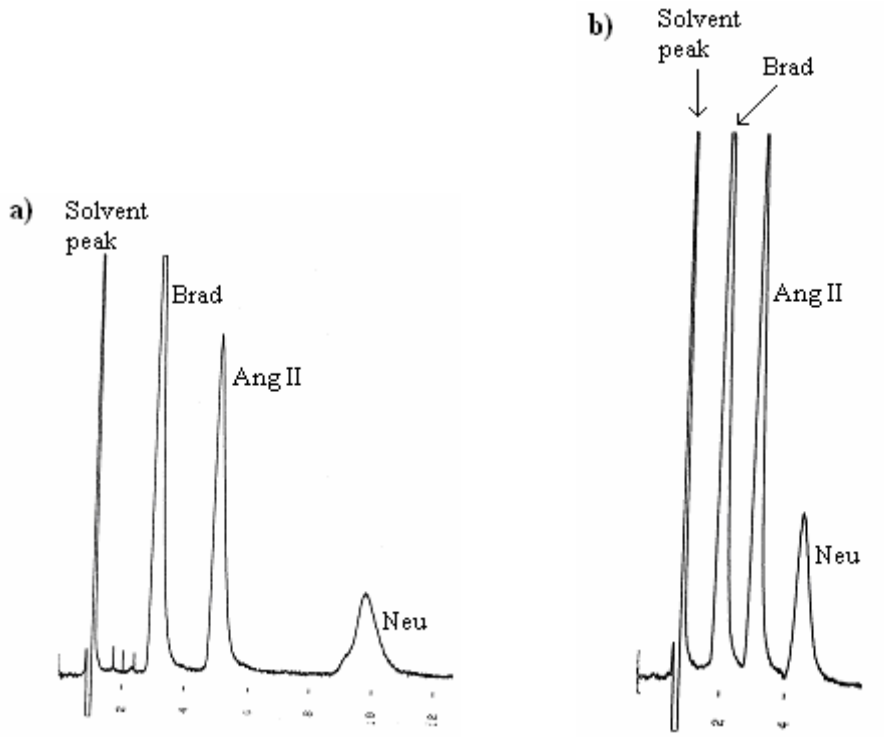

Figure A1: Chromatograms of the test solution (mixture of ~0.1 mg/mL bradykinin, angiotensin II, neurotensin dissolved in water). 50 nL of the peptide mixture was injected into an isocratic capillary LC-UV system with a Kromasil C_{18} column (0.5 x 0.3 mm (ID), 3.5 µm). The mobile phases containing **a)** AcN – H_2O (23:77, v/v) with TFA added (99.9:0.1, v/v) and **b)** AcN – H_2O (25:75, v/v) with TFA added (99.9:0.1, v/v) were delivered at a flow rate of 0.005 mL/min. UV detection was performed at a wavelength of 210 nm.

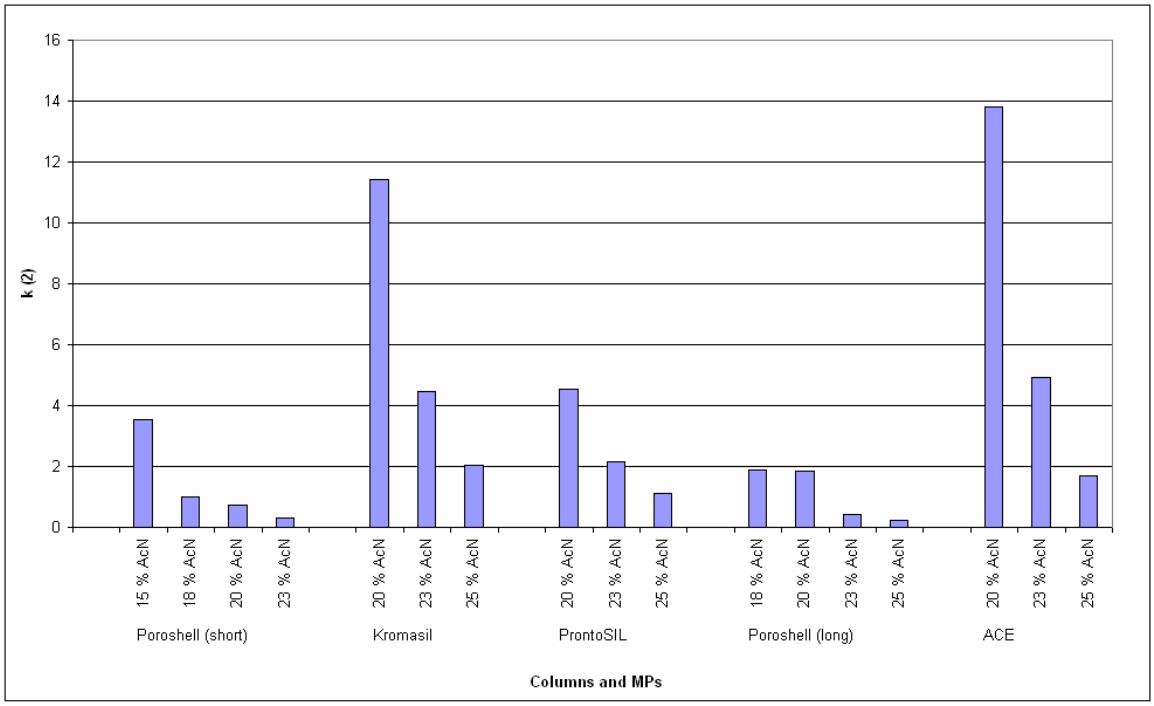

Figure A2: k – values for the second peak, k(2).

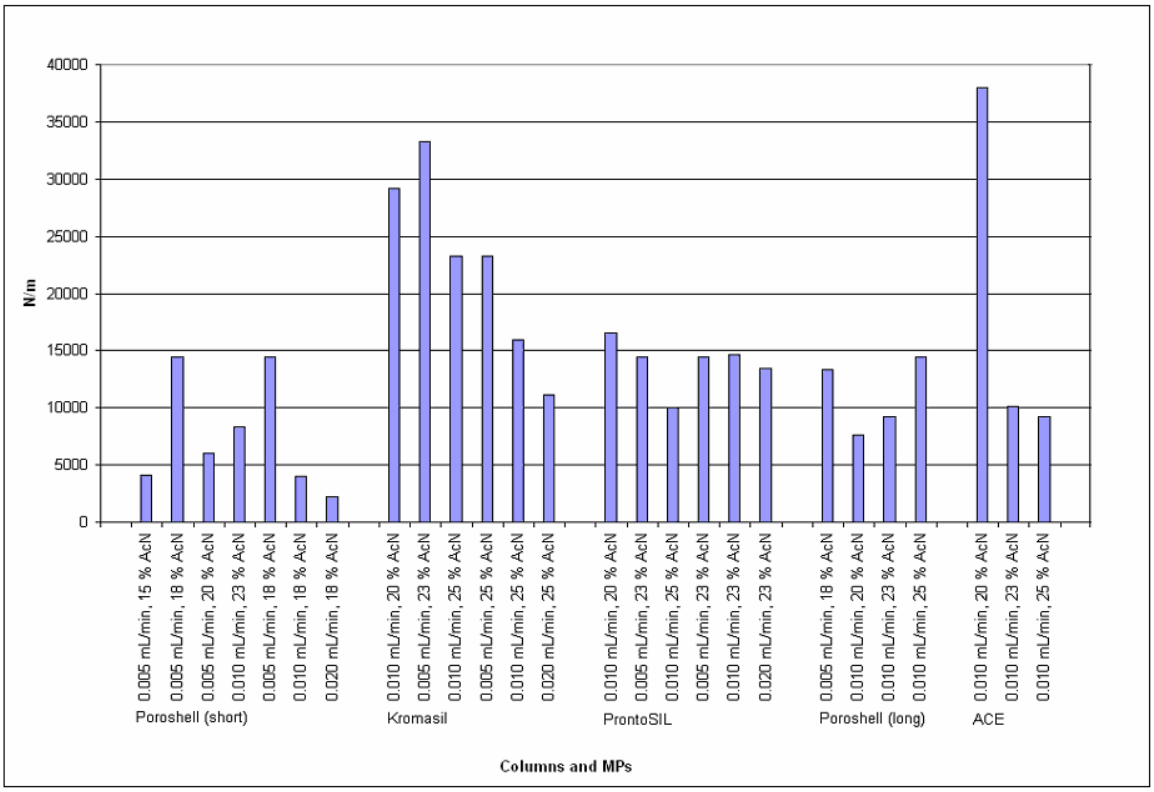

Figure A3: Plate number per meter for the 5 different columns tested using mobile phases with different AcN-content and flow rates.

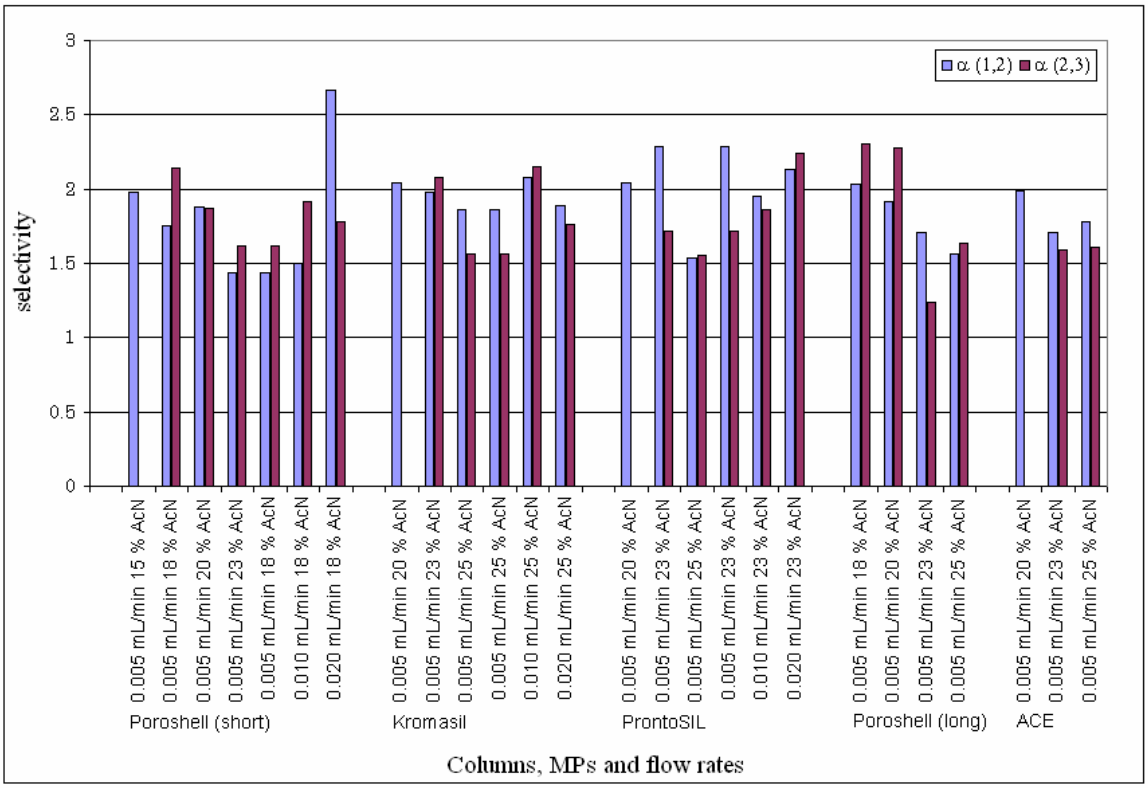

Figure A4: The selectivity between peak 1 and 2, and between peak 2 and 3 using different columns and MP compositions.

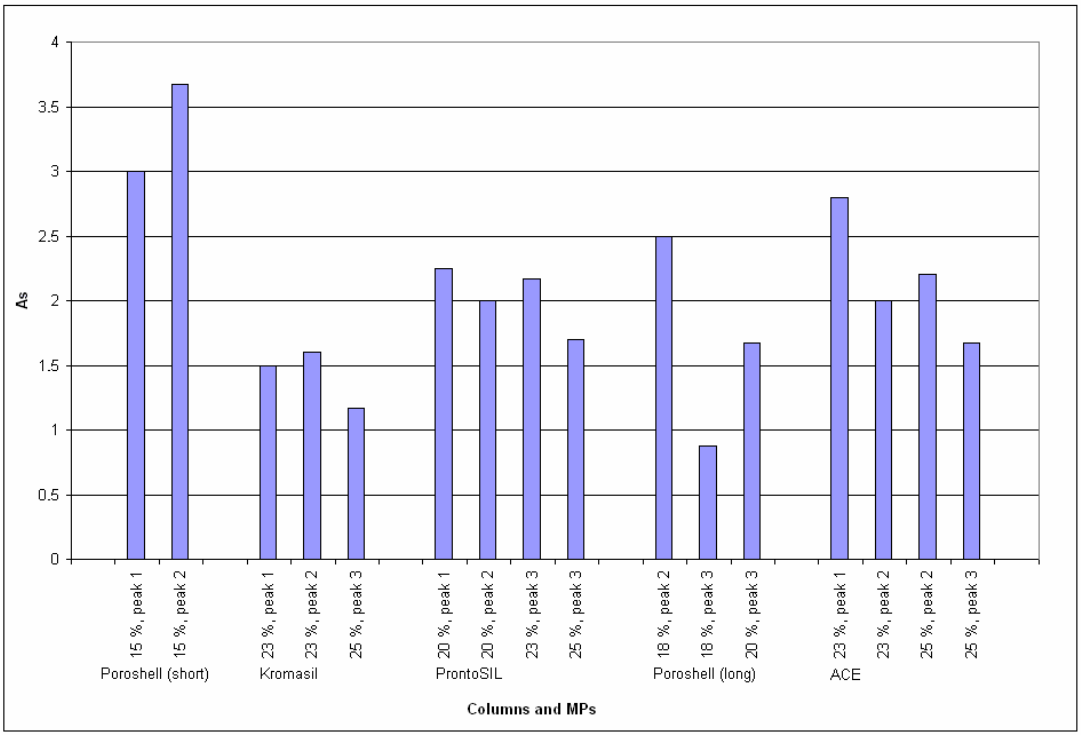

Figure A5: The peak asymmetries for the separated peptides obtained on the 5 columns tested using mobile phases with different AcN-content.

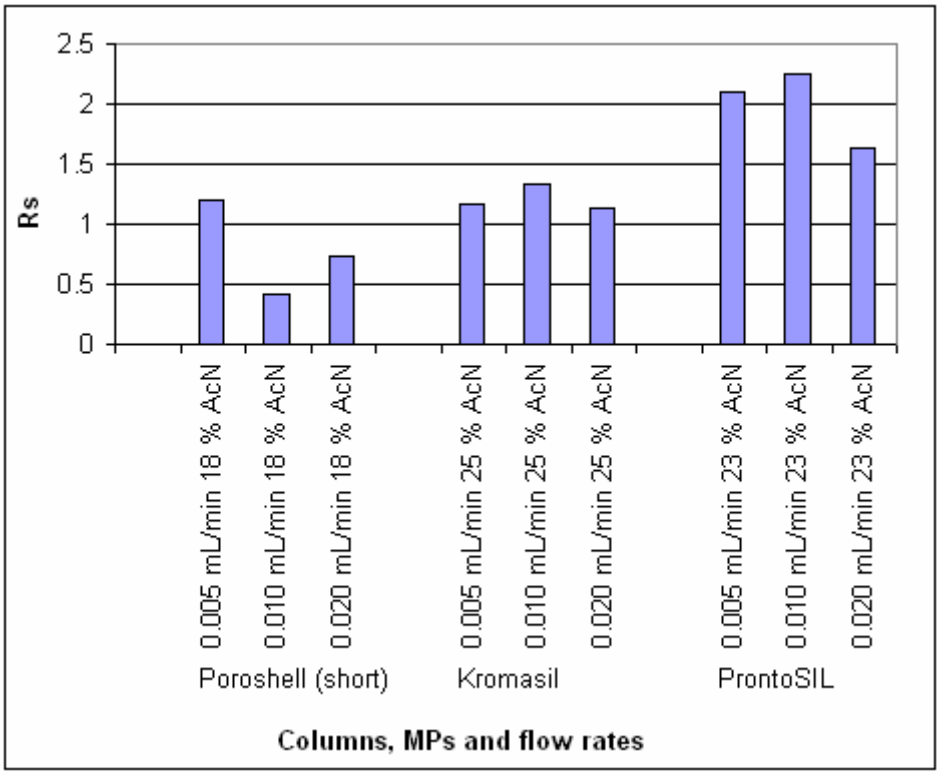

Figure A6: The peak resolution (Rs) for the peptides on the 5 columns using different mobile phase flow rates.

Three of the columns (no 1. Poroshell (short), no 2. Kromasil and no 3. ProntoSIL) were also tested with MP delivered at flow rates of 0.010 and 0.020 mL/min. Mixtures of AcN/H_2O (with 0.1 % TFA added) that gave similar k-values for the second peak (Figure A2) on each column were used as mobile phase. Examples of chromatograms are shown in Figure A7. The differences in N/m-values between the columns are shown in Figure A3 (the MP contents are also given in the figure).

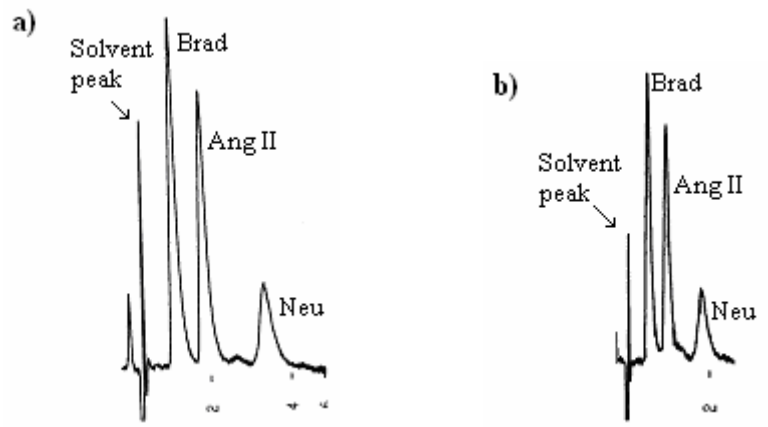

Figure A7: Chromatograms of the test solution (mixtures of ~0.1 mg/mL bradykinin, angiotensin II, neurotensin dissolved in water) injected into an isocratic capillary LC-UV system with a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm). The injection volume was 50 nL and detection was performed at 210 nm. The mobile phase (AcN – H_2O (25:75, v/v) with TFA (99.9:0.1) added) was delivered at a flow rate of **a)** 0.010 mL/min and **b)** 0.020 mL/min.

Finally, the efficiencies of the columns were investigated using toluene. Toluene is a relatively inert neutral compound: shows no secondary interactions with the rest-silanol groups, has reasonable retention on C_{18} material and has UV absorbance. Since it also is easily available at most labs it is commonly used as test substance when investigating columns.

The test solutions consisted of 1 mg/mL toluene dissolved in H_2O (for column no 3 the toluene was dissolved in a mixture of AcN and H_2O (70:30, v/v)). Since toluene was detected at a wavelength of 261 nm, uracil had to be added to the MP as a t_0-tracer because the mobile phase did not give any solvent peak at that wavelength. Figure A8 shows a representative chromatogram. The k-values obtained with toluene as analyte are given in Figure A9 and the N/m-values in Figure A10 (the MP contents are shown in both figures).

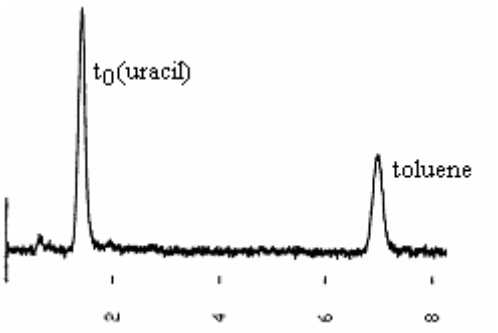

Figure A8: Chromatogram of 50 nL 1 mg/mL toluene injected into a capillary LC-UV system with a Kromasil C_{18} column (50 x 0.3 mm (ID), 3.5 µm). The mobile phase (AcN – H_2O (45:55, v/v)) was delivered at a flow rate of 0.005 mL/min. UV detection was performed at 210 nm.

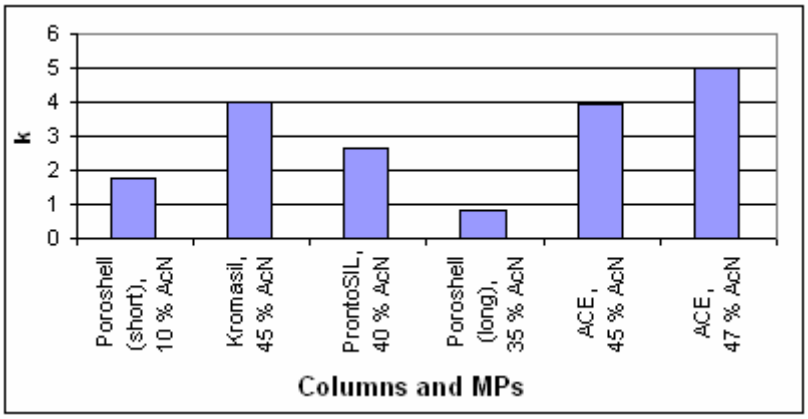

Figure A9: k-values for toluene using different columns and different mobile phases.

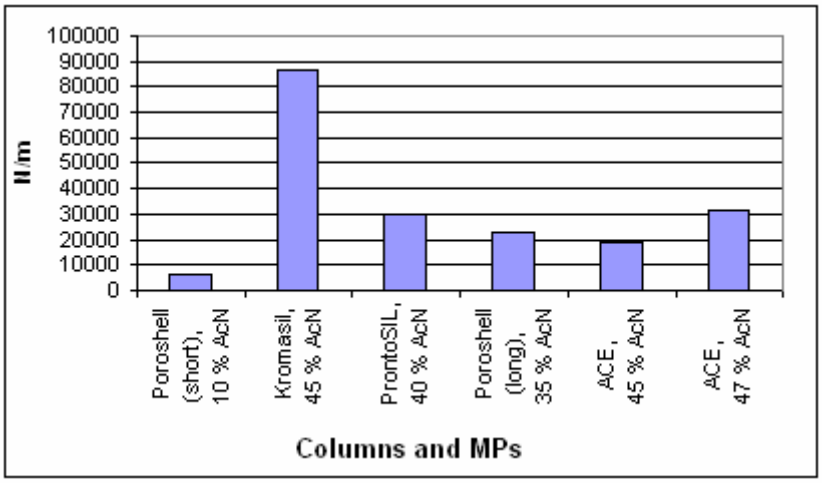

Figure A10: N/m-values obtained using different columns and different mobile phases.

The Kromasil and the ACE columns obtained the highest plate number per meter, except at high flow rates, when a mixture of three peptides was injected (Figure A3). The Kromasil column gave an N/m-value of 33 000 (when tested with 23 % AcN), and the ACE column gave an N/m-value of 38 000 (with 20% AcN as eluting solvent). In a column switching system high efficiency is favourable for the analytical column. The problem with the ACE column however, was that the retention time (t_R) using 20 % AcN was too long (t_R(brad) ~12.5 min, t_R(ang II) ~23.5 min and even longer retention time for neu).When toluene was used as test substance Kromasil had a significantly higher plate number per meter (N/m ~ 87 000). Kromasil also showed low asymmetry values; 1.17-1.60 for the peaks that were investigated (Figure A5). The ACE and Kromasil columns also obtained the highest k-values for the second peak (Figure A2), and thus the longest retention time, making them suitable as pre-columns in a column switching system. All five columns showed selectivity (Figure A4) between 1.5 and 2.5 (except the α(1,2) for 7.5 cm Poroshell column and α(2,3) for 15 cm Poroshell column both using 23 % AcN).

When the short Poroshell, Kromasil and ProntoSIL columns were tested at high flow rates, the ProntoSIL showed no loss in efficiency and high resolution at all flow rates (Figure A3 and A6) in contrast to the other columns.

When using a flow rate of 0.005 mL/min the Kromasil and ACE columns are the best choice, due to good selectivity and high N/m. The Kromasil, however, also gave lower asymmetry and more suitable retention times. At higher flow rates the ProntoSIL column is a good alternative because it maintains the efficiency and high selectivity.

6.1.3 Preliminary testing of mobile phases

Since the aim of the study was first, to develop a chromatographic method for the determination of bradykinin, lysyl-bradykinin and arginyl-bradykinin and then, to measure their concentrations in muscle tissue from human and rats, the method had to deal with the large salt concentration in the real samples caused by the dialysis liquid,

Ringer's acetate, used to extract the peptides from the tissue. Different properties of Ringer's acetate were investigated.

The pH was measured in 5 different mobile phases (Table A1), and the absorbance of the three first MP (Table A1) was measured using a molecular absorption spectrophotometer (described in Section 2.3.3.1). The absorbencies of the mobile phases are shown in Table A2-A4 and Figure A11-A13. Finally the influence of the MP on the peak shape and retention time of ang II was investigated using System P (Section 6.1.1). The chromatograms are given in Figure A14.

Table A1: pH in different mobile phases.

Mobile phase	**pH**
AcN-H_2O (14:86, v/v) with OHAc (99.9/0.1, v/v) added	3.3
AcN-Ringer's acetate (14:86, v/v) with OHAc (99.9/0.1, v/v) added	5.2
AcN-Ringer's acetate (14:86, v/v)	7.4
AcN-H_2O (14:86, v/v) with 20 mM $NH_4^+HCOO^-$ added	6.1
AcN-H_2O (14:86, v/v) with 20 mM $NH_4^+CH_3COO^-$ added	6.7

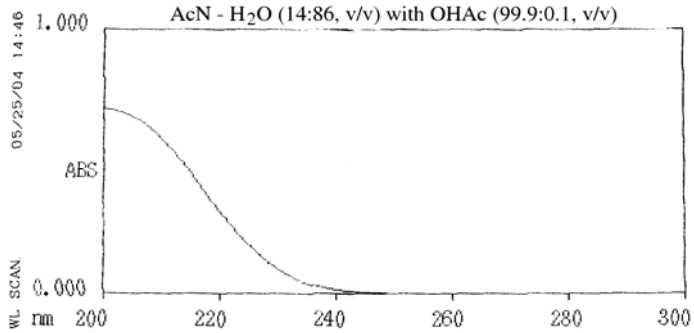

Figure A11: The absorbance of AcN-H_2O (14:86, v/v) with OHAc added (99.9/0.1, v/v).

Table A2: The absorbance of AcN-H_2O (14:86, v/v) with OHAc added (99.9/0.1, v/v).

Wavelength [nm]	**ABS**
300.0	0.000
290.0	0.001
280.0	0.001
270.0	0.001
260.0	0.001
250.0	0.002
240.0	0.014
230.0	0.093
220.0	0.311
210.0	0.587
200.0	0.700

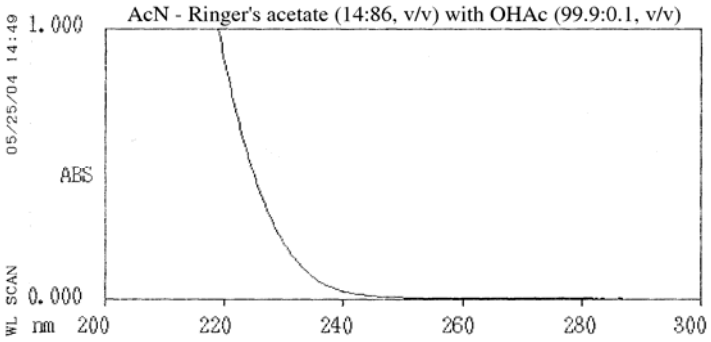

Figure A12: The absorbance of AcN-Ringer's acetate (14:86, v/v) with OHAc added (99.9/0.1, v/v).

Table A3: The absorbance of AcN-Ringer's acetate (14:86, v/v) with OHAc added (99.9/0.1, v/v).

Wavelength [nm]	**ABS**
300.0	0.001
290.0	0.002
280.0	0.003
270.0	0.003
260.0	0.004
250.0	0.007
240.0	0.030
230.0	0.209
220.0	0.878
210.0	2.465
200.0	3.428

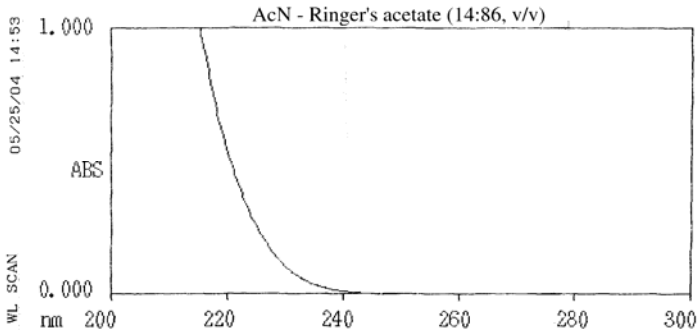

Figure A13: The absorbance of AcN-Ringer's acetate (14:86, v/v).

Table A4: The absorbance of AcN-Ringer's acetate (14:86, v/v).

Wavelength [nm]	**ABS**
300.0	0.000
290.0	0.000
280.0	0.001
270.0	0.001
260.0	-0.002
250.0	-0.003
240.0	0.009
230.0	0.102
220.0	0.532
210.0	1.893
200.0	3.337

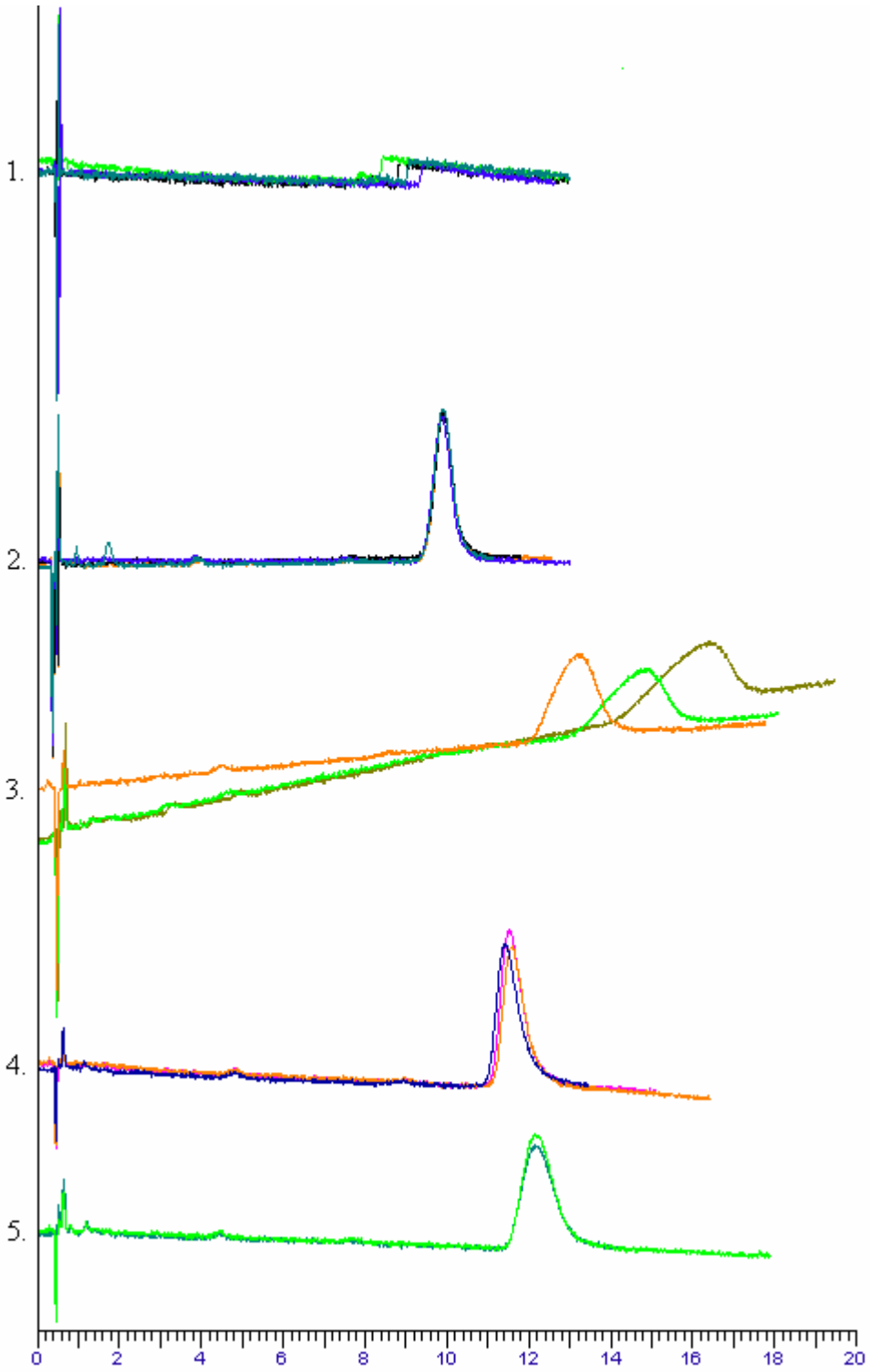

Figure A14: Chromatograms of 20 nL injections of ang II. A capillary LC-UV system was used. Different mobile phases were investigated: 1. AcN-H_2O (14:86, v/v) with OHAc added (99.9/0.1, v/v), 2. AcN-Ringer's acetate (14:86, v/v) with OHAc added (99.9/0.1, v/v), 3. AcN-Ringer's acetate (14:86, v/v), 4. AcN-H_2O (14:86, v/v) with 20 mM $NH_4^+HCOO^-$ added and 5. AcN-H_2O (14:86, v/v) with 20 mM $NH_4^+CH_3COO^-$ added. All MPs were delivered at a flow rate of 0.005 mL/min. UV detection was performed at 210 nm.

6.2 Raw data

6.2.1 Injection volumes

The peak areas for different injection volumes of ang II (Table A5) and brad (Table A6) were measured and the average, $\overline{X}$, and the absolute and relative standard deviation, *s* and *RSD*, respectively, were calculated. The relative standard deviations for each volume varied from 0.25 - 3.3 %, which shows that the system gives relatively repeatable results.

The data below was used in the plots shown in Figure 8-9 in the section on Injecting volume (Section 3.3):

Table A5: Peak areas for different injection volumes of ang II. (Inj. = injection)

Volume	Inj. 1	Inj. 2	Inj. 3	Inj. 4	Inj. 5	$\overline{X}$	S	RSD [%]
50	1069308	1037539	1039798	1077419	--	$1.056 \cdot 10^6$	$2.0 \cdot 10^4$	1.9
75	1628941	1584546	1559416	--	--	$1.59 \cdot 10^6$	$4 \cdot 10^4$	2.2
100	2018853	1992433	2146211	1991409	1999806	$2.03 \cdot 10^6$	$7 \cdot 10^4$	3
150	2879793	2876834	2979688	--	--	$2.91 \cdot 10^6$	$6 \cdot 10^4$	2.0
200	4332657	4210616	4112921	--	--	$4.22 \cdot 10^6$	$1.1 \cdot 10^5$	2.6
300	5207197	5399127	--	--	--	5303162	--	--

Table A6: Peak areas for different injection volumes of brad.

Volume	Inj. 1	Inj. 2	Inj. 3	$\overline{X}$	S	RSD [%]
50	1070122	1121118	1140962	$1.11 \cdot 10^6$	$3.7 \cdot 10^4$	3
75	1528276	1576672	1523047	$1.543 \cdot 10^6$	$3.0 \cdot 10^4$	1.9
100	1898515	1889157	1895658	$1.894 \cdot 10^6$	$5 \cdot 10^3$	0.25
150	2514498	2549729	2684862	$2.58 \cdot 10^6$	$9 \cdot 10^4$	4
200	3764320	3798265	3894527	$3.82 \cdot 10^6$	$7 \cdot 10^4$	1.8

6.2.2 Peak areas and retention times obtained during the stability testing

All peak areas and retention times from the stability testing are showed in Tables A7-A10.

Grubbs' test [81], recommended by ISO in preference to Dixon's test was used to exclude eventual outliers:

$$G = \frac{\left|\text{suspected value} - \overline{X}\right|}{s}$$ (The suspected value is included when s is calculated.)

Table A.6 in "Statistics and Chemometrics for Analytical Chemistry" [81] gives the critical values of G for P = 0.05. The suspected value is treated as an outlier if the calculated G-value is larger than the critical value.

Table A7: Peak areas for ang II stored in different matrices at different temperatures.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 14	Day 19	Day 24
AngII in H2O R	61311	60941	59243	62384	62998	53870	55734	53545		40656
AngII in H2O R	62461	62035	61944	62528	64447	57393	60660	51001		
AngII in H2O R										
Mean	61886	61488	60593	62456	63722	55632	58197	52273		40656
St.dev.										
RSD										
AngII in H2O K	61311	47620	47993	49891	43730	43229	43442	44891	46137	44141
AngII in H2O K	62461	51701	48166	48416	47435	42925	43661	45882	44438	42709
AngII in H2O K		47698	48907							
Mean	61886	$4.90 \cdot 10^4$	$4.84 \cdot 10^4$	49153	45583	43077	43551	45387	45287	43425
St.dev.		$2.3 \cdot 10^3$	$5 \cdot 10^2$							
RSD		5	1.0							
AngII in H2O F	61311	50438	50604	51480	45499	45624	45125	53274	44113	45826
AngII in H2O F	62461	54463	57442	46403	44870	49841	46060	47866	46069	46894
AngII in H2O F		49446		53100				50262		
Mean	61886	$5.14 \cdot 10^4$	54023	$5.0 \cdot 10^4$	45185	47732	45593	$5.05 \cdot 10^4$	45091	46360
St.dev.		$2.7 \cdot 10^3$		$3 \cdot 10^3$				$2.7 \cdot 10^3$		
RSD		5		7				5		
AngII in Ringer R	50095	43096	42198	51825	46444	40562	42218	36724	29216	29828
AngII in Ringer R	47147	45546	46951	51283	45559	41054	42690	37872	31450	29537
AngII in Ringer R						42476				
Mean	48621	44321	44575	51554	46001	$4.14\ 10^4$	42454	37298	30333	29682
St.dev.						$1.0 \cdot 10^3$				
RSD						2.4				
AngII in Ringer K	50095	24519	27965	34069	29279	24767	28165	26574	25259	24514
AngII in Ringer K	47147	25589	35353	23973	28798	29644	27717	23187	26007	24389
AngII in Ringer K			30177	24057						
Mean	48621	25054	$3.1 \cdot 10^4$	$2.7 \cdot 10^4$	29038	27206	27941	24880	25633	24451
St.dev.			$4 \cdot 10^3$	$6 \cdot 10^3$						
RSD			12	21						
AngII in NH4HCOO R	67313	64348	65880	71891	66227	67010	65087	67808	61975	62884
AngII in NH4HCOO R	64257	63794	67132	69866	65779	67647	66908	68083	65471	62494
AngII in NH4HCOO R										
Mean	65785	64071	66506	70878	66003	67329	65997	67945	63723	62689
St.dev.										
RSD										
AngII in NH4HCOO K	67313	60068	69025	68442	65323	64396	63724	64240	60113	62860
AngII in NH4HCOO K	64257	66533	65260	67940	61952	61414	61179	64303	61869	61968
AngII in NH4HCOO K		65421								
Mean	65785	$6.40 \cdot 10^4$	67143	68191	63637	62905	62451	64271	60991	62414
St.dev.		$3.5 \cdot 10^3$								
RSD		5.4								

Table A8: Peak areas for brad stored in different matrices at different temperatures.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 8	Day 15	Day 21	Day 24
Brad in H2O R	64497	64617	64317	64602	64944	71257	67208	23450		11273
Brad in H2O R	71035	66479	66838	60366	63674	72796	67004	25497		9679
Brad in H2O R										
Mean	67766	65548	65577	62484	64309	72026	67106	24473		10476
St.dev.										
RSD										
Brad in H2O K	64497	66062	57340	54136	64112	59832	61141	66260	49128	58160
Brad in H2O K	71035	67600	62928	61720	64173	61551	67235	67410	52075	58890
Brad in H2O K				64737						
Mean	67766	66831	60134	$6.0 \cdot 10^4$	64142	60692	64188	66835	50602	58525
St.dev.				$5 \cdot 10^3$						
RSD				9						
Brad in H2O F	64497	65296	61972	55335	66193	62772	63539	50721	49292	52677
Brad in H2O F	71035	67257		62561	66933	60681	63156	59485	56943	56783
Brad in H2O F								61180	53176	
Mean	67766	66276	61972	58948	66563	61727	63347	$5.7 \cdot 10^4$	$5.3 \cdot 10^4$	54730
St.dev.								$6 \cdot 10^3$	$4 \cdot 10^3$	
RSD								10	7	
Brad in Ringer R	59608	68046	65470	71472	65546	65444	64323	61118	55449	55564
Brad in Ringer R	59651	63803	63671	69518	65554		62346	62522	59955	56193
Brad in Ringer R	63340								61465	
Mean	$6.09 \cdot 10^4$	65924	64571	70495	65550	65444	63335	61820	$5.9 \cdot 10^4$	55878
St.dev.	$2.1 \cdot 10^3$								$3 \cdot 10^3$	
RSD	4								5	
Brad in Ringer K	59608	58864	62607	66530	58965	49362	61813	67044	62721	56864
Brad in Ringer K	59651	59755	67251	56117	54807	47567	56609	62479	60318	55279
Brad in Ringer K	63340									
Mean	$6.09 \cdot 10^4$	59309	64929	61323	56886	48464	59211	64761	61520	56071
St.dev.	$2.1 \cdot 10^3$									
RSD	4									
Brad in NH4HCOO R	72397	78154	78883	82597	80966	79003	80542	82337	81299	74308
Brad in NH4HCOO R	74061	75689	76640	81283	80524	79339	79573	73608	82808	81227
Brad in NH4HCOO R		72720						84653		
Mean	73229	$7.55 \cdot 10^4$	77761	81940	80745	79171	80057	$8.0 \cdot 10^4$	82053	77767
St.dev.		$2.7 \cdot 10^3$						$6 \cdot 10^3$		
RSD		4						7		
Brad in NH4HCOO K	72397	70477	79049	79473	78767	76174	79557	80734	80925	80188
Brad in NH4HCOO K	74061	78341	76258	78588	81447	77016	81690	82209	79875	76717
Brad in NH4HCOO K										
Mean	73229	74409	77653	79031	80107	76595	80623	81472	80400	78452
St.dev.										
RSD										

Table A9: Retention times for angII stored in different matrices at different temperatures.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 14	Day 19	Day 24	Mean	St.dev.	RSD
AngII in H2O R	5.883	5.833	6.033	6.017	5.983	5.667	5.883	5.883		5.884			
AngII in H2O R	5.900	5.833	6.017	5.965	5.900	5.733	5.817	5.883			5.89	0.10	1.7
AngII in H2O R													
AngII in H2O K	5.883	5.847	6.117	6.025	5.983	5.800	5.933	5.883	5.635	5.883			
AngII in H2O K	5.900	5.850	6.017	5.933	5.967	5.783	5.883	5.883	5.722	5.883	5.90	0.12	2.0
AngII in H2O K		5.893	6.147										
AngII in H2O F	5.883	5.850	6.033	6.000	5.983	5.800	5.917	5.877	5.653	5.848			
AngII in H2O F	5.900	5.867	6.033	5.977	6.017	5.860	5.900	5.867	5.672	5.883	5.89	0.10	1.7
AngII in H2O F		5.933		5.967				5.917					
AngII in Ringer R	5.917	5.917	6.017	5.967	5.983	5.676	5.900	5.867	5.600	5.836			
AngII in Ringer R	5.917	5.917	6.017	5.967	5.950	5.783	5.900	5.917	5.583	5.800	5.87	0.12	2.1
AngII in Ringer R						5.867							
AngII in Ringer K	5.917	5.917	5.983	5.933	5.933	5.817	5.900	5.917	5.700	5.833			
AngII in Ringer K	5.917	5.883	6.000	5.920	5.992	5.850	5.917	5.917	5.683	5.884	5.90	0.08	1.4
AngII in Ringer K			5.983	5.952									
AngII in NH4HCOO R	5.917	5.917	5.933	5.933	6.000	5.833	5.933	5.883	5.600	5.733			
AngII in NH4HCOO R	5.950	5.933	6.000	5.933	5.967	5.833	5.900	5.867	5.650	5.937	5.88	0.11	1.8
AngII in NH4HCOO R													
AngII in NH4HCOO K	5.917	5.833	5.983	5.917	6.000	5.817	5.899	5.883	5.667	5.900			
AngII in NH4HCOO K	5.950	5.933	5.967	5.950	6.000	5.817	5.933	5.883	5.683	5.883	5.89	0.09	1.5
AngII in NH4HCOO K		5.956											

Table A10: Retention times for brad stored in different matrices at different temperatures.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 8	Day 15	Day 21	Day 24	Mean	St.dev.	RSD
Brad in H2O R	5.717	5.700	5.748	5.700	5.800	5.617	5.617	5.650		5.633			
Brad in H2O R	5.717	5.700	5.750	5.683	5.783	5.600	5.600	5.433		5.600	5.67	0.09	1.5
Brad in H2O R													
Brad in H2O K	5.717	5.683	5.764	5.667	5.800	5.550	5.617	5.333	5.567	5.467			
Brad in H2O K	5.717	5.700	5.767	5.633	5.783	5.567	5.600	5.583	5.550	5.567	5.63	0.11	2.0
Brad in H2O K				5.583						5.600			
Brad in H2O F	5.717	5.700	5.739	5.617	5.675	5.617	5.600	5.650	5.583	5.617			
Brad in H2O F	5.717	5.700		5.617	5.772	5.567	5.617	5.617	5.568	5.650	5.64	0.06	1.1
Brad in H2O F								5.600	5.583				
Brad in Ringer R	5.200	5.733	5.733	5.683	5.767	5.583	5.600	5.667	5.600	5.433			
Brad in Ringer R	5.300	5.683	5.750	5.650	5.767		5.617	5.650	5.567	5.483	5.59	0.15	2.7
Brad in Ringer R	5.417								5.583				
Brad in Ringer K	5.200	5.700	5.767	5.617	5.767	5.600	5.583	5.633	5.550	5.517			
Brad in Ringer K	5.300	5.700	5.767	5.633	5.783	5.583	5.583	5.617	5.567	5.567	5.59	0.15	2.6
Brad in Ringer K	5.417												
Brad in NH4HCOO R	5.483	5.650	5.767	5.567	5.783	5.600	5.593	5.650	5.550	5.617			
Brad in NH4HCOO R	5.533	5.700	5.741	5.617	5.783	5.567	5.617	5.683	5.517	5.583	5.63	0.09	1.5
Brad in NH4HCOO R		5.700						5.583					
Brad in NH4HCOO K	5.483	5.717	5.740	5.650	5.767	5.617	5.517	5.633	5.567	5.600			
Brad in NH4HCOO K	5.533	5.717	5.717	5.583	5.817	5.583	5.583	5.617	5.550	5.600	5.63	0.09	1.6
Brad in NH4HCOO K													

6.2.3 Carryover

Table A11: Results carryover. (Inj. seq. = Injection sequence, Inj. = Injection; Bl. = Blank)

Inj. seq.	**Rheodyne valve**		**Rheodyne valve (rinsed)**		**Valco valve (rinsed)**	
	Peak area	Peak area relative to the bradykinin peak	Peak area	Peak area relative to the bradykinin peak	Peak area	Peak area relative to the bradykinin peak
Inj.	56272	100 %	57414	100 %	75164	100 %
Bl.1	5193	9.2 %	5942	10.4 %	1530	2.0 %
Bl.2	1889	3.4 %	3420	6.0 %	454	0.60 %
Bl.3	1823	3.2 %	2329	4.1 %	--	--
Bl.4	1336	2.4 %	1818	3.2 %	--	--

6.2.4 Peak areas and retention times obtained using capillary LC-ESI-TOF-MS

Table A12: The retention times and peak areas for 10 µg/mL bradykinin dissolved in AcN – FA – H_2O (20 : 0.05 : 79.95, v/v).

Column	**Injection**	**t_R [min]**	**Peak area**
Kromasil C_{18}; 50 x 0.3 mm (ID), 3.5 µm	1	5.82	31
	2	5.80	31
	3	5.75	33
	$\overline{X}$	5.79	31,7
	S	0.04	1.2
	RSD [%]	0.6	4
Zorbax SB-C_{18}; 150 x 0.5 mm (ID) 5 µm	4	7.94	21
	5	7.90	21
	6	7.90	20
	$\overline{X}$	7.910	20.7
	S	0.023	0.6
	RSD [%]	0.29	2.9

6.3 Statistic

6.3.1 F-test and pooled two-sample t significance test

The peak areas using old and new pre-columns (Table A13) were compared using statistical tests.

Table A13: The peak areas for 1 µg/mL bradykinin with an old versus a new pre-column of the same type.

	Old pre-column (population 2)	**New pre-column** (population 1)
	Peak area	Peak area
1	78083.2	77172.0
2	75513.0	79272.0
3	77304.5	79519.0
4	77654.5	80355.0
5	78051.0	--
6	77047.5	--
$\overline{X}$	$7.73 \cdot 10^4$	$7.91 \cdot 10^4$
S	$1.0 \cdot 10^3$	$1.4 \cdot 10^3$
RSD [%]	1.2	1.7

A hypothesis is established; $H_0: \sigma_1^2 = \sigma_2^2$ (where σ_1^2 and σ_2^2 are the variances for the peak areas obtained with the new and old pre-columns, respectively), saying that the difference between two sample variances is not significant. To investigate whether the hypothesis could be retained or not, the statistic F was calculated:

$$F_{calc} = \frac{s_1^2}{s_2^2} = \frac{1354^2}{955^2} \approx \underline{2.010}$$

The number of degrees of freedom are $n_1 - 1 = 4 - 1 = 3$ (numenator) and $n_2 - 1 = 6 - 1 = 5$ (denominator).

There is no reason to expect in advance that the variance of one population should be greater than the other, so a two-sided test is chosen. To find the critical value of F with a probability of 0.05 Table A.4 in "Statistics and Chemometrics for Analytical Chemistry" written by Miller and Miller [81] was used giving a $F_{tab\ (3,5)} = 7.764$. Since $F_{tab} < F_{tab}$, the H_0 is retained; there is no significant difference between the two variances at the 5 % level, thus pooled two-sample t procedures can be used to compare the experimental means.

The pooled s is calculated:

$$s_p{}^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 + n_2 - 2)} = \frac{(4-1)1354^2 + (6-1)955^2}{(4+6-2)} = 1257509.125$$

$$\underline{s_p \approx 1121}$$

The statistic t is calculated:

$$t = \frac{(\overline{X}_1 + \overline{X}_2)}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} = \frac{(79080 - 77276)}{1121\sqrt{\frac{1}{4} + \frac{1}{6}}} \approx \underline{2.493}$$

The degree of freedom (df) is estimated to be:

$$\text{df} = n_1 + n_2 - 2 = 4 + 6 - 2 = \underline{8}$$

giving the critical value, $t_{tab(8)} = 2.306$ (obtained from TABLE D in "Introduction to the Practice of Statistics" written by Moore and McCabe [82]) for P = 0.05. Since $t_{calc} > t_{tab}$ the H_0 is rejected; the two means of the peak areas ($\overline{X}_1$ and $\overline{X}_2$) are significantly different at the 5 % level. However, for P = 0.01 the critical value, $t_{tab(8)} = 2.896$, thus the H_0 is retained, and the two means of peak areas using an old and a new pre-column are not significantly different at the 1 % level.

6.3.2 ANOVA

Analysis of variance can be used to distinguish between variations which are caused by changes in method parameters and random errors. The retention times obtained using MP of different ages is listed in Table A14.

Table A14: The retention times for 1 µg/mL bradykinin using analytical mobile phase of varying age 1) old MP, 2) new MP in chamber A but old MP in chamber B and 3) new MP in both chambers were measured. The injection volume was 200 µL. The loading MP (AcN – H_2O (2:98, v/v) with 20 mM $NH_4^+CH_3COO^-$ added) and analytical MP (AcN – H_2O (gradient) with 20 mM $NH_4^+HCOO^-$ and 0.05 % FA added) were delivered at a flow rate of 0.200 and 0.005 mL/min, respectively.

	Old MP	**New MP in A**	**New MP in A and B**
Ret time brad (inj. 1)	5.73 min	5.68 min	5.60 min
Ret time brad (inj. 2)	5.70 min	5.65 min	5.65 min
$\overline{X}$	5.715 min	5.665 min	5.625 min

ANOVA was performed, using a CASIO CFX-9850GC PLUS graphic calculator, to see whether the difference in sample means is due to random error or caused by the changes of MP. Since only one parameter (change of MP) may affect the result of the experiment (retention time of brad), one-way ANOVA can be used. The calculations obtained were:

F = 5.6744	calculated F-value (F_{calc})
P = 0.095599	P-value
χpón-1 = 0.02677	
Fdf = 2	Degree of freedom (between-sample)
SS = $8.1333 \cdot 10^{-3}$	Sum of squares (between-sample)
MS = $4.0666 \cdot 10^{-3}$	Mean square (between-sample)
Edf = 3	Degree of freedom (within-sample)
SSe = 4.3521	Sum of squares (within-sample)
MSe = 1.4507	Mean square (within-sample)

The critical F-value, $F_{2,3} = 9.552$ (found in Table A.3 in "Statistics and Chemometrics for Analytical Chemistry" [81]). Since $F_{calc} < F_{2,3}$ the sample means do not differ significantly, thus the variations in sample means is due to random error.