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Degree Course Life Technologies

Option Analytical Chemistry

Diploma 2007

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Influences of different HPLC phases on separation of synthetic peptides

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Sion, 23 November 2007

I would like to dedicate this work to my father

Étude de l'influence de différents HPLC-Phasen sur la séparation des peptides de synthèse.

Untersuchung des Einfluss verschiedener HPLC-Phasen auf der trennung von synthetischen Peptiden.

Objectif

Différentes phases HPLC et UPLC sont testées dans l'optique de généraliser l'approche de la séparation des peptides synthétiques.

Résultats

Concernant le peptide de 20 acides aminés ± 2 glycines, les meilleures résultats de séparations sont obtenus avec l'UPLC à pH 3 avec une solution de perchorate 100 mM, mais la phase HPLC Waters HILC permet pour une séparation moins performante (R_s 1.06 contre 1.60) de réduire le temps d'analyse d'un facteur 1.5.

Pour les diastéréomères, petits peptides dérivatisés, les phases Atlantis C18 et HALO C18 permettent une très bonne résolution en HPLC, mais le système UPLC permet de réduire d'un facteur 2 à 3 le temps d'analyse pour une résolution identique.

Mots-clés

HPLC, UPLC, peptides, séparation.

Ziel

Verschiedene HPLC und UPLC Phasen wurden getestet hinsichtlich der Suche nach einem allgemeinen Ansatz für die Trennung von synthetischen Peptiden.

Resultate

Die Peptide von 20 Aminosäuren ± 2 Glyzinien, wurden die besten Trennung Ergebnisse mit der UPLC bei pH 3 mit einer Perchorat Lösung 100 mM erreicht, aber die HILC Phase kann mit einer etwas geringerer Trennleistung (R_s 1.06 gegen 1.60) die Analysedomen um den Faktor 1.5 verkürzen.

Die Diastereoisomere, kleine geschützte Peptiden, kann mittels Atlantis C18 und HALO C18 Phasen ermöglichen eine sehr gute Auflösung in HPLC, aber das UPLC System reduziert um einen Faktor 2 bis 3 die Zeit Analyse für eine ziemliche identische Auflösung.

Schlüsselwörter

HPLC, UPLC, Peptiden, Trennung.

Table of content

1. INTRODUCTION

Today, the pharmaceutical industry is particularly interested in using rapid and efficient procedures for qualitative and quantitative analysis in order to cope with a large number of samples and to reduce the time required for delivery of results. The method of choice in pharmaceutical analysis for determining the concentration of drug substances in various matrices remains liquid chromatography coupled with different universal and/or selective detectors.
However, conventional analysis times are usually longer than 10 min with columns of 15 cm length and 4.6 or 2.1mm internal diameter. In order to perform rapid or ultra-rapid procedures (i.e., cycle times lower than 5 or 1 min, respectively), different strategies can be applied [1].

The purpose of the current study purpose is to compare qualitatively different HPLC and UPLC columns for the separation of underivatized 20-mer peptides, deletions, hits and on derivatized diastereomers 4-mer peptides.

Peptides are composed of chains of less than 50 amino acids and posses many different aspects. Thus, in addition to the highly variable polarity of these compounds, they may, in proportion to the length of the string, adopt secondary and tertiary structures that could influence retention on different HPLC supports [2].

The basic molecule of peptides is alanine (Figure 1) where R is a methyl group. R may take 20 (Annex 1) different forms in “natural peptides” and many more in synthetic peptides.

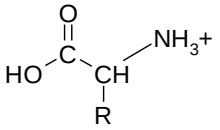

Figure 1

Amino acids are linked through a peptide bond (figure 2). The analyzed samples come from a production of the Lonza AG. The target compound consists of a 20 amino acid peptide ending with 4 glycines and the challenge is to show the influence of the different phases on the separation of this peptide and the different impurities that could appear in the synthesis of this peptide: “deletions and hits”, where hits are additions of one glycine and deletions are the same peptides with a glycine less. These deletions and hits appear during the synthesis of the peptide when for example a dimer in place of a monomer is attached to the growing chain (figure 6).

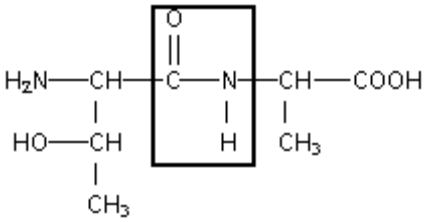

Figure 2

Different HPLC columns are compared in order to find a good compromise between speed and quality of separation for analysis of impurities in the synthesis of a peptide.

Column types:

- RP (C18 classical ; C18 wide pore)
- UPLC (C18 sub 2 µm ; C18 sub 2 µm wide pore)
- Monolithic column C18
- PS-DVB (polystyrene divinylbenzene polymers)
- HILIC (hydrophilic interaction liquid chromatography)

HPLC RP phase

RP HPLC is one of the most widely used techniques in the analysis of peptides. Indeed, in addition to its resolution ability, it allows the use of a wide variety of mobile phases, for example ACN and water mixed with TFA. The limit for the application of reverse phases is the time needed for analysis of polar molecules. Indeed, they cannot be separated as a result of weak interaction with the stationary phase [3].

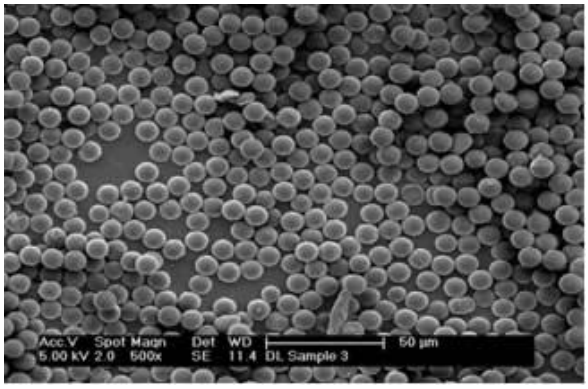

Figure 3

Techniques like RP-HPLC with monolithic column and UPLC are rapid methods, an analysis time 3 to 8 times faster than classical HPLC with 3 to 5 µm particles (figure 3) [1]. However, they are based on the same interactions as RP HPLC. The major difference is the change of structure of the phase: UPLC uses particles of less than 2 µm, as a result of wich the back pressure increases (Δ P: ≤ 700 bar) and the use of a specific HPLC system is required. The pore size ranges varies between 100 and 300 Å.

The structure of a monolithic column illustrated in Figure 4 uses a polymerized silica C18 phase, with a two dimensional structure: macropores of 2 µm and mesopores of 130 Å. This structure allows the use of high flow rates while maintaining a reasonable back pressure.

In fact, the loss of pressure is related to the diameter of the particles and the viscosity [4] as described by equation 1.

$$\Delta P = \frac{\eta \cdot L^2 \cdot u \cdot \phi}{d_p^2 \cdot t_0} \qquad \text{Equation (1)}$$

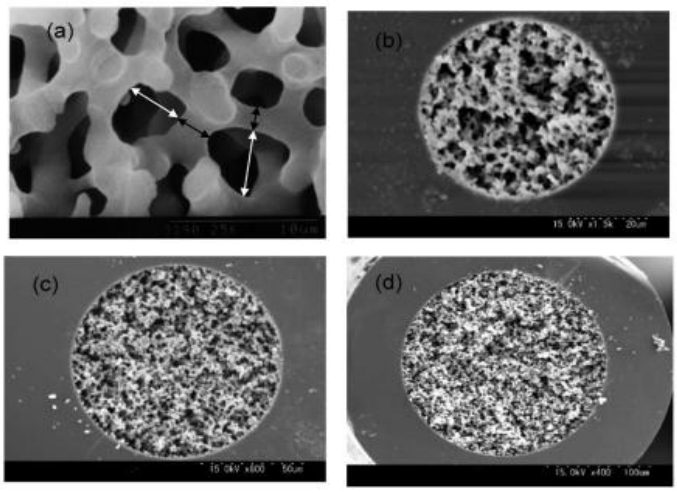

Figure 4

The study by Beth L. and al [a] of two identical reverse phase columns with different pore sizes (150 and 300 Å, Acquity, Waters, 2.1 x 100mm, 1.7 µm) on peptide mapping of 2 different proteins shows that with different porosity, different retention times are obtained for the same peptide. Retention times are not always correlated with the molecular weight, other phenomena are involved when the pores size is changed.

HILIC phase

Under the name HILIC is actually hidden a multitude of different phases. HILIC is the acronym of Hydrophilic Interaction LIquid Chromatography. This is a new name to describe a normal or reverse-reverse phase. Manufacturers sell under the name HILIC normal phase (silica) columns and phases grafted with polar groups such (PVA polyvinyl alcohol, primary amine propyl, diols,… cyano). This method offers an attractive alternative to the reverse phase for polar compounds because they often have short retention times in RP. Figure 5 illustrates the separation mode of a HILIC column.

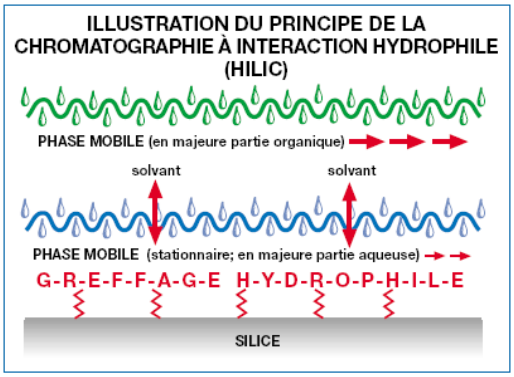

Figure 5

T. Yoshida [3] present a model (equation 3) based on equation 2 [4] which yields a linear relationship between the factor of retention and the proportion of solvent (in this case, water in

ACN). In the equations 2 and 3, k' is the retention coefficient, k_0 the coefficient of retention of water, S is the elution strength and Φ_B the proportion of the polar solvent

$$\text{Log } (k') = \text{Log } (k_0) - S\ (\Phi_B) \text{ Reverse phase} \qquad \text{Equation (2).}$$

$$\text{Log } (k') = \text{Log } (k_0) - S \text{ Log } (\Phi_B) \text{ normal phase} \qquad \text{Equation (3).}$$

Other ways to separate complex mixtures involve a two-dimensional separation. For example, by combining a RP HPLC column and analyzing the fractions unseparated by RP with a second HILIC type column [3]. There is also the possibility of another way by separating first through a phase of SCX type, and then by carrying out a second separation by RP [5].

Derivatization

The derivatization consists of adding a chromophore (fluorescamine, o-phthaldialdehyde for example) when UV-VIS detection is not sensitive enough or when the synthesis needs a protector group like the peptide synthesis. Figure 6 shows a widely used scheme of solid phase peptide synthesis; it is dominated by the use of BOC/benzyl and Fmoc/tert-butyl protection schemes.

The derivatization can be done before or after the chromatographic separation.

- Post derivatization: this allows for the separation of peptides in their native forms.
- Ante derivatization: it allows a change the behavior of peptides for the chromatographic separation.

The chromophores are detected by LASER fluorescence (FLD). The derivatization thus improves the detection sensitivity by a factor 1000 compared with UV-VIS detection [2].

Derivatization also allows differentiating enantiomers. When an enantiomer mixture is derivatized with an enantiomericaly pure mixture one obtains two diastereomers.

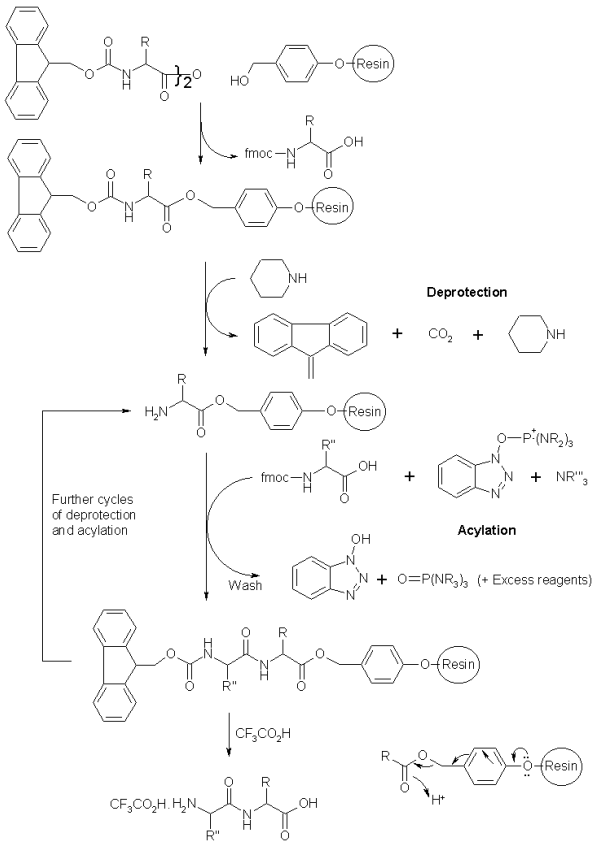

Figure 6

1.1. Analysis of results and comparisons of columns

The criteria for comparing columns are:

$$\text{Reduced length: } l = \frac{L}{d_p} \qquad \text{Equation (4)}$$

$$\text{Reduced plate height: } h = \frac{H}{d_p} = \frac{L}{N \cdot d_p} \qquad \text{Equation (5)}$$

$$\text{Reduced flow rate: } \upsilon = \frac{u \cdot d_p}{D_m} = \frac{l \cdot d_p^2}{t_0 \cdot D_m} \qquad \text{Equation (6)}$$

These reduced characteristics allow adapting the methods to columns of various sizes while keeping the same resolution. Equation 7 and 8 allow adapting the flow rate according to the ratio of the diameters of two columns with equivalent lengths [2] and the injection volume.

$$Flow\ a = \left(\frac{r_a}{r_b}\right)^2 \cdot Flow\ b \qquad \text{Equation (7)}$$

$$\text{Inj vol}_1 = \text{Inj vol}_2\ (r_2/r_1)^2\ (L_2/L_1) \qquad \text{Equation (8)}$$

The criteria for comparing the columns are first, the analysis time which is a decisive argument in industry and second, the resolution as expressed in equation 9 which is determinant for comparing the separation of two pairs of components [4].

$$R_s = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha-1}{\alpha}\right) \cdot \left(\frac{k'}{1+k'}\right) \qquad \text{Equation (9)}$$

Equation 9 involves the capacity factor of equation 13 as well as N, the number of theoretical plates (Equation 10) and the selectivity (Equation 11). H is the height of the peak, t_r its retention time and A its area. There is another way to calculate N as shown above, like the moment method which consist of the surface of the peak calculated by integration of it, 5σ or half height method.

$$N = \frac{2\pi \cdot h^2 t_r^2}{A^2} \qquad \text{Equation (10)}$$

Or

$$N = \frac{\mu_1^2}{\mu_2}$$

Or

$$N = 25\left(\frac{t_R}{W_{5\sigma}}\right)^2 \; or \; 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$$

α, the selectivity is given by equation 11.

$$\alpha = \frac{k'_2}{k'_1} \qquad \text{Equation (11)}$$

k', the capacity factor, is given by equation 12.

$$k' = \frac{t_{r-t_0}}{t_0} \qquad \text{Equation (12)}$$

Equation 13 calculates the plate height. This can be calculated with the help of the total length of the column L and the plate count N or with the Van Deemter equation. Where υ is the reduced flow rate, A is the Eddy diffusion, B is the longitudinal diffusion and C the mass transfer.

$$H = \frac{N}{L} = Au \quad + \frac{B}{u} + Cu \qquad \text{Equation (13)}$$

The study of these different parameters allows through graphic representations such as H = f(u) and h = f(υ) to interpret the effects of various physical parameters such as pore diameter, particle size or separation of the considered peptides [1]. In order to take into account the viscosity of the eluent, according [6, 1] to the graphic representation (kinetic plots) with respectively separation impedance $N2/t_R$ = f(N) or retention impedance $N2/t_m$ = f(N) can be used to visualize the optimal plate count and time of analysis for one component.

The impedance formula is given by equation 14. It allows taking into account of the viscosity of the eluent and the diffusion coefficient of the analyte. In this study, viscosity was measured, but

diffusion coefficient was calculated with a linear regression with an approximative mass of target peptide of 2600 kDa (20 AA at an average mass of 130 Da and two references ribonuclease (13700 kDa and $0.12X10^{-9}$ m^2/s) and serum albumin (65000 kDa and $0.059X10^{-9}$ m^2/s) in water. This approximation gives a diffusion coefficient of $7.239X10^{-12}$ m^2/s [7]

$$E = \frac{t_R \cdot \Delta P}{N^2 \cdot \eta \cdot (1+k)} \qquad \text{Equation (14)}$$

E should, for a spherical particle optimally filled column, turn around 3000 and 5000 [4].

2. EXPERIMENTAL

2.1. APPARATUS

2.1.1. HPLC SYSTEMS

Spectra-physics Analytical.

Degasser :	SCM 1000.
Pump :	P 4000.
Auto-sampler :	AS 3000.
Detector :	UV 2000.
Interface :	Agilent, 35900 E.
Program :	Chemstation for LC systems Agilent tech. 2001-2006 version Rev. B. 02. 01. [244].
Dwell volume :	0.59 ml

1100 series :

Degasser :	G1322A
Pump :	G1311A
Auto-sampler :	G1329A
Detector :	UV-VIS (DAD) G1315B 1024 array, cell : 13µl volume, 10 mm optic pathway.
Detector :	Fluorescence laser detector (FLD) G1321A array, cell : 8 µl volume (0.5 X 80 mm).
Program :	Chemstation for LC systems Agilent tech. 2001-2006 version Rev. B. 02. 01. [244].
Dwell volume :	0.94 ml

Waters Acquity UPLC system :

Lonza n°:	ZDUPLC314
Pump :	A05UPJ488M
Auto-sampler :	M04UPB5001M
Detector :	UV-VIS (WAJ081142) Waters, cell : 500 nl volume, 10 mm optical pathway 20 Hz sample rating.
Program :	Empower build 1154 copyright 2002.

2.1.2. HPLC RP COLUMNS

Table 1. Types of HPLC-RP columns.

Manufacturer / Name	Part n°/lot	Grafting	Column sizes d x l [mm]	Particle size [µm]	Pores size [Å]	% carbon	Pore area m^2/g
Agilent / Zorbax	863953-902/B05103	SB-C 18	4.6 x 150	3.5	80	12.5	180
Waters / XBridge	186003055/ 010135237124 10	C 8	4.6 x 150	3.5	135	-	-
Waters / Atlantis	186001317/ 0114350261130 8	dC 18	3.9 x 150	3.0	100	12	330
Phenomenex / Onyx	CHO-7644/ 050150-50	Monolytic C 18	4.6 x 50	-	Meso-pores: 130Å Macro-pores: 2µm	-	-
Advanced material technology / Halo	USGW001284 / AH72215	C18	4.6 x 75	2.7	90	-	-
Waters / YMC	BA99S051504FT / 90306051	Alkylsilanes ≤ C8, neutral silica	4.0 x 150	5	Wide pore	7	-
Hamilton	79444	PS-DVB	4.1 x 150	5	100	-	-

2.1.3. UPLC-RP COLUMNS

Table 2. Types of UPLC columns.

Manufacturer / Name	Part n°/lot	Grafting	Column sizes d x l [mm]	Particle size [µm]	Pores size [Å]	% carbon	Pore area m^2/g
Waters / Acquity	30003	C18	2.1 x 150	1.7	130	17.41	183
Waters / Acquity	186003556	C18 special peptide	2.1 x 150	1.7	130	17.93	197
Waters / Acquity	30049	C18	2.1 x 150	1.7	300	12.15	88

2.1.4. HPLC-NP COLUMN

Table 3. Types of HPLC-NP columns.

Manufacturer / Name	Part n°/lot	Grafting	Column sizes d x l [mm]	Particle size [µm]	Pores size [Å]
Waters / Atlantis	186002019 / 0102361813107	Silice	3 x 100	3.0	-

2.1.5. SAMPLES

Table 4. Samples.

Sample name	Structure	LIMS ID	Remarques
Problem 1: Double hits / Deletions			
Peptide_4G	20mer with 4 consecutive Gly	39379	Target compound
Peptide _3G	20mer with 3 consecutive Gly	67103	
Peptide_2G	20mer with 2 consecutive Gly	4375142	
Peptide_5G	20mer with 5 consecutive Gly	1414847	
Peptide_6G	20mer with 6 consecutive Gly	44247	
Problem 2: diastereomers			
Pep-L-phe	Fmoc-Cys(trt)-Gly-L-Phe	V-1705-1	
Pep-L-phe	Fmoc-Cys(trt)-Gly-D-Phe	V-1707-1	

2.1.6. LABORATORY SPECIFIC MATERIAL

Analytical balances:

Mettler AE 240

Sartorius CP255D

Viscosity measurements:

Capillary viscosimeter SCHOTT Geräte AVS 310.

Capillary Oc SCHOTT Geräte.

Water bath SCHOTT Geräte D 6238.

PKa measurement:

Metrohm Dosimat 655.

Turbidimetry measurement:

UV-VIS spectrophotometer libra S12 biochrom.

Plastibrand cuvettes N° 7590 15.

2.1.7. REAGENTS

Perchloric acid:	Fluka 77232
Hydrochloric acid:	Fluka 84436
Thiourea:	Fluka 88810 puriss. ≥ 99.0 %
TFA:	Fluka 91707 puriss ≥ 99.0 %
Sodiumperchlorate:	Fluka 71852 puriss ≥ 99.0 %
Amonium formate:	Fluka 09739 puriss > 97 %
Formic acid:	Fluka 06440 puriss > 98 %
Sodium hydrogen sulfate:	Fluka 71656 puriss > 93 %
Ethanol:	Fluka 02860 puriss ≥ 99.8 %

2.1.8. ELUENTS

H_2O Milli-Q Millipore Q-Gard 2

Perchlorate solution 100 mM pH 3.0

Acetate Buffer 10 mM pH 4

Phosphate buffer 10 mM pH 2 and 3

Formate buffer 10 mM pH 2

ACN Lab-Scan for HPLC C17C11X

Methanol for HPLC Merck 1.06018.2500

Ethanol

2.2.METHODS

2.2.1. VISCOSITY MEASUREMENTS

The viscosity measurements of eluents were conducted with the intention to characterize columns in order to calculate the impedance during isocratic test mode. First, the density was determined by weighing three times a sample of 5 ml of the mixture 75/25 ACN / phosphate buffer 10 mM pH 2 and 3 for HILIC and 25/75 ACN / phosphate buffer 10 mM pH 2 and 3 for RP-HPLC. After 5 minutes of acclimatization at 25 °C, the viscosity (transit time) was measured 3 times with samples of about 20 ml in the capillary viscosimeter.

2.2.2. TURBIDIMETRY MEASUREMENTS

2 ml samples of mixtures 95/5 ACN / phosphate buffer (100 mM and 10 mM) pH 2 and mixtures 85/15 ACN / phosphate buffer (100 mM and 10 mM) pH 2 were measured twice at 750 nm to determine if there was formation of a precipitate. Zero was fixed through water.

2.2.3. DWELL TIME

Dwell time was measured twice on both systems, spectra physics and Agilent 1100, with a solution of toluene 5.6 mg/l in water at a flow rate of 0.2 ml/min detection at 215 nm. For the spectra physical system the measurement was made at 2.2 ml/min.

2.2.4. PK_A DETERMINATION

pK_a determination of the target compound was carried out with 1 ml of a solution 2.57 g/l in water of the peptide 4gly with an addition of 2 ml of NaOH 0.1 mol/l.

2.2.5. KINETIC AND KNOX PLOTS

For HILIC: a sample of target compound (4gly) of 2.03 mg was dissolved in 110 µl formic acid and 890 µl ACN [3] and analyzed with a dilution factor of 2. The dead time of the column was measured with a solution of 56 mg/l of toluene. The blank was composed of 110 µl formic acid and 890 µl ACN. The flow rates were varied from 0.1 ml/min to 1.6 ml/min. 10 µl were injected and the run isocratically with 75 / 25 ACN / buffer. Detection was at 215 nm.

For RP-HPLC: a sample of the target compound (4gly) of 10.51 mg and 8.36 mg of thiourea (dead time) were dissolved in 5ml with in water and diluted by a factor 20 the blank is composed of water. Flow rates were varied between 0.1 ml/min to 1.5 ml/min. and between 1.0 to 5 ml/min for ONYX monolithic column. Analyses were run in isocratic mode with 25 / 75 ACN / buffer. Detection was at 215 nm.

Measures were made with Agilent 1100 HPLC at 25°C and sample rate was 1 point per second. Integration parameters were: slope sensitivity 5, peak width 0.05, area reject 5 height reject 1. N was calculated by chemstation by the half height of the peak method.

2.2.6. DIASTEREOMERES ANALYSIS

For HPLC analysis, samples were dissolved in ethanol solution of peptide L-phe (1.053 mg/ml) and peptide D-phe (1.203 mg/ml) were prepared. A spiking 1:1 of both diastereomers was also prepared. These samples were injected (5 µl) on Waters Atlantic C18 with a gradient from 50 / 50 to 10 / 90 buffers pH 2, 3, 4, 5 / ACN in 30 minutes at 40 °C with a flow rate of 0.8 ml/min . The blank was ethanol and dead time was measured with a solution (0.0107 mg/ml) of thiourea in methanol. Acquisition was made with Spectra physics HPLC system and parameters were: rise time : 3.0, and range one and two at 1.0 detection was carried out at 215 nm. N was calculated by chemstation with the half height of the peak method. .

For UPLC analysis, samples were dissolved in ethanol solution of peptide L-phe (1.053 mg/ml) and peptide D-phe (1.203 mg/ml) were prepared. A spiking 1:1 of both diastereomers was also prepared. These samples were injected (2 µl) on a gradient from 40 /60 to 10 /90 acetate buffer at pH 4 / ACN in 17.20 minutes at 40 °C and 60 °C with a flow rate of 0.4 ml/min. The blank was ethanol and dead time was measured with a solution (0.0107 mg/ml) of thiourea in methanol. Acquisition was made with Waters Acquity UPLC system and parameters were: 20 points per sec., filter time constant, 0.5 sec., detection was carried out at 215 nm. N was calculated by EMpower with the 5σ method.

2.2.7. NOTE

All chromatograms and calculation that are not presented in the results section, are available in the annex 1, 2, 3, 4, 5 at laboratory F103 at HES-SO, route du Rawyl 64, CH-1950 Sion.

3. RESULTS AND DISCUSSION

3.1. CHARACTERIZATION OF THE PEPTIDE

3.1.1. pK_A MEASURES OF PEPTIDE 4GLY :

The pK_a of peptide was measured to determine the pH range in which target peptide (4 gly), is in a stable form. For this purpose, a reverse titration was carried out.

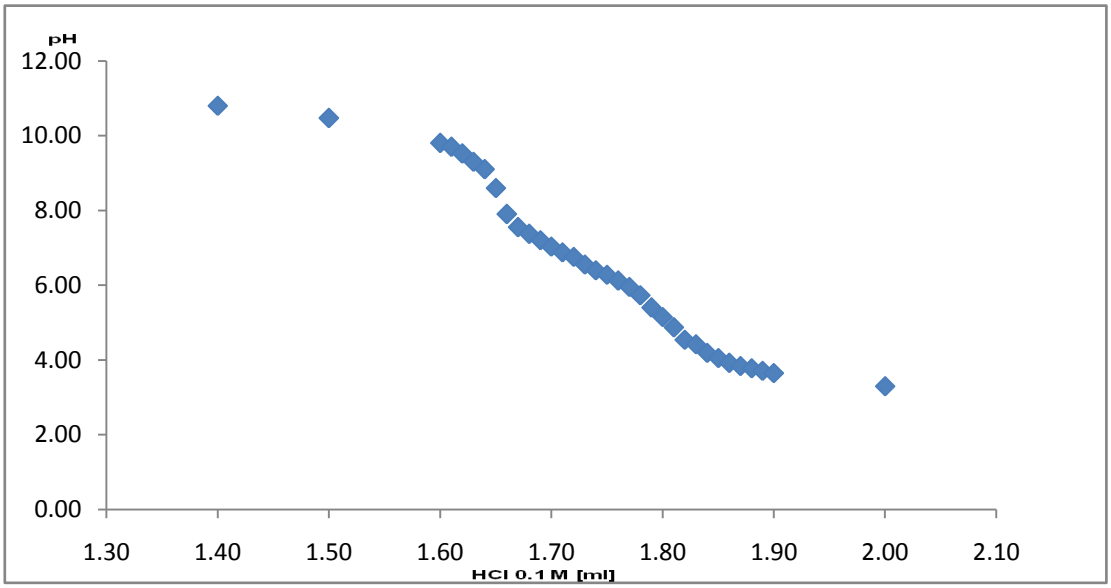

Figure 7: Analyses n°1 of the titration of peptide 4 gly with HCl 0.1 M

Figure 7 shows that between pH 2 and 3 the peptide is in a stable protonated form. Table 5 shows the average of the two analyses. The pK_a 2 at 4.65 involves that part of the peptide is in an unprotonated form. Chromatographic analyses should therfore be carried out below pH 3 or above pH 10.5 for more reproducible time of retention and peak sharpness. But few silica based phases are capable to resist above pH 8 because of hydrolysis. Therefore PH 2 and 3 were retained to carry out the analysis.

Table 5. Average of pK_a analyses 1 and 2.

	pK_a 1	pK_a 2
Analyse 1	7.82	4.53
Analyse 2	7.65	4.78
Average	**7.74**	**4.65**
SD	0.12	0.18
CV	1.52	3.81

3.2. CHARACTERIZATION OF THE MEASUREMENT SYSTEM

3.2.1. TURBIDIMETRY MEASUREMENTS:

Turbidimetry measurements were made because phosphate buffer (10 mM) was chosen to carry out measurements and at a high concentration of ACN phosphate salt precipitates. Phosphate buffer facilitates the measurements because of its UV transparency, compared to formate acetate or glycine buffers. Results of analyses of mixtures of 95/5 and 85/15 ACN phosphate buffer 100 and 10 mM are shown in table 6.

Table 6. Turbidimetry analyses of different eluent solutions.

	Absorbance at 750mn [-]	
Solutions	Analyse 1	Analyse 2
95/5 ACN / water	0.086	0.086
95/5 ACN / 100 mM buffer	0.200	0.230
95/5 ACN / 10 mM buffer	0.086	0.083
85/15 ACN / water	0.062	0.063
85/15 ACN / 100 mM buffer	0.291	0.272
85/15 ACN / 10 mM buffer	0.062	0.065

Table 5 shows clearly that at concentrations of 10 mM there is no precipitation the values are under the limit of detection. By contrast, at high concentrations it is possible to observe a precipitate.

3.2.2. VISCOSITY MEASUREMENTS:

Viscosity measurements were carried out to determine the impedance that reflects the quality of the filling of the column. For HILIC and RP columns, results are summarized in table 7. The high content of volatile ACN can explain the small difference between the HILIC and RP-HPLC standard deviation. A complete table of results is presented in annex 6 and 7.

Table 7. Viscosity analyses of different eluent mixtures.

	Type of eluent	pH	η [Pa*s]	Standard deviation
HILIC	75/25 ACN / phosphate buffer (10 mM)	2	$4.90 \text{ X}10^{-4}$	$1 \text{ X}10^{-6}$
	75/25 ACN / phosphate buffer (10 mM)	3	$5.12 \text{ X}10^{-4}$	$6 \text{ X}10^{-6}$
RP-HPLC	25/75 ACN / phosphate buffer (10 mM)	2	$9.05 \text{ X}10^{-4}$	$4 \text{ X}10^{-6}$
	25/75 ACN / phosphate buffer (10 mM)	3	$9.04 \text{ X}10^{-4}$	$3 \text{ X}10^{-6}$

3.3. CHARACTERIZATION OF THE HPLC PHASES

To characterize the different phases, the kinetic plot method [6] was chosen for its abilities to show at the same time the optimum plate count and the optimal time for the analysis and for the different possibilities to present it graphically.

3.3.1. VAN DEEMTER CURVES AND KINETIC PLOTS

First the reduced value plots, figure 8 and 9, show that the best reduced plate height is obtained with the HILIC phase for a reduced flow rates of over 900. It represents a flow rate of 0.9 ml/min. For these specific plots, the diffusion coefficient used for the calculation of reduced flow rate is $7.239X10^{-12}$ [m^2/s] as calculated in the introduction. All chromatogram and tables of results are presented in annex 1.

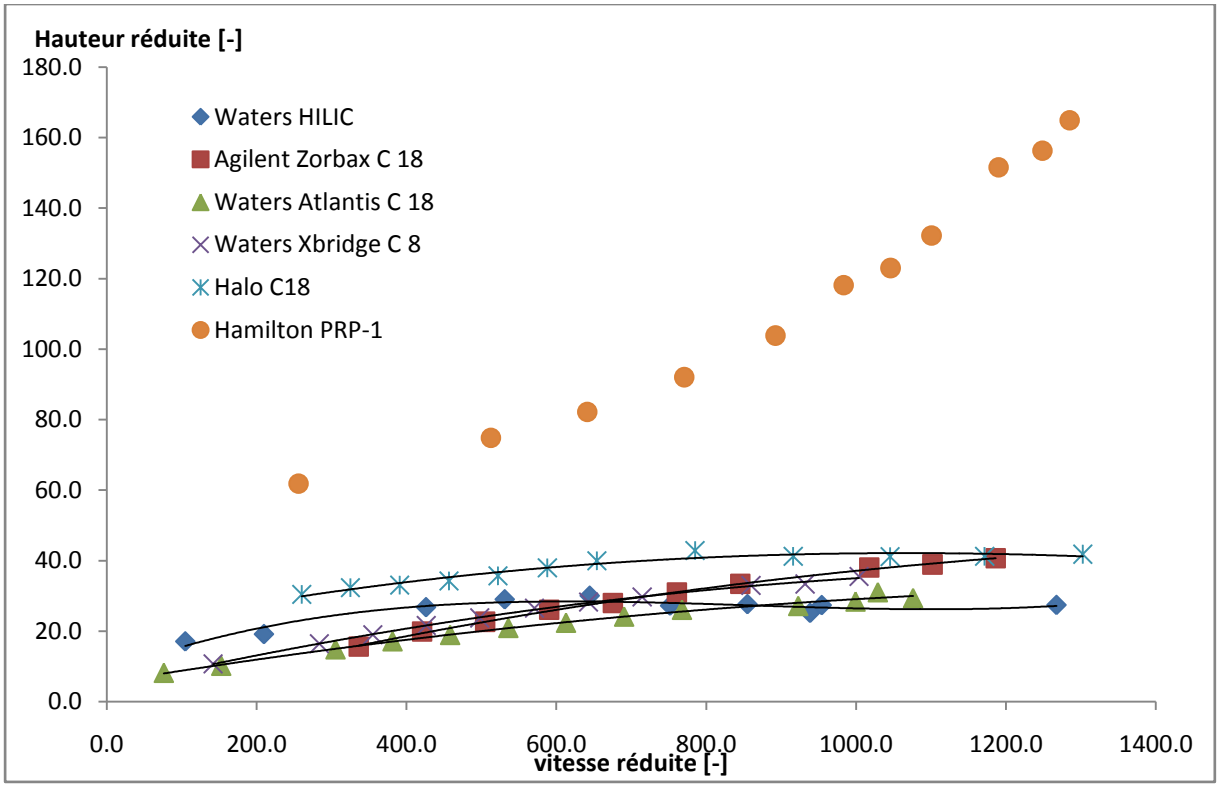

Figure 8. Reduced plate height in function of the reduced flow rate at pH 2.

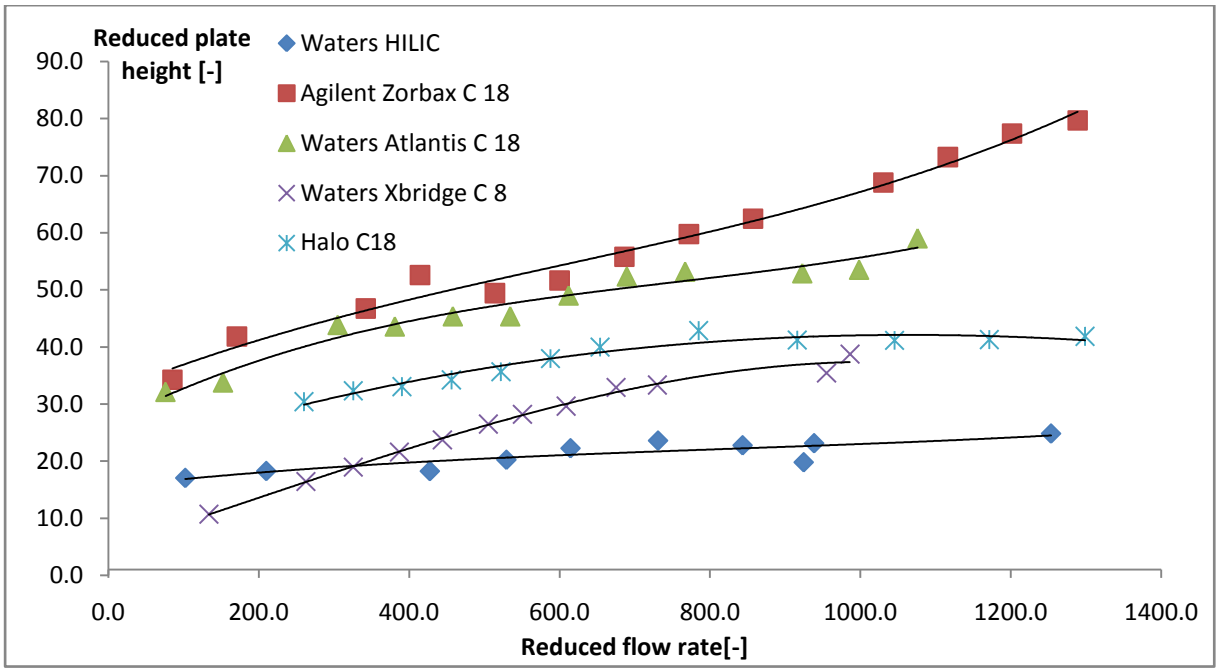

Figure 9. Reduced plate height in function of the reduced flow rate at pH 3.

Hamilton PRP-1 phase does not appear on figure 9 because the tailing effect was so strong that it was not representative enough to be shown graphically.

It is important to note that ONYX monolithic column does not figure in these figures as its internal structure is made of polymerized silica, without particles.

For this reason a second plot method was chosen to represent the optimal phase for peptide 4gly separation.

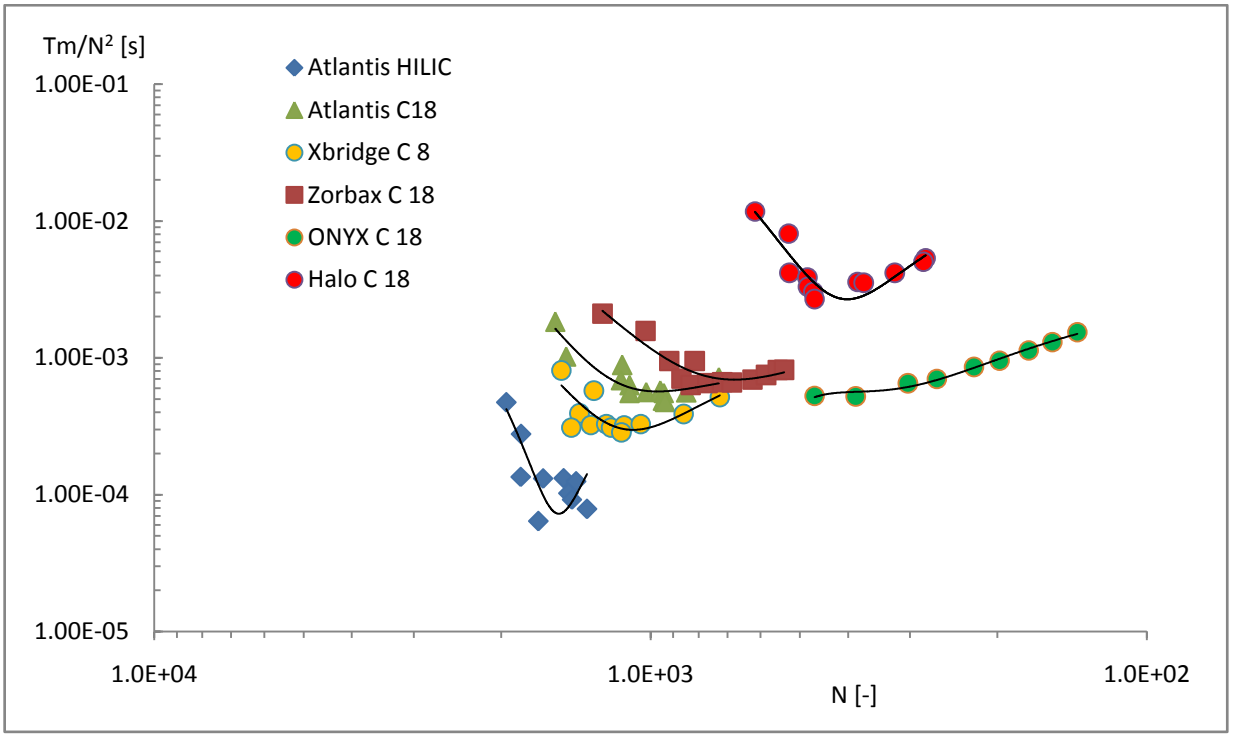

Figure 10. Separation impedance of peptide 4 gly at pH 3 for the different phases.

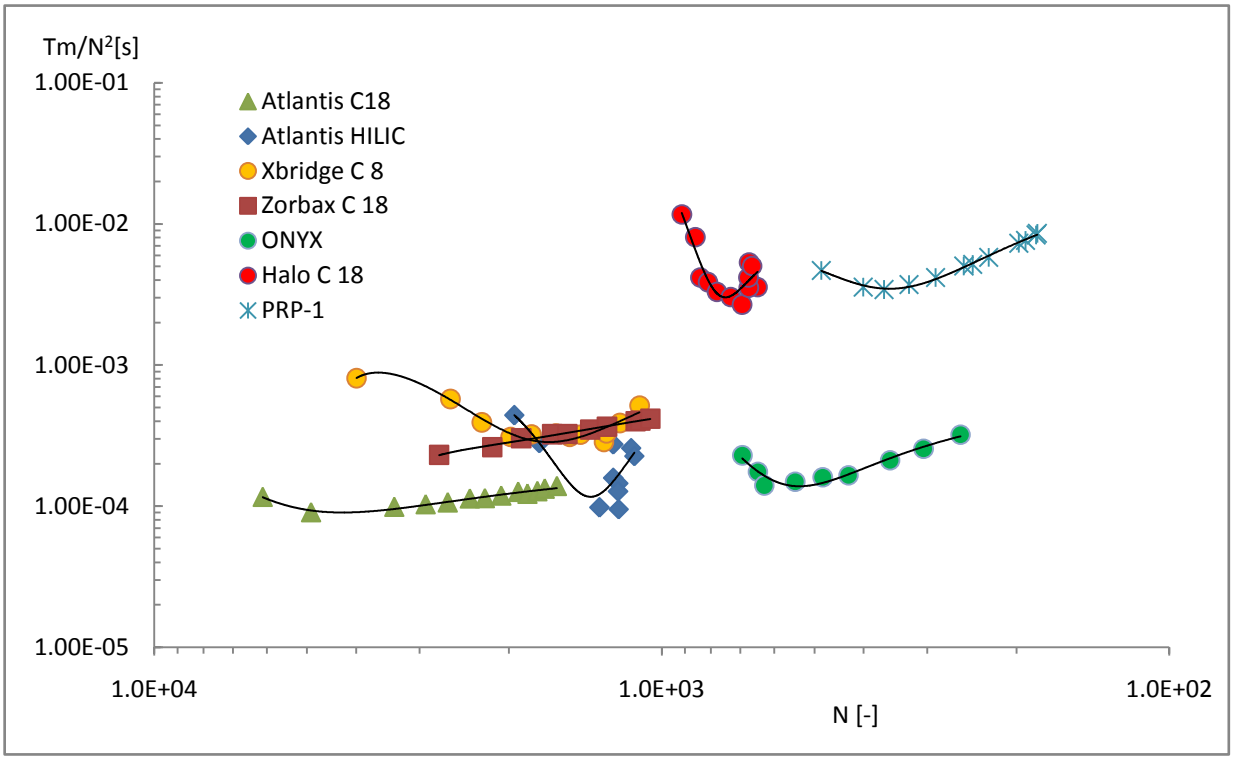

Figure 11. Separation impedance of peptide 4 gly at pH 2 for the different phases.

Figure 10 and 11 show the retention impedance of the different phases at pH 2 and 3 [9]. The minima of the curves indicate the obtained optimal plate count. From left to right the shift of the optimum shows a shorter analysis time and a lower plate count.

Two columns stand out from the lot at pH 2. Waters Atlantis C18 and Atlantis HILIC give the best plate count. The fastest column is the ONYX monolithic column but it has the inconvenience of having a lower plate count than the two others and significant eluent consumption due to fast flow rate. The Atlantis C18 gives the longest t_m for the best plate count. And in the middle there is the HILIC phase with a slightly shorter t_m and lower plate count.

At pH 3 there is no compromise, the best plate count is obtained with the Waters Atlantis HILIC phase. This is due to a tailing effect apparently induced by the use of phosphate buffer and reverse phases.

3.3.2. TESTS WITH HILIC AND GRADIENT ELUTION

The results obtained with kinetic plot method were confirmed with gradient tests. Figure 12 and 13 show results obtained with Waters Atlantis HILIC and a gradient elution from 85/15 to 65/35 ACN / 10 mM phosphate buffer pH 2 and 3 in 20 minutes with a flow rate of 1.0 ml/min. A sample of a mix of each peptide was injected. All chromatogram and tables of results are presented in annex 2.

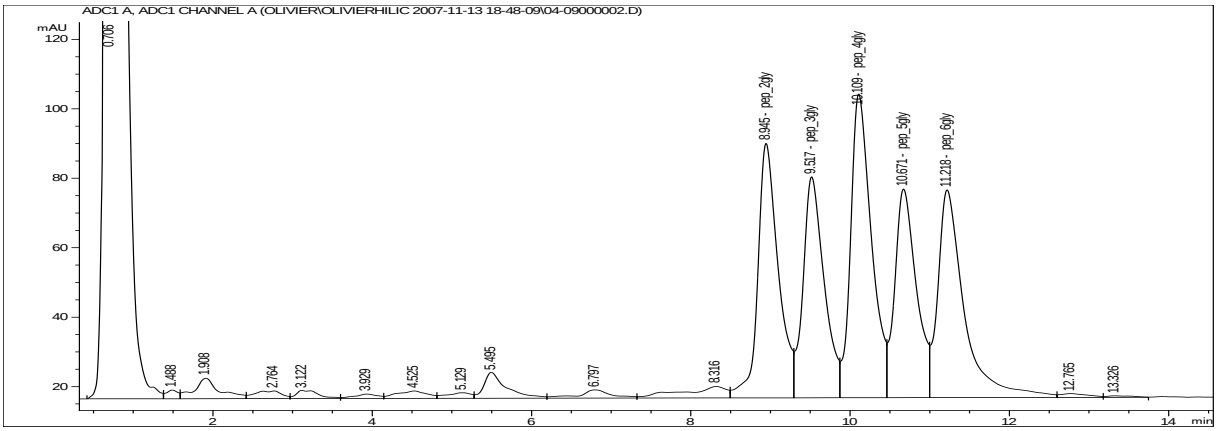

Figure 12. Chromatogram of a spiking with peptide 2, 3, 4, 5, 6 gly with HILIC phase at pH 2, second injection.

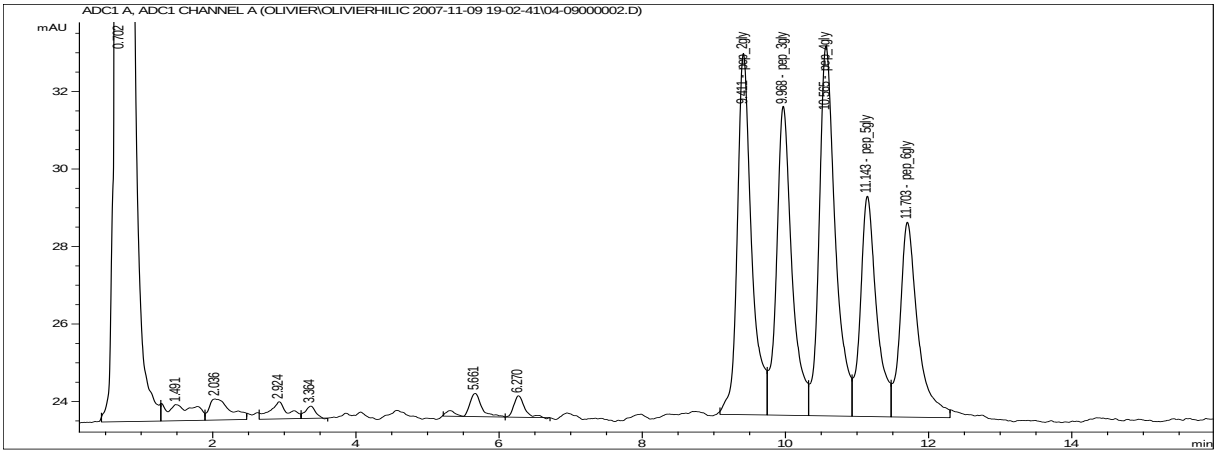

Figure 13. Chromatogram of a spiking with peptide 2, 3, 4, 5, 6 gly with HILIC phase at pH 3 second injection.

These results confirm the kinetic plot method and show that at pH 3 the retention time obtained are slightly longer than at pH 2. But the average resolution is the same, 1.06, for the two different pH.

3.3.3. TESTS WITH RP-HPLC AND GRADIENT ELUTION

The chromatograms obtained with columns ONYX and Atlantis C18 are presented in figure 14 and 15. The gradient elution details are presented in annex 2 and consist of a step gradient elution from 20/80 to 95/5 ACN / 10 mM phosphate buffer pH 2. This gradient is an adaptation of the gradient used by Lonza A.G. with the UPLC system (annex 3) and was adapted to the HPLC system with. A mix of a spiking of each peptide was injected.

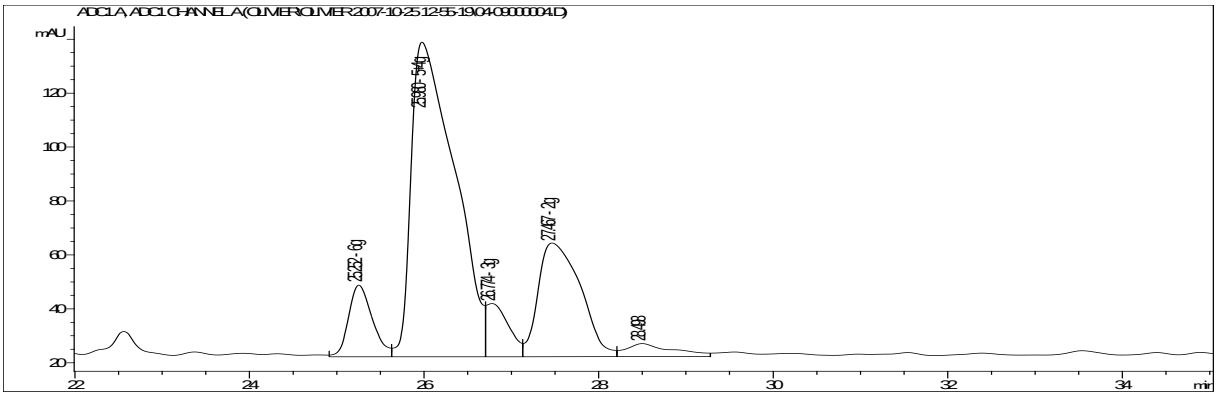

Figure 14. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Atlantis C18 phase at pH 2 second injection.

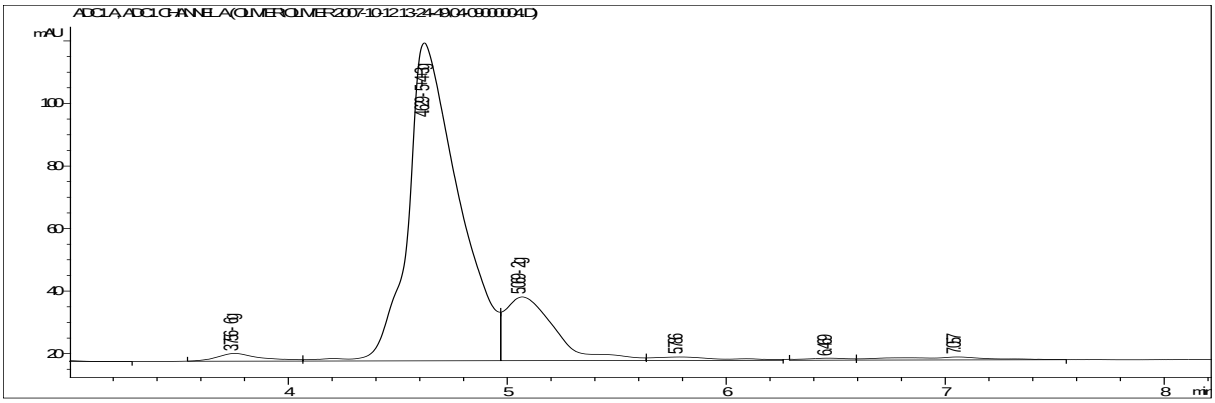

Figure 15. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with ONYX monolithic C18 phase at pH 2 second injection.

Two problems were encountered; the first one was the poor resolution, insufficient to resolve the 5 different peptides and the relatively poor sharpness of the peak.

These problems were partially resolved for the Atlantis C18 phase with the replacement of the phosphate buffer by the perchlorate solution at pH 3 with the same elution gradient.

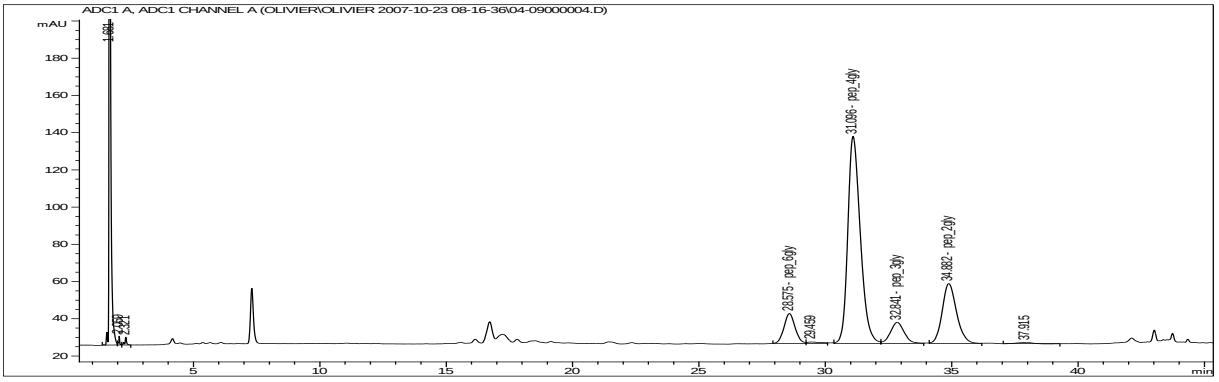

Figure 16. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Atlantis C18 phase with perchlorate solution pH 3.

Average resolution obtained for the separation increases from 1.6 to 2.5. This is an interesting result for the adaptation of an optimized gradient in UPLC on an HPLC system. The only

disadvantage is that the separation occurs in about 32 minutes with a 60 minute gradient. As shown in the kinetic plot, the Atlantis gives a better plate count as the HILIC phase but with a longer analysis time. Moreover with perchlorate solution, batch to batch retention time reproduction is not assured.

Other phases were tested with gradient elution, Waters YMC C8 wide pore, Halo C18 and Waters Xbridge C8. Results were disappointing, even with buffered eluent, retention time was not reproducible as expected and tailing effect was strong. These analyses are presented in annex 2

One problem has to be highlighted. The one by one injection of the different peptides shows that peptide 4 gly and 5 gly were irresolvable; they have the same retention time. This problem has been solved by replacing the original batch of peptide 5 gly by a new one. The results obtained with the HILIC phase in figure 12 and 13 shows the 5 peptides resolved.

3.4. CHARACTERIZATION OF THE UPLC PHASES

To characterize the different UPLC phases another approach was chosen as the phases differ only in pore size, the same method (annex 3) was used to observe the differences in retention time and resolution between the columns. The method consist of a step gradient elution from 20/80 to 95/5 ACN / 100 mM perchlorate solution at pH 3 with a flow rate of 0.25 ml/min. The next four figures (17 to 20) present the second injection on the three different columns BEH C18, BEH130 and BEH300. It is important to note that BEH 130 is sold in the commerce as a special column for peptide separation but it has the same specification as the BEH C18 column. The only changes are in carbon % and pore area that are slightly important for BEH 130 phase.

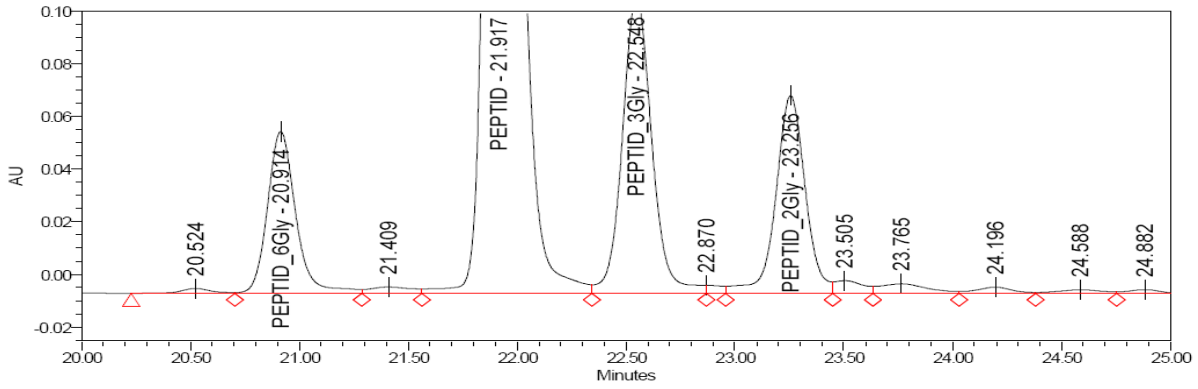

Figure 17. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Waters BEH C18 phase with perchlorate solution at pH 3 First injection.

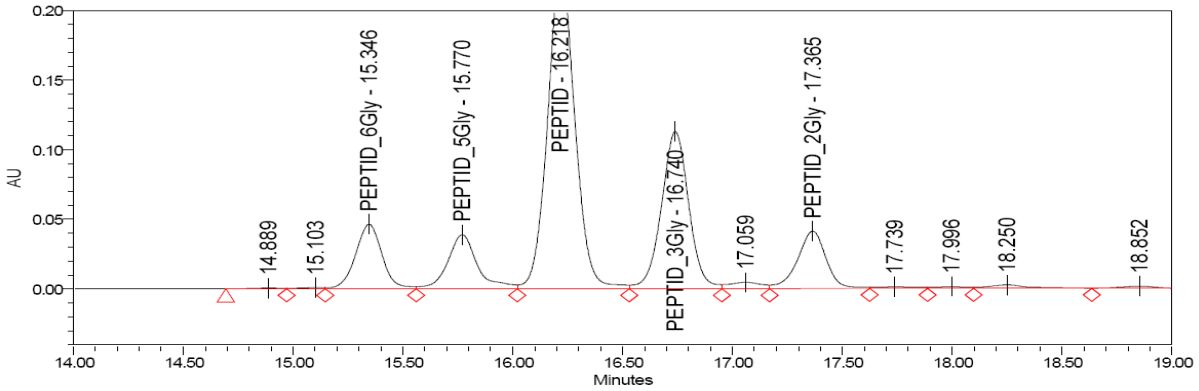

Figure 18. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Waters BEH C18 phase with perchlorate solution at pH 3 second injection.

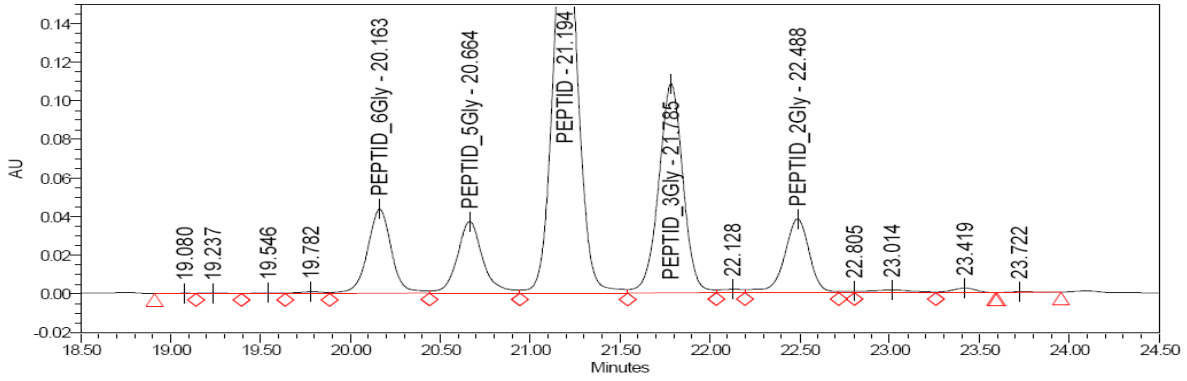

Figure 19. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Waters BEH 130 C18 phase with perchlorate solution at pH 3 second injection.

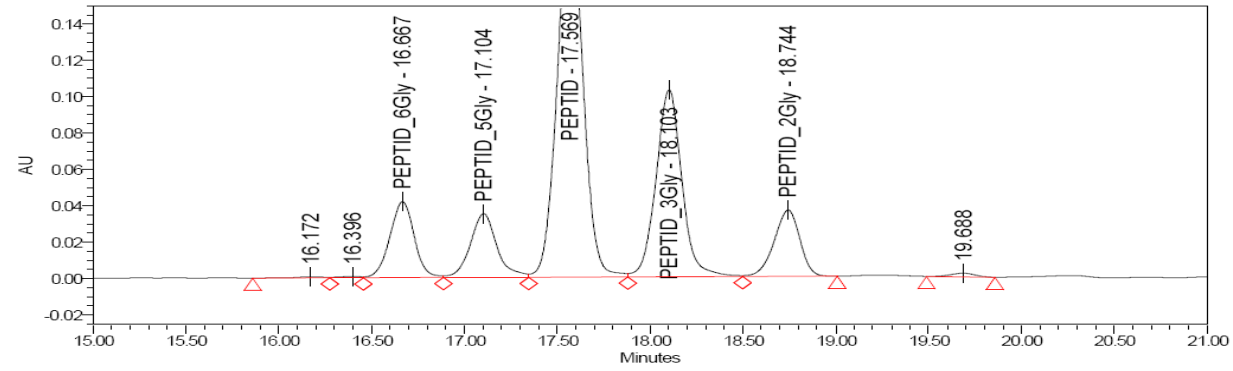

Figure 20. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Waters BEH300 C18 phase with perchlorate solution at pH 3 second injection.

Table 8. Values obtained for resolution with the three different phases and perchlorate solution.

Peaks	BEH C18	BEH 130 C18	BEH 300 C18
6 gly	1.77	1.85	1.79
5 gly	1.82	1.91	1.86
4 gly	2.06	2.07	2.07
3 gly	2.37	2.38	2.40
2 gly	1.39	1.74	3.36

The resolution obtained (table 8) for the three different columns shows no significant differences. The only difference noted was in the results obtained with the BEH 130 column but this column was a brand new phase lent by waters especially for this study that also could explain the differences between the retention times. The different values of Rs (resolution) were calculated with the average plate counts as shown in annex 3

In order to try to suppress the variability of the retention times caused by unbuffered perchlorate solution, tests were made with formate buffer at pH 2. The figures below show the different analyses carried out with formate buffer with the same gradient as with perchlorate solution (annex 7). We can see a better reproducibility between retention times is observed but no correlation between retention time and pore size for example. By contrast it is interesting to note that BEH 130 and 300 induce a strong tailing effect. In addition, BEH 130 and BEH C18 have the same properties. It is to notice that due to the use of formic acid, the response of the system is reduced by a factor of 40. The detection was performed at 260 nm.

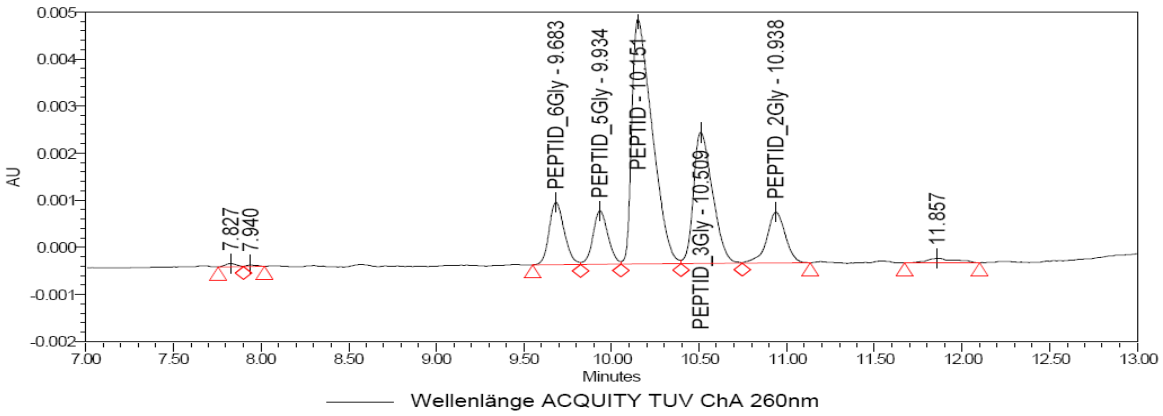

Figure 21. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Waters BEH C18 phase with formate buffer at pH 2 first injection.

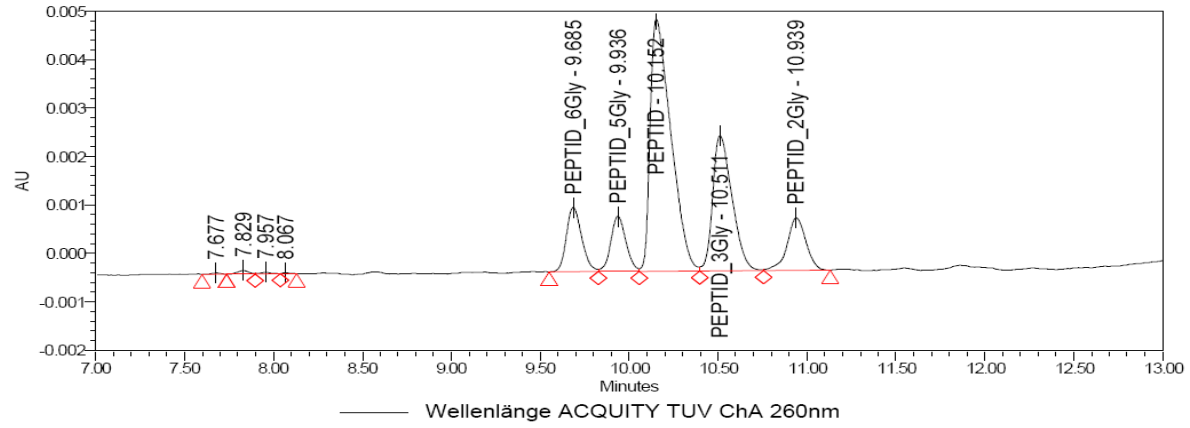

Figure 22. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Waters BEH C18 phase with formate buffer at pH 2 second injection.

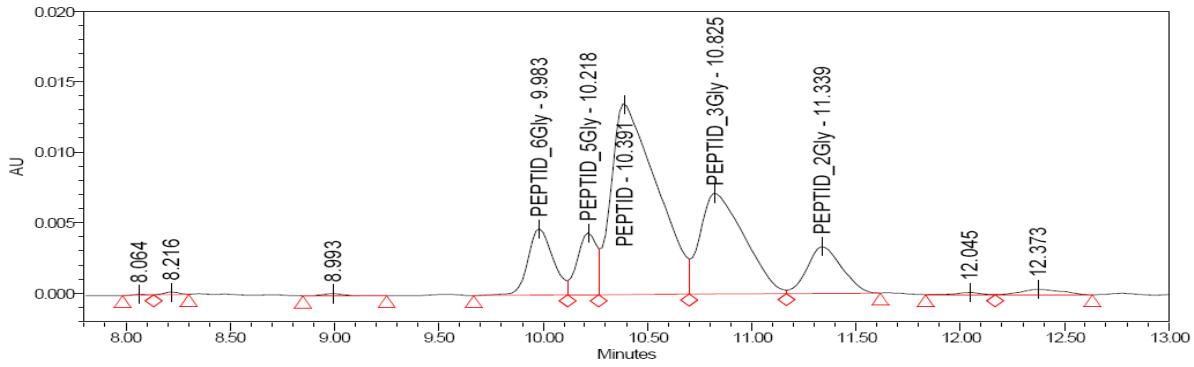

Figure 23. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Waters BEH130 C18 p hase with formate buffer at pH 2 second injection.

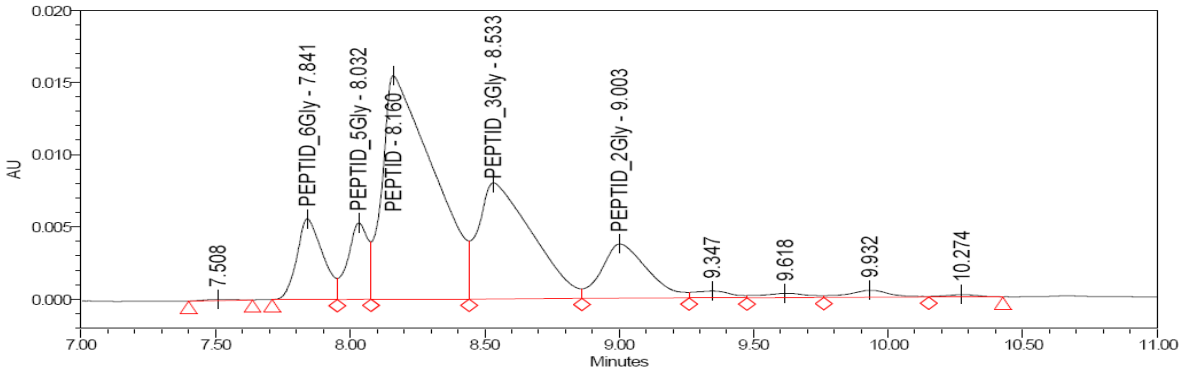

Figure 24. Chromatogram of a spiking of peptide 2, 3, 4, 5, 6 gly with Waters BEH300 C18 phase with formate buffer at pH 2 second injection.

Table 9. Values obtained for resolution with
The BEH C18 phases and formate buffer pH 2 solution.

Peaks	BEH C18
6 gly	1.291
5 gly	1.088
4 gly	1.743
3 gly	1.995

Resolution obtained with formate buffer pH 2 is slightly lower than with perchlorate solution pH 3 but with an adaptation of the gradient this could be improved. The only shadow of this system is the low response due to formate's strong absorbance in UV domain. Moreover the tailing effect for BEH 130 and 300.

To summarize this part, first, the kinetic plot method is a powerful tool to find optimal conditions for the use of the phases. It gives fine tendencies to guide through the choices of an incredible number of available phases. The main disadvantage is that it that it takes long time to precede to the test of the columns. The second disadvantage is that it works for the compound tested in the condition tested and not for other compounds as seen in the second problem for the separation of smaller protected peptides.

With an increase of 4 to 10 times more plate count, UPLC is for the moment the quickest way to analyze these middles sized peptides with the best resolution.

3.5.HPLC AND DIASTEREOMER PROBLEM

The first problem encountered with the diastereomers was the relatively poor solubility of these compounds in water and ACN (certainly due to the apolar protector groups trt and fmoc). Tests were made to find the right solvent. These were methanol, ethanol and dimethylformamide. Ethanol was chosen for these analyses.

The first optimized parameter was pH through a test with Atlantis C18 column with a classical 50 / 50 to 95 / 5 ACN / buffers pH 2, 3, 4 and 5 in 30 minutes with a flow rate of 0.8 ml/min. As shown in figure 25, optimal phase separation is obtained at pH 4 with a resolution of 1.4. The details of the methods used are presented in annex 4.

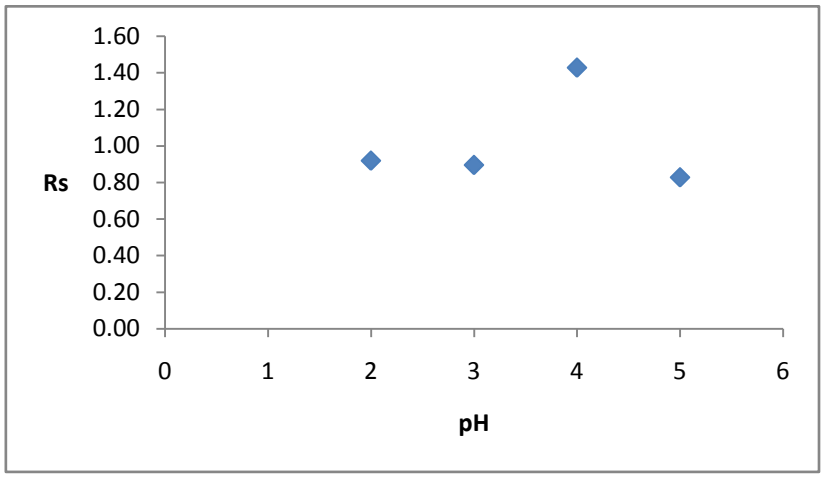

Figure 25. Resolution in function of different pH values.

Figures 26 to 28 show the effect of the different HPLC phases on diastereomer separation.

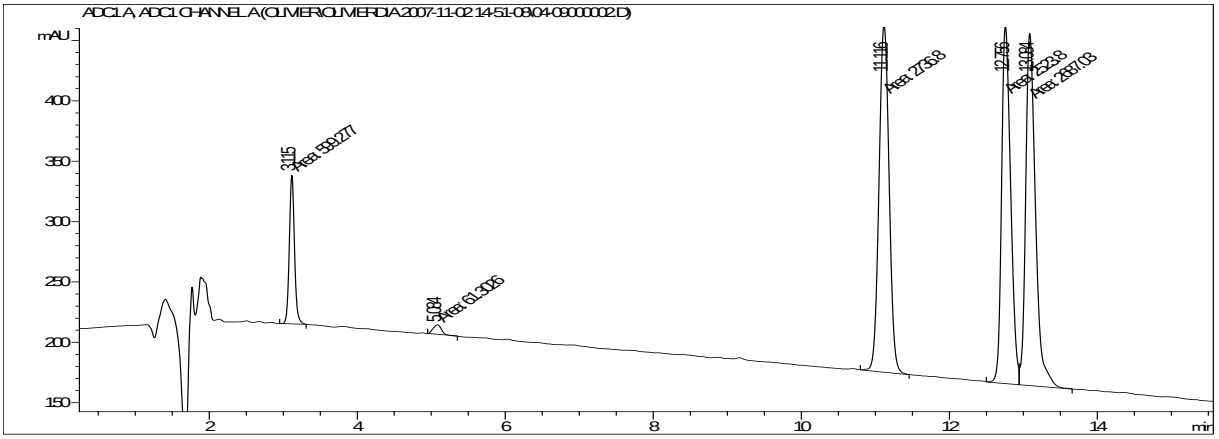

Figure 26. Chromatogram of a spiking of peptide L-phe and D-phe with Waters Atlantis C18 phase with acetate buffer at pH 4 second injection.

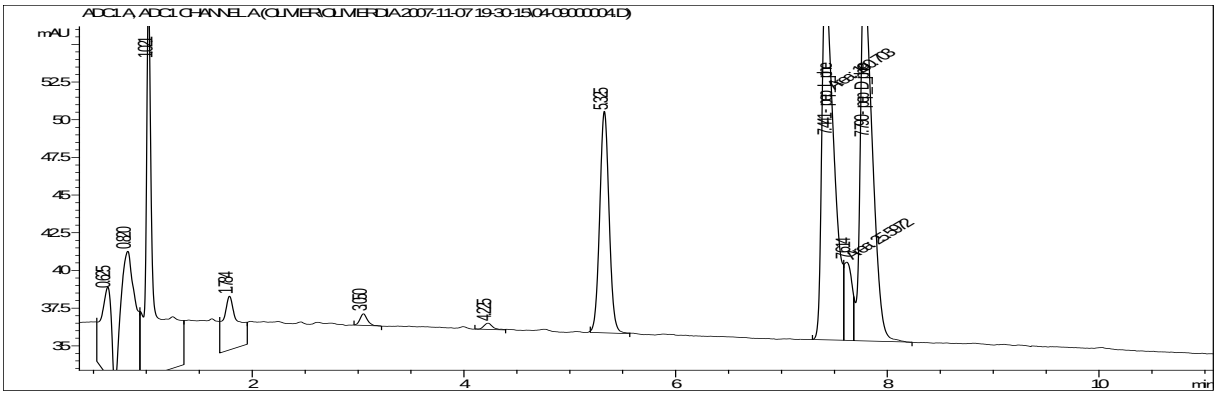

Figure 27. Chromatogram of a spiking of peptide L-phe and D-phe with HALO C18 phase with Acetate buffer at pH 4 second injection.

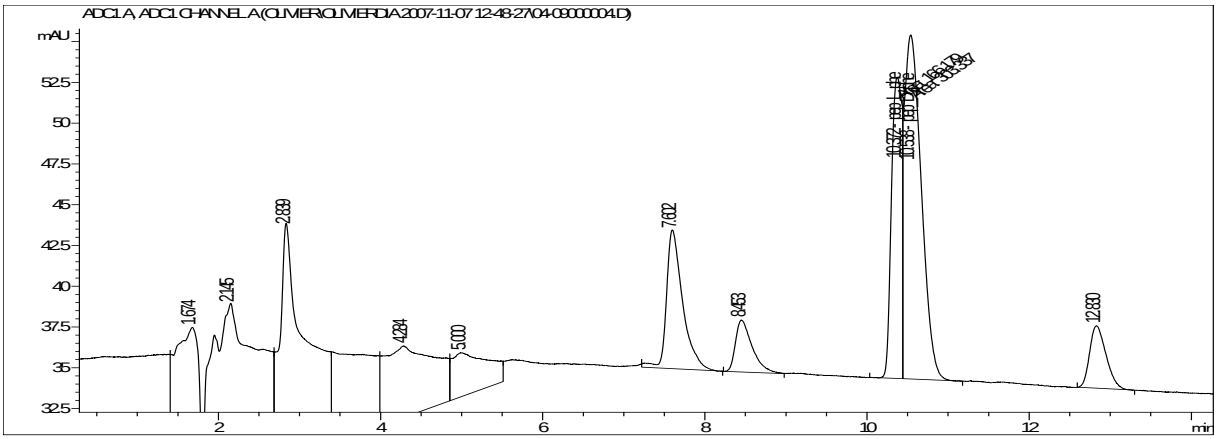

Figure 28. Chromatogram of a spiking of peptide L-phe and D-phe with YMC C8 phase with Acetate buffer at pH 4 first injection.

The resolution obtained with Waters Atlantis phase at pH 4 is 1.43. HALO phase resolution is better with 1.68. The smaller particle size could explain this gain in resolution even if HALO is 75 mm shorter than the Waters Atlantis column.

YMC phase seems to be inappropriated for this separation. It is certainly due to its larger particles, its C8 grafting and its pore size. Moreover a tailing effect appears with this column.

3.6.UPLC AND DIASTEREOMER PROBLEM

The gradient used for analysis was calculated by Veuthey's calculator tool (Lonza software) in order to adapt the HPLC gradient and the injection load to UPLC system. The method consist of a step gradient elution from 60/40 to 90/10 ACN / 10 mM acetate buffer at pH 4 in 17 .2 minutes with a flow rate of 0.40 ml/min

First tests were made at 60 °C at pH 4 and another series of assays were carried out at 40 °C to check the effect of temperature on the separation of the diastereomer. The only series presented is the one carried out at 40 °C the other one is in presented in annex 5.

Figures 29 to 31 show the effect of the different UPLC phases on diastereomers separation.

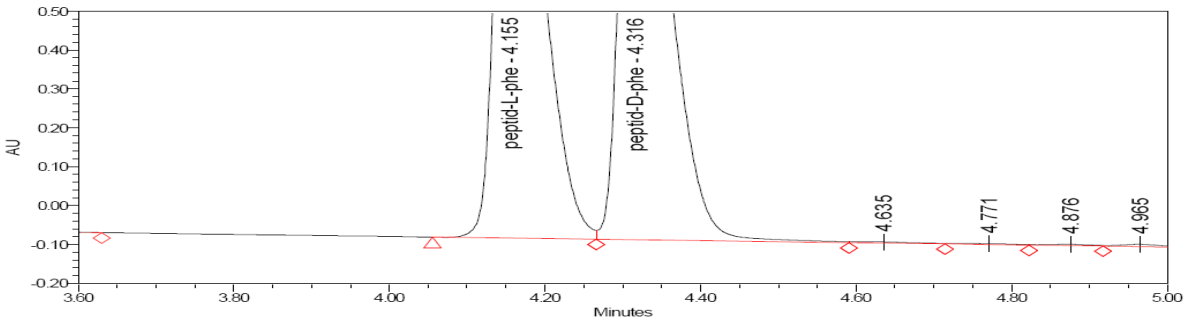

Figure 29. Chromatogram of a spiking of peptide L-phe and D-phe with BEH C18 phase with Acetate buffer at pH 4 second injection 40 °C.

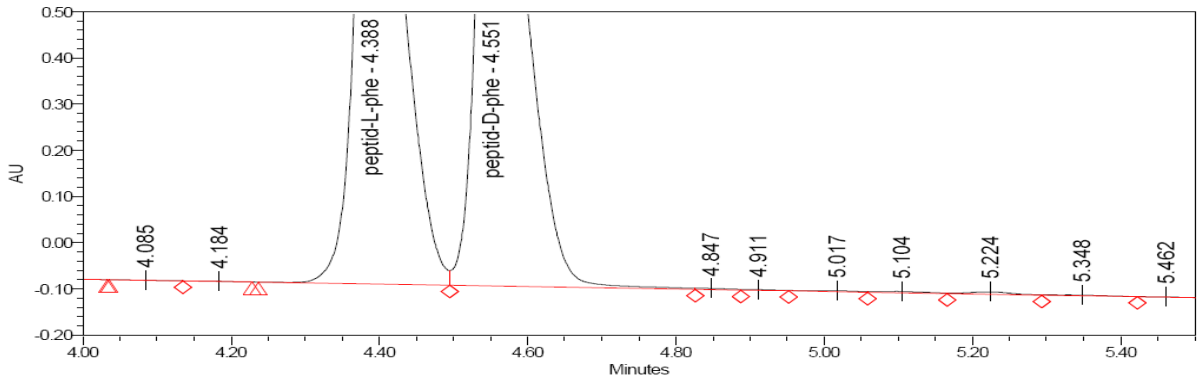

Figure 31. Chromatogram of a spiking of peptide L-phe and D-phe with BEH130 phase with Acetate buffer at pH 4 second injection 40 °C.

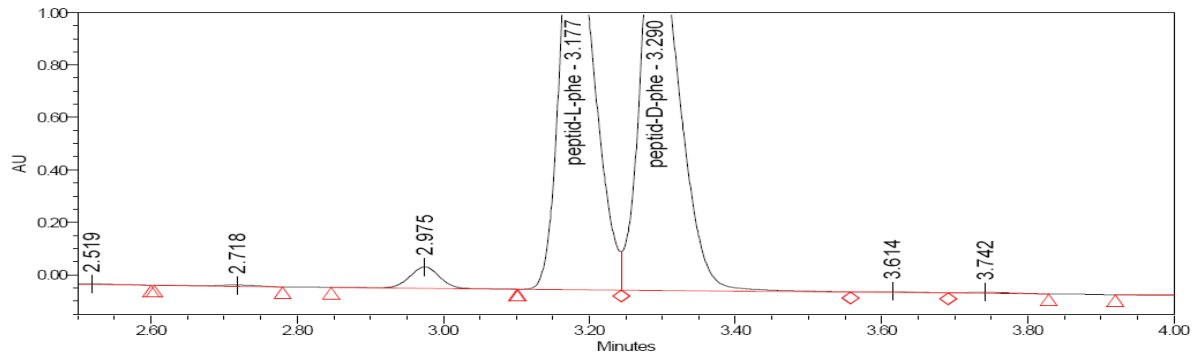

Figure 31. Chromatogram of a spiking of peptide L-phe and D-phe with BEH300 phase with Acetate buffer at pH 4 second injection 40 °C.

Table 10. Values obtained for resolution with UPLC phases at different temperatures.

	Resolution	
Column	40 °C	60 °C
BEH C18	1.49	1.28
BEH 130	1.37	1.10
BEH 300	1.30	0.97

Table 10 shows that the resolution increases at the lower temperature and with a reduction of the pore size.

The resolution obtained (table 10) for the three different columns show slight differences. The BEH 130 especially designed for peptide separation has a significantly poorer resolution than the BEH C18 “standard”. Moreover, if pore size has a slight effect on resolution, a temperature increase has a strong negative effect on the diastereomer separation. The different tables of results and calculation are presented in annex 5.

To summarize, with an analysis 2 times faster, UPLC is for the moment the quickest way to separate these small size diastereomer peptides. HPLC methods with the HALO phase is a good compromise between time analysis and resolution. Thus, it would be useful to test, a 150 mm HALO phase.

4. CONCLUSION AND PERSPECTIVES

The Kinetic plot method is powerful method for the characterization of very different phases like in the first problem with the middle size peptide. It is perhaps not the fastest method for the phases analysis, moreover its results are valuable only for the conditions tested. Indeed, when phosphate was changed to perclorate solution as done with Waters Atlantis C18, an increase of resolution was observed. But the results obtained with this method allow to get an overview of the optimal phases to proceed to the separation. The second disadvantage is that it works for the compound tested and not for other compounds as seen in the second problem for the separation of smaller protected peptides.

For the separation of the medium size peptide, UPLC is for the moment the method that gives the best resolution. There may be a solution to be tested with the use of an HLIC phase especially made for UPLC it may be a reasonable way to enhance time analysis. Otherwise no significant differences were found between the three UPLC columns.

For the small peptides diastereomers problem, the differences between the phases are more visible. First the particle size seems to influence more the separation than for the first problem. This is certainly due to the more apolar character of this compound. Here, smaller particle size, smaller pore size and a temperature not over 40 °C seem to enhance the separation of the diastereomers. A way to improve separation could be the use of a HALO phase of 150 mm length in place of the 75 mm one tested.

5. NOMENCLATURE

HILIC :	Hydrophilic interaction liquid chromatography.
HPLC RP :	High performance liquid chromatography reverse phase.
HPLC NP :	High performance liquid chromatography normal phase.
Peptide :	chain of more no more than 50 amino acids [4].
UPLC :	Ultra performance liquid chromatography.
SCX :	Strong Cation Exchange.
ACN :	Acetonitrile.
TFA :	Trifluoroacetic acid.
MeOH :	Methanol.
FLD :	Fluorescence Laser Detector.
A :	Knox term for Eddy diffusion
B :	Knox term for longitudinal diffusion
C :	Knox term for mass transfer resistance
dp :	particle size in packed bed
$Dm_{A,B}$:	diffusion coefficient of solute A at very low concentration in solvent B
E :	separation impedance
h :	reduced height equivalent of a theoretical plate
hopt :	optimal reduced height equivalent of a theoretical plate
H:	height equivalent of a theoretical plate
Hopt :	optimal plate height leading to highest efficiency
K :	retention factor of a compound
Kv :	column permeability based on interstitial velocity
L :	column length
N :	plate number or plate count
tr :	retention time of a compound
tm :	dead time
η :	dynamic mobile phase viscosity
trt :	triphenylethyl aminoacid side chain protector group.
Fmoc :	(F)luorenyl-(m)eth(o)xy-(c)arbonyl

6. ACKNOWLEDGEMENTS

Author would like to thank HES-SO Valais - Wallis and Lonza A.G. for the scientific support, and gratefully acknowledge Waters for the loan of Acquity column.

Sion, November 23, 2007.

Olivier Gaudard

7. BIBLIOGRAPHY

[1] D. Guillarme et al., « Recent developments in liquid chromatography-Impact on qualitative and quantitative performance » J. Chromatogr. A (2006), pp. 2-7.

[2] R. A. Meyers Wiley, « Encyclopedia of analytical chemistry », New York, (2000), p 11262.

[3] T. Yoshida, « Peptide separation by hydrophilic interaction chromatography: a reviev », J. biochemical and biophysica methods, (2004), p 268.

[4] R. Ciccarelli, « Chromatography en phase liquide », HES-SO vs, Sion, (2005-2006).

[5] M. T. Davis et al., « Automated LC-LC-MS-MS platform using binary ion exchange and gradient reversed phase chromatography for improved proteomic analyses », J. Chromatogr. B (2001), p 282.

[6] D. Clicq et al., « use of kinetic plot method to analyze commercial high temperature liquid chromatography systems part I and II » J. Chromatogr. A (2007), p 197.

[7] Daniel C. Harris., « Quantitative chemical analysis » 5th edition, W. H. Freemann and company, New York, 1999, p. 656.

[8] O. Gaudard, « Kinetic plot, results and chromatograms of the different phases analyses », HES-SO vs, Sion, (2007).

[9] O. Gaudard, « HPLC gradient method, results and chromatograms of the different phases analyses with 20-mer peptide », HES-SO vs, Sion, (2007).

[10] O. Gaudard, « UPLC gradient method, results and chromatograms of the different phases analyses with 20-mer peptide », HES-SO vs, Sion, (2007).

[11] O. Gaudard, « HPLC, gradient method, results and chromatograms of the different phases analyses for the diastereomer problem », HES-SO vs, Sion, (2007).

[12] O. Gaudard, « UPLC, gradient method, results and chromatograms of the different phases analyses for the diastereomer problem », HES-SO vs, Sion, (2007).

7.1. INTERNET LINKS

[a] http://www.lcgceurope.com/lcgceurope/content/printContentPopup.jsp?id=388985, (04.05.2007).

[b] http://www.phenomenex.com/phen/products/onyx/, (04.05.2007).

[c] http://www.waters.com/WatersDivision/pdfs/73600064CU.pdf, (21.11.2007).

[d] http://www.hamiltoncompany.com/HPLC/columnTechSpecs.asp, (21.11.2007).

[e] http://www.waters.com/watersdivision/pdfs/715001371CU.pdf, (04.05.2007).

8. ANNEXES

Annex 1 [8]

Annex 2 [9]

Annex 3 [10]

Annex 4 [11]

Annex 5 [12]

Annex 6 Tables of viscosity measurements for HILIC at pH 2 and 3

Annex 7 Tables of viscosity measurements for RP-HPLC at pH 2 and 3.

Annex 8 Tableau comparatif des différents types de phases de leurs avantages et de leurs inconvénients [1].

Annex 6. Tables of viscosity measurements for HILIC at pH 2 and 3

75 / 25 ACN Tampon phosphate 10 mM pH 2

	ρ [Kg/m3]	Temps de passage [s]	Constante K	Correction de Hagenbach	Viscosité cinétique [mm2/s]	Viscosité dynamique [Pa*s]
essai 1	851	194.18	0.003	1.77	0.57723	4.91E-04
essai2	848	194.34	0.003	1.77	0.57771	4.90E-04
essai 3	846	194.27	0.003	1.77	0.5775	4.89E-04
moyenne						4.90E-04
ecart type						1E-06

75 / 25 ACN Tampon phosphate 10 mM pH 3

	ρ [Kg/m3]	Temps de passage [s]	Constante K	Correction de Hagenbach	Viscosité cinétique [mm2/s]	Viscosité dynamique [Pa*s]
essai 1	871	199.31	0.003	1.77	0.59262	5.16E-04
essai2	857	199.26	0.003	1.77	0.59247	5.08E-04
moyenne						5.12E-04
ecart type						6E-06

Annex 7. Tables of viscosity measurements for RP-HPLC at pH 2 and 3.

75 / 25 ACN Tampon phosphate 10 mM pH 2

	ρ [Kg/m3]	Temps de passage [s]	Constante K	Correction de Hagenbach	Viscosité cinétique [mm2/s]	Viscosité dynamique [Pa*s]
essai 1	956	317.34	0.003	0.725	0.949845	9.079E-04
essai2	949	317.32	0.003	0.725	0.949785	9.015E-04
moyenne						9.05E-04
ecart type						4E-06

75 / 25 ACN Tampon phosphate 10 mM pH 3

	ρ [Kg/m3]	Temps de passage [s]	Constante K	Correction de Hagenbach	Viscosité cinétique [mm2/s]	Viscosité dynamique [Pa*s]
essai 1	946	317.87	0.003	0.725	0.951435	9.00E-04
essai2	951	317.88	0.003	0.725	0.951465	9.05E-04
essai 3	952	317.91	0.003	0.725	0.951555	9.06E-04
moyenne						9.04E-04
ecart type						3E-06

ANNEXE 8. TABLEAU COMPARATIF DES DIFFÉRENTS TYPES DE PHASES, DE LEURS AVANTAGES ET DE LEURS INCONVÉNIENTS [1].

Type de colonne	Type de greffage	Avantages	Inconvénients	Domaine d'utilisation / Fabricant et nom
Monolithiques	C8, C18, Si	Faible perte de charge Différents types de squelettes disponibles	Technique récente, Faible résistance de la colonne. Stabilité de la phase stationnaire	pH : 2-7.5 ΔP : ≤200 bar T : ≤ 35° C Surface spécifique: 300 m^2/g Taille des pores: - Mesopores: 130Å - Macropores: 2µm Volume des pores: 1 mL/g Porosité: > 80% [b] / Phenomenex, Onyx
HPLC	HILIC [3]	Permet de retenir les acides aminés polaires	Temps d'analyse	pH : 1-5 ΔP : ≤400 bar T : 20 à 40° C Taille des particules: 3 µm [c]
HPLC	C 18 et C 18 wide pore	Méthode éprouvée et largement utilisée	Rapidité Difficulté de séparation des peptides polaires	pH : 2-7 ΔP : ≤ 400 bar T : 20-40 °C Taille des pores: 100 Å 300 Å (wid pore) Taille des particules: 3 µm [c]
PS-DVB	-	Grande résistance au pH	Temps de rétention des composés polaires	pH : 1 à 13 ΔP : ≤ 400 bar T : 5-60 °C Taille des pores: 100 Å Taille des particules: 5 µm [d]
UPLC	C8, C18, shield RP18, HILIC, phényl, sub 2 µm C18 sub 2 µm wide pore	Possibilité de réduire considérablement le temps d'analyse	Appareillage spécifique ΔP~1000 bar Peu de phases stationnaires stables disponibles Compressibilité des solvants	pH : 1-12 (dépend du gréphage) ΔP : ≤ 700 bar T : ≤ 70 C Taille des pores: 130 Å 300 Å (wid pore) Surface spécifique: 180 m/g Volume des pores: 0.7 mL/g Taille des particules: 1.7 µm [e] Waters, acquity