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Scuola di Dottorato in Biotecnologie Industriali

Quantification Challenge: 2 Matrices for a Complex Protein

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Riassunto

I recenti sviluppi in molte discipline biologiche, sono strettamente collegati agli sviluppi ed alle applicazioni sviluppate nel campo della proteomica, rese possibili in gran parte dagli sviluppi della spettrometria di massa.

L'orbitrap è una recente aggiunta nel set di "strumenti" utilizzabili per gli studi di proteomica ed è da subito diventato la miglior scelta per questo tipo di applicazioni, grazie alla sua abilità di determinare con grande accuratezza bassissime differenze di massa ad un'alta risoluzione.

L'orbitrap, come suggerisce il nome è una trappola, che opera in maniera non convenzionale; gli ioni sono intrappolati in un campo elettrostatico, in cui si muovono in un complesso moto a spirale e la trasformata di Fourier viene impiegata per trasformare le frequenze di oscillazione in accuratissimi m/z. L'utilizzo dell'orbitrap ha reso possibile lo sviluppo di un metodo quantitativo in grado di andare a catturare e rivelare una proteina presente in 2 matrici biologiche, il siero ed il liquido sinoviale. Lo sviluppo del processo analitico è stato dettato da un imput dell'FDA, dato che la proteina ricombinante utilizzata per scopi terapeutici da Merck, è una modifica di una proteina nativa, naturalmente presente nel corpo umano. Al momento dell'inizio di questo lavoro, Merck utilizzava come

metodo quantitativo solo una determinazione via ELISA, ma dato che le 2 proteine si differenziano solo per qualche modifica in sequenza primaria all'N-terminal ed al C-terminale, la FDA ha richiesto che venisse sviluppato un metodo quantitativo ortogonale, con una detection di tipo fisico e non di tipo immunologico. Attualmente in letteratura non sono presenti metodi analitici che riescano a determinare i ng/mL o i pg/mL su una proteina intatta, vista la complessità nel trattare con una proteina intera. Nonostante questo, i primi passi nello sviluppo delle analisi si sono rivolti in questa direzione. La prima complicazione nello sviluppo di questa metodica è dato dal fatto che una delle matrici biologiche in cui è possibile trovare il campione è il liquido sinoviale, che presenta una elevata viscosità e quindi risulta incompatibile con molti metodi di purificazione. Si è deciso quindi di iniziare a sviluppare dei metodi di purificazione utilizzando le beads magnetiche. Questo tipo di beads hanno diversi coating esterni e quindi si possono realizzare diversi tipi di separazione, in base al tipo di beads scelta. Come primo approccio sono state utilizzate 2 metodologie, la prima è lo scambio cationico, data la natura della proteina, in cui 10 % della sequenza sono lisine e quindi la proteina possiede un punto isoelettrico maggiore di 9.5. La seconda tecnologia è invece basata sull'ELISA; viene infatti preso l'anticorpo primario usato in ELISA e legato alle beads con differenti strategie: biotinilato alle beads streptavidinate e tal quale legato alle beads ricoperte da proteina-G. questi approcci hanno mostrato una preferenza per la coppia Ab biotinilato/beads streptavidinate in quanto il legame con lo scambio cationico risultava troppo forte, mentre l'approccio con la proteina G ha mostrato risultati inferiori da un punto di vista del recovery finale. Le beads si sono dimostrate efficenti nella cattura e nella separazione della nostra proteina in entrambe le matrici biologiche. È stato anche prodotto un internal standard per effettuare le analisi di tipo quantitativo. Purtroppo date le caratteristiche fisiche della proteina, tutti i processi soffrivano di grossi fenomeni di binding aspecifico. Questo ha

influenzato in modo particolare la sensibilità strumentale, che purtroppo si è attestata a circa 12 ng/mL in buffer, mentre per il metodo completo (purificazione da matrice+ analisi) non si è mai riusciti a scendere sotto il µg/mL. Visti questi presupposti e visto che le concentrazioni nei campioni clinici sono al di sotto di questi livelli di concentrazione si è dovuti passare all'analisi quantitativa di un peptide derivante dalla nostra proteina. Nello sviluppo dell'analisi quantitativa sul peptide si è dovuto tener conto di 2 fattori nella scelta dell'enzima per eseguire il taglio: l'unicità del peptide, ossia il peptide generato doveva possedere una sequenza attribuibile solo alla nostra proteina e la specificità, ossia questo peptide doveva essere differente tra la forma nativa e la forma ricombinante della proteina, quindi si è dovuto andare a scegliere forzatamente peptidi generati da N o C terminale. La nostra prima scelta è ricaduta sul C-terminale, in quanto le differenze in struttura primaria erano maggiori. Purtroppo anche il C-Terminale presenta molte lisine nella sua sequenza e un PI di circa 9.2. Su questo peptide sono state fatte ricerche preeliminari che hanno portato ad una sensibilità strumentale di circa 480 ng/mL e mai nessun metodo completo è stato provato per questo tipo di peptide. Si è deciso quindi di andare sull'N-terminale, che presentava una sequenza più corta di quello ottenuto al C-terminale, ma soprattutto un PI di 4.2, che lo rende immune da fenomeni di binding aspecifico. È stato quindi effettuato un primo studio teorico sugli enzimi che ci potessero dare il peptide di nostro interesse. Una volta effettuato questo screening teorico, abbiamo testato in pratica i diversi enzimi, andando a scegliere la tripsina per motivi di resa di reazione e costo dell'enzima. La sensibilità strumentale in buffer si è dimostrata subito molto buona dato che ci ha permesso di raggiungere i 300 pg/mL. Data l'alta efficienza strumentale si è deciso di tenere buono questo enzima e questo tipo di peptide e siamo andati a produrre un internal standard, che viene richesto nei metodi analitici quantativi in spettrometria di massa validati per FDA, questo standard ha una modifica di 1 AA nella

zona del peptide di interesse in modo da avere una differenza di massa ed inoltre possiede 2 siti di clevage che ci peremttono di valutare anche l'efficienza della digestione. Questo internal standard viene aggiunto al campione prima della digestione Una volta sviluppato il metodo in buffer siamo andati a testare la nostra soluzione in siero. Abbiamo effettuato diverse prove di digestione enzimatica classica in soluzione, con la tripsina disciolta in siero, ma nonostante le diverse condizioni provate, non siamo mai riusciti ad ottenere nessun segnale relativo al nostro peptide. Questo è dovuto alla presenza dell'alfa-1-antitripsina, una delle proteine più abbondanti nel siero, che è un diretto inibitore dell'enzima da noi scelto per effettuare il clevage enzimatico. Essendo tutti gli altri enzimi nello screening anch'essi delle proteasi seriniche, abbiamo dovuto trovare una soluzione per questo problema, non essendo in grado di fare, come comunemente si effettua per le analisi quantitative in siero, la deplezione delle proteine più abbondanti. La deplezione non è possibile a causa delle caratteristiche della nostra proteina che durante questo passaggio di purificazione andrebbe persa a causa del forte binding aspecifico. Questi fattori ci hanno portato a provare con successo una digestione su fase solida, utilizzando della tripsina immobilizzata su dei puntali, forniti dalla Proteogen Bio, grazie ai quali siamo riusciti ad ottenere la digestione su siero intero senza la deplezione dell'inibitore. Un forte sviluppo dal protocollo di base è stato necessario, perché questi puntali sono sviluppati per analisi di proteomica di tipo qualitativo a livello di µg/ml e non per applicazioni quantitative a livello di pg/mL. Il maggior sviluppo in termini di segnale è stato fornito dall'utilizzo del Rapidgest (Waters), che è una miscela di tensioattivi in grado di non denaturare gli enzimi addetti al clevage. Per le future analisi dei campioni clinici è stato anche sviluppato un metodo su una stazione automatica liquid handler della Hamilton, in modo tale che il processo di digestione venga standardizzato ed automatizzato. Una volta sviluppato questo metodo di digestione, sono stati provati 3 metodi di purificazione del

campione dalla matrice. Il primo ad essere sviluppato è stato un metodo con estrazione in fase solida, che mostrava una buona pulizia del campione, ma una bassa resa, portando il limite di quantificazione totale del metodo a circa 400 ng/mL. Il secondo metodo di purificazione utilizzato è stata la protein precipitation, che si è dimostrato anch'esso molto valido e ci ha abbassato il LOQ a circa 100 ng/mL. Purtroppo questo metodo si è dimostrato troppo sporco per essere utilizzabile in routine, dato che si sono verificati molti fenomeni di intasamento a livello della colonna cromatografica e di tutto il sistema HPLC e si è deciso quindi di abbandonare questo tipo di tecnica. L'ultima strategia ha riguardato l'uso di un anticorpo policlonale contro il nostro peptide attaccato alle beads magnetiche. L'anticorpo si è dimostrato capace di catturare anche l'internal standard e di abbassare il LOQ del metodo a livello di pg/mL. Purtroppo questo metodo è molto sensibile ed i risultati fino a qui ottenuti non hanno permesso una validazione a livello di pg/mL, ma il metodo si mostra molto efficiente a livello di ng/mL. Per il liquido sinoviale tutte le tecniche sviluppate in siero si sono dimostrate altrettanto efficienti per il liquido sinoviale, l'unica modifica è stata l'aggiunta di un pre-trattamento della matrice con enzimi addetti al taglio dei polimeri di acido ialuronico, che permettono una maggior fluidità della matrice e quindi agevolano il passaggio della stessa all'interno dei puntali trispinizzati. Il metodo sviluppato permette quindi l'analisi di proteine o peptidi derivanti da esse, senza modifiche sostanziali alla matrice e l'approccio con l'immunoaffinity permette un buon clean up dopo la digestione. Teoricamente questa metodica è applicabile universalmente ad ogni tipo di proteina/peptide e potrebbe essere la nuova procedura standard per l'analisi entità biologiche in diversi tipi di matrice.

Introduction to mass spectrometry

Mass spectrometry arose to an outstanding position among analytical methods for its characteristics: unequalled sensitivity, detection limits, speed and diversity of its applications. In analytical chemistry, the most recent applications are mostly oriented towards biochemical issues, such as proteome, metabolome, high throughput in drug discovery and metabolism, and so on. Other analytical applications are routinely applied in pollution control, food control, forensic science, natural products or process monitoring. Mass spectrometry has progressed extremely rapidly during the last years, between 1995 and 2005. This progress has led to the advent of entirely new instruments and so the development of new applications. [1]

History of mass spectrometry

The history of mass spectrometry dates back to more than one hundred years and has its roots in physical and chemical studies regarding the nature of matter. The study of gas discharges in the mid 19th century led

to the discovery of anode and cathode rays, which turned out to be positive ions and electrons. Improved capabilities in the separation of these positive ions enabled the discovery of stable isotopes of the elements. The first discovery in this analytical field was related to the atom neon, which was shown by mass spectrometry to have at least two stable isotopes: Ne^{20} with 10 protons and 10 neutrons and Ne^{22}, with 10 protons and 12 neutrons. Mass spectrometers were used in the Manhattan Project for the separation of isotopes of uranium necessary to create the atomic bomb.

Discovery of isotopes

In 1913, as part of his exploration into the composition of canal rays, J. J. Thomson channelled a stream of ionized neon through a magnetic and an electric field and measured its deflection by placing a photographic plate in its path. Thomson observed two patches of light on the photographic plate which suggested two different parabolas of deflection.

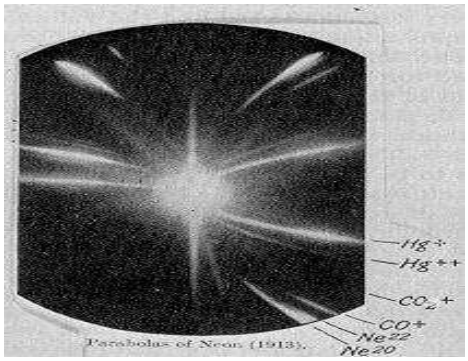

Fig 01: In the bottom right corner of this photographic plate are markings for the two isotopes of neon: neon-20 and neon-22.

Mellerio G.: Following the traces of Mass spectrometry.

Thomson concluded that the neon gas was composed of atoms of two different atomic masses: Ne^{20} and Ne^{22}[2]. Francis William Aston continued the research, building the first full functional mass spectrometer in 1919. He was able to identify isotopes of chlorine (35 and 37), bromine (79 and 81), and krypton (78, 80, 82, 83, 84 and 86), proving that these natural occurring elements are composed of a combination of isotopes. His work on isotopes also led to his formulation of the “Whole Number Rule” which states that "the mass of the oxygen isotope being defined and all the other isotopes have masses that are very nearly whole numbers".

Manhattan Project

A Calutron is a sector mass spectrometer that was used for separating the isotopes of uranium developed by Ernest O. Lawrence[3] during the Manhattan Project and was similar to the Cyclotron invented by Lawrence. They were implemented for industrial scale uranium enrichment at the Oak Ridge, Tennessee Y-12 plant established during the war and provided much of the uranium used for the nuclear weapon, which was dropped onto Hiroshima in 1945.

Development of chromatography-mass spectrometry

The use of a mass spectrometer as the detector in chromatography was developed during the 1950s by Roland Gohlke and Fred McLafferty.[4-5] The development of affordable and miniaturized computers has helped in the simplification of the use of this instrument, as well as allowed great improvements in the amount of time it takes to analyse a sample.

Principles

The first step in the mass spectrometric analysis of compounds is the production of gas-phase ions, for example by electron ionization:

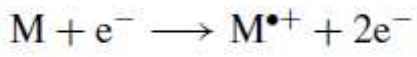

Fig02a: Electronic ionization scheme.

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This molecular ion normally undergoes fragmentations. Because it is a radical cation with an odd number of electrons, it can fragment to give either a radical or an ion with an even number of electrons, or a molecule and a new radical cation. We stress the important difference between these two types of ions and the need to write them correctly:

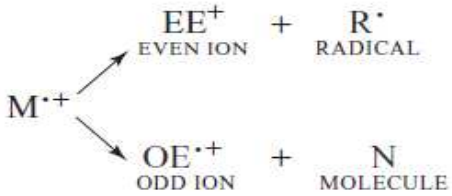

Fig02b: Electronic ionization scheme.

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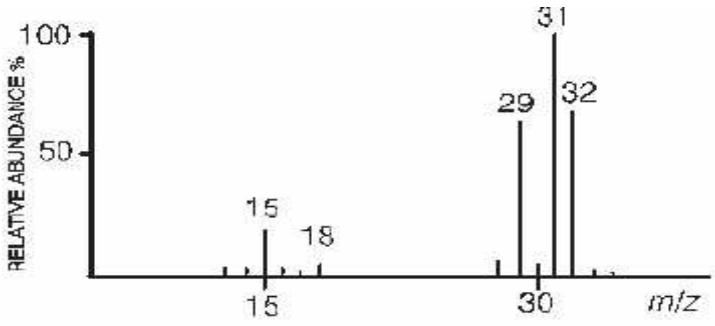
These two types of ions have different chemical properties. Each primary product ion derived from the molecular ion can, in turn, undergo fragmentation, and so on. All these ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It provides this result as a plot of ion abundance versus mass-to-charge ratio.



m/z	Relative abundance (%)	*m/z*	Relative abundance (%)
12	0.33	28	6.3
13	0.72	29	64
14	2.4	30	3.8
15	13	31	100
16	0.21	32	66
17	1.0	33	0.73
18	0.9	34	~ 0.1

Fig 03: Example of mass spectrometry data

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As illustrated in Fig.03 mass spectra can be presented as a bar graph or as a table. In either presentation, the most intense peak is called the base peak and is arbitrarily assigned the relative abundance of 100 %. The abundances of all the other peaks are given their proportionate values, as percentages of the base peak. Many existing publications label the y axis of the mass spectrum as number of ions, ion counts or relative intensity. Most of the positive ions have a charge corresponding to the loss of only one electron, but for large molecules, multiple charged ions also can be obtained. Ions are separated and detected according to the mass-to-charge ratio.

There are different ways to define and thus to calculate the mass of an atom, molecule or ion. For stoichiometric calculations, chemists use the average mass calculated using the atomic weight, which is the weighted average of the atomic masses of the different isotopes of each element in the molecule. In mass spectrometry, the nominal mass or the mono-isotopic mass is generally used. The nominal mass is calculated using the mass of the predominant isotope of each

element rounded to the nearest integer value that corresponds to the mass number, also called nucleon number. But the exact masses of isotopes are not exact whole numbers. They differ weakly from the summed mass values of their constituent particles that are protons, neutrons and electrons. These differences, which are called the mass defects, are equivalent to the binding energy that holds these particles together. Consequently, every isotope has a unique and characteristic mass defect. The mono-isotopic mass, which takes into account these mass defects, is calculated by using the exact mass of the most abundant isotope for each constituent element. The difference between the average mass, the nominal mass and the mono-isotopic mass can amount to several Da, depending on the number of atoms and their isotopic composition. The type of mass determined by mass spectrometry depends largely on the resolution and accuracy of the analyser. Let us consider CH_3Cl as an example. Actually, chlorine atoms are mixtures of two isotopes, whose exact masses are respectively 34.968 Da and 36.965 903 u. Their relative abundances are 75.77% and 24.23 %. The atomic

weight of chlorine atoms is the balanced average: (34.968…×0.7577+36.965…×0.2423) =35.453 Da. The average mass of CH3Cl is [12.011… + (3×1.00) + 35.453…]= 50.4878 Da, whereas its mono-isotopic mass is [2.000 + (3×1.007…) + 34.968852…]= 49.992 327 u. When the mass of CH3Cl is measured with a mass spectrometer, two isotopic peaks will appear at their respective masses and relative abundances. Thus, two mass-to-charge ratios will be observed with a mass spectrometer. The first peak will be at m/z (34.968…+12.000+3×1.007….) = 49.992. The mass-to-charge value of the second peak will be (36.965…+12.000+3×1.007….) = 51.989. To better explain the differences between monoisotopic mass and average mass we could imagine two simple alkanes molecules ($C_{20}H_{42}$ and $C_{100}H_{202}$).

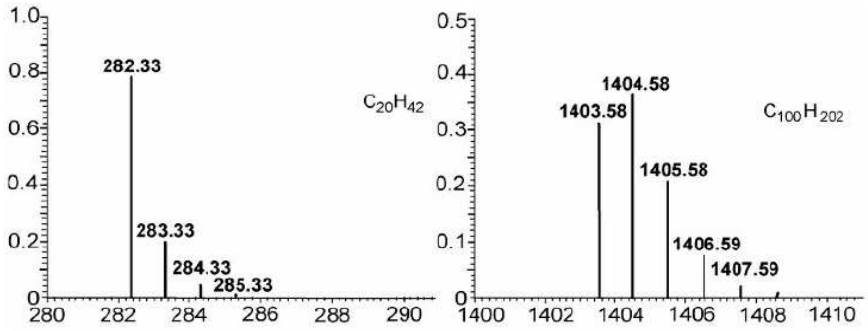

Fig 04: The monoisotopic mass is the lighter mass of the isotopic pattern whereas the average mass, used by chemists in stoichiometric calculations, is the balanced mean value of all the observed masses.

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In conclusion of this brief introduction, a mass spectrometer should always perform the following processes:

1. Produce ions from the sample in the ionization source.
2. Separate these ions according to their mass-to-charge ratio in the mass analyser.
3. Eventually, fragment the selected ions and analyze the fragments in a second analyser.
4. Detect the ions emerging from the last analyser and measure their abundance with the detector that converts the ions into electrical signals.

5. Process the signals from the detector that are transmitted to the computer and control the instrument through feedback.[1]

The Orbital Trap

The increasing complexity of biological samples encountered in proteomic and metabolomic studies continues to push the technological limits of analytical instrumentation. Mass spectrometry (MS) has increasingly become an analytical tool of choice in these areas owing to its speed, wide dynamic signal range, quantitative capability and the facility to interface with chromatographic separation methods. Reliable identification of metabolites, sequences and post-translational modifications of proteins in complex mixtures necessarily requires robust mass spectrometers with high resolving power, mass accuracy, sensitivity and dynamic range. These bioanalytical demands were addressed by such hybrid instruments as the quadrupole/time-of-flight (QqTOF, where Q refers to a mass-resolving quadrupole, q to a

radio frequency (RF)-only quadrupole or hexapole collision cell and TOF to a time-of-flight mass spectrometer)[6] and the linear quadrupole ion trap/Fourier transform ion cyclotron resonance (LIT/FT-ICR)[7] that performs in the high resolution (>10,000) and high mass accuracy (<5 ppm) regimes[8-10]. The QqTOF, which can be regarded as a triple quadrupole (QqQ) instrument where the third quadrupole is replaced by an orthogonal TOF, was originally used for rapid de novo peptide sequencing[11] but has found application in many areas of research including metabolite[12], nucleic acid[13] and glycoprotein[14] analysis. However, for scan-types in which a single ion is monitored such as precursor ion and neutral loss scans (important experiments for structural characterization), the sensitivity of the QqTOF (5–30% duty cycle) is lower than QqQ instruments because more ions are lost in TOF compared with a third quadrupole. These losses occur during transfer into the orthogonal TOF, on grids and at the detector. For my experimental work I had the possibility to work on the latest generation of mass spectrometry instrument, the Orbitrap, provided by Thermo Fisher. The Orbitrap,

presented by Alexander Makaraov in 2005, brings the high resolution MS to a novel rising era, due to the fact, that this kind of spectrometer allow an high resolution detection with a small and “cheap” instrumentation, compared to the normal High resolution MS, based on cyclotronic resonance.[15]

The “Old” high resolution

Fourier-transform ion cyclotronic resonance mass spectrometry FT-ICR is often thought of as being the most complex method of mass analysis and detection. The technique of ICR-MS was first published in the mid. 1950's [16] where it was demonstrated for measurement of very small mass differences at very high precision. The technique remained a largely academic tool until the application of FT methods [17] by Alan Marshall and Melvin Comisarow in the early 1970's [18]. It is now one of the most sensitive methods of ion detection in existence and has almost unlimited resolution. The ions are generated in the source (as usual) and then pass through a series of pumping stages at increasingly high vacuum. When ions enter the cell (ion trap), pressures are in the range of 10^{-10} to

10^{-11} mBar with temperatures close to absolute zero. The cell is located inside a spatial uniform static superconducting high field magnet (typically 4.7 to 13 Tesla) cooled by liquid helium and liquid nitrogen. When the ions pass into the magnetic field they are bent into a circular motion in a plane perpendicular to the field by the Lorentz Force. They are prevented from processing out of the cell by the trapping plates at each end. The frequency of rotation of the ions is dependent on their *m/z* ratio. At this stage, no signal is observed because the radius of the motion is very small. Excitation of each individual *m/z* is achieved by a swept RF pulse across the excitation plates of the cell. Each individual excitation frequency will couple with the ions natural motion and excite them to a higher orbit where they induce an alternating current between the detector plates. The frequency of this current is the same as the cyclotron frequency of the ions and the intensity is proportional to the number of ions. When the RF goes off resonance for that particular *m/z* value, the ions drop back down to their natural orbit (relax) and the next *m/z* packet is excited. Although the RF sweep is made up of a series of stepped frequencies, it

can be considered as all frequencies simultaneously. This results in the measurement of all the ions in one go producing a complex frequency *vs*. time spectrum containing all the signals; the deconvolution of this signal by FT methods results in the deconvoluted frequency *vs*. intensity spectrum which is then converted to the mass *vs*. intensity spectrum by equation. It is also usual to correct for mass errors at this stage by applying a calibration

$$\mathbf{F} = z\mathbf{v} \times \mathbf{B} \quad \text{.....(1)}$$

$$\omega_c = \frac{z\mathbf{B}}{2\pi m} \quad \text{.....(2)}$$

$$m/z = \frac{\mathbf{B}}{2\pi\omega_c} \quad \text{.....(3)}$$

- **F** is the Lorentz Force observed by the ion when entering the magnetic field
- **B** is the magnetic field strength (constant)
- **v** is the incident velocity of the ion
- w_c is the induced cyclotron frequency
- m is the mass of the ion
- z is the charge on the ion

Fig 05a: equation that rules the FT-ICR

http://www.chm.bris.ac.uk/ms/theory/fticr-massspec.html

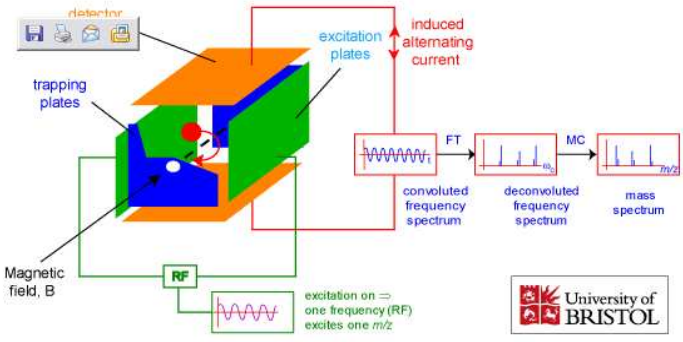

Fig 05b: FT-ICR scheme

http://www.chm.bris.ac.uk/ms/theory/fticr-massspec.html

The Orbitrap

As we could see before, this kind of instrumentation is expensive, not only for to the initial price, but also for the work and maintaining condition, that requires a lot of energy and a lot of expensive materials. The Orbitrap mass analyzer bears a similarity to an earlier ion storage device, the Kingdon trap, as well as to two types of ion-trapping mass analyzers, the Paul trap (quadrupole ion trap), and the Fourier transform ion cyclotron resonance instrument. The first step in the set of inventions that led to the Orbitrap was the implementation of orbital trapping, a method of ion trapping, which can itself be used for mass analysis. Makarov invented a new “Knight-style’ Kingdon trap” with specially shaped inner and outer electrodes. Despite its relatively recent commercial introduction, the LTQ-Orbitrap has already proven to be an important analytical tool with a wide range of applications. The high resolving power (>150,000) and excellent mass accuracy [15], significantly reduce false positive peptide identifications in bottom-up protein

analyses. The orbitrap mass analyzer (which can also be considered a refined Knight-style Kingdon trap) is composed of a spindle-like central electrode and a barrel-like outer electrode. A DC voltage is applied between the two axially symmetric electrodes, resulting in the following electrostatic potential distribution.[19-21] .

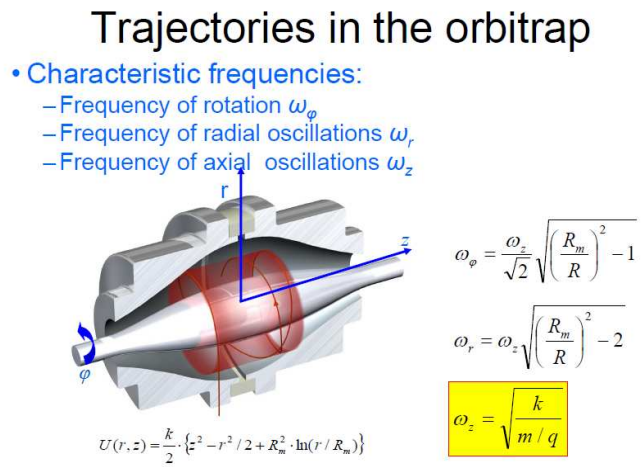

Fig 06: Equations that rule the Orbital trap, the axial oscillation directly depends on the mass of the ions

J. Mass Spectrom. 2005; **40**: 430–443

When ions are injected into the orbitrap at a z-position offset, the ion packet begins coherent axial oscillation without the need for any additional excitation.[21] Ions are injected into the orbitrap after the voltage on the central electrode is turned on (typically 50–90 msec)

but before the voltage has reached its final value. Consequently, as the ions enter the orbitrap they experience a monotonic increase in electric field strength, a process termed ‘‘electrodynamic squeezing’’. [20-21] This has the effect of contracting the radius of the ion cloud, as well as pulling the ion packet closer to the z-axis (i.e., reducing the rotational radius), thereby preventing collisions with the outer electrode as the packets begin their axial oscillations. The rise-time of the field strength (typically 20–100 msec) determines the trapped m/z range.

Since ions of different m/z values are injected at different times, with larger m/z ions arriving later, electrodynamics squeezing results in larger final amplitude of axial oscillation as well as larger mean orbital radius for ions of larger m/z ratio. Both effects will tend to increase the induced ion image current for larger m/z, although this effect may be partially or completely offset by the dependence on the axial frequency. Squeezing is stopped when there is no more possibility of losing ions to collisions with the outer electrode, which is maintained at virtual ground.

After ions of all m/z values have entered the orbitrap, the voltage on the central electrode and deflector is held constant to prevent mass shifts during detection. The deflector is switched to a voltage level that compensates for fringing fields caused by the injection slot. [22] This is necessary to ensure that ions experience the harmonic axial potential throughout the volume of the orbitrap, thereby minimizing differences in frequency for ions of a given m/z value, which in turn could result in mass errors, peak splitting and lower resolution. After both the central electrode and deflector voltages are stabilized, image current detection may take place.

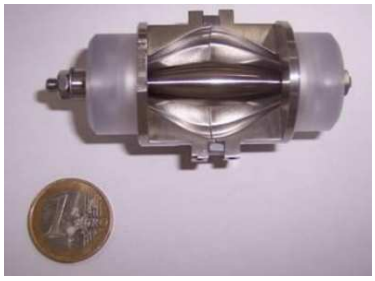

Fig 07: Real dimension of Orbital trap.

Provided by Thermo fisher scientific.

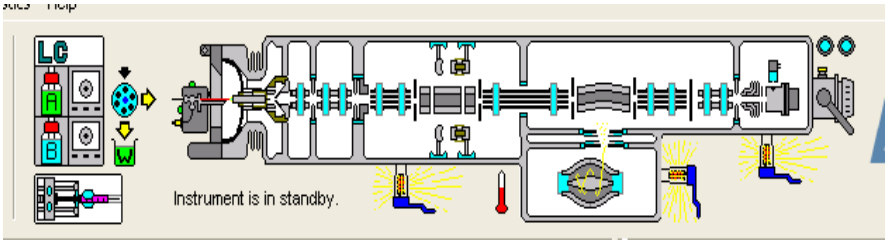

Fig 08: schematic representation of the LTQ XL Orbitrap

Picture obtained from LTQ Tune software, Thermo Fisher scientific.

High performance mass spectrometers continue to play significant roles in many areas of scientific investigation. For example, high performance liquid chromatography (HPLC) retention times and MS/MS information in conjunction with accurate mass measurements can greatly limit peptide candidates to just a few sequences [23] and also facilitate elucidation of elemental composition. These capabilities can also provide valuable structural information about the location and identification of PTMs. So, the high mass accuracy (better than 1 ppm with internal calibration), resolving power (up to 150,000) and MS^n capabilities of the LTQ-Orbitrap make it a valuable instrument for chemical analysis. The orbitrap is the first new mass analyzer to be introduced as a commercial instrument (LTQ-Orbitrap hybrid mass spectrometers) in the last 20 years. Its high mass accuracy (2–5 ppm) makes it useful

for high performance analyses of complex mixtures; its relative low cost and complexity allows it to be used in a variety of laboratory settings. A number of methods and instruments have been developed to inject ions into the orbitrap: (i) electrostatic acceleration lenses, (ii) external linear quadrupole ion trap featuring axial ejection of ions, and (iii) the LTQ-C-trap, with radial ejection of ions. Combining the orbitrap with an external accumulation region such as the LIT of the LTQ provides MS^n capability for the elucidation of analyte structure and allows coupling with continuous ionization sources such as ESI and dual nESI configurations for implementation of ETD. In addition, precursor ions can be fragmented in the C-trap (LTQ-Orbitrap Discovery and XL) or an additional linear octapole located at the rear end of the C-trap (LTQ-Orbitrap XL), which provides additional MS^n versatility. As demonstrated in many recent publications, the orbitrap mass analyzer supports a wide range of applications ranging from routine compound identification to the analysis of trace-level components in complex mixtures and is expected to make

significant contributions to many areas of scientific exploration.[24]

Protein analysis

The ability to identify proteins and to determine their covalent structures has been central to the life sciences. The amino acid sequence of proteins provides a link between proteins and their coding genes via the genetic code, and, in principle, a link between cell physiology and genetics. The identification of proteins provides a window into complex cellular regulatory networks. Before the genomics revolution, chemical or enzymatic methods were used to probe the covalent structure of single, highly purified proteins, and typically, the products of such reactions were detected by ultraviolet (UV) absorbance or fluorescent spectroscopy. For example, polypeptides were sequenced by stepwise chemical degradation from the N-terminus to the C-terminus (Edman degradation), with subsequent identification of the released amino acid derivatives by UV absorbance spectroscopy. Gradually over the past two decades, mass

spectrometers were interfaced with a number of protein chemistry assays to create detectors providing superior information. With the increased performance and versatility of the instrumentation, new protein analytical strategies have emerged in which mass spectrometry is the central element. For example, by the mid-1990s, a variety of mass spectrometry–based strategies had essentially replaced the Edman degradation as the as the mainstream method for determining the amino acid sequences of polypeptides. The trend toward mass spectrometry as the technique of choice for identifying and probing the covalent structure of proteins was accelerated by the genome project. Genomics demonstrated the power of high-throughput, comprehensive analyses of biological systems. Genomics also provides complete genomic sequences, which are a critical resource for identifying proteins quickly and robustly by the correlation of mass-spectrometric measurements of peptides with sequence databases. The systematic analysis of all the proteins in a tissue or cell was popularized under the name proteomics, with mass spectrometry central to most proteomic strategies. The analysis of a full

proteome presents a formidable task and, in spite of recent technical developments, remains to be achieved for any species. The task is challenging because proteomes have a large and unknown complexity. What is certain is that the number of proteins in a species proteome exceeds by far the number of genes in the corresponding genome. This diversity arises from the fact that a particular gene can generate multiple distinct proteins as a result of alternative splicing of primary transcripts, the presence of sequence polymorphisms, posttranslational modifications, and other protein processing mechanisms. Moreover, proteins span a concentration range that exceeds the dynamic range of any single analytical method or instrument. For example, it has been estimated that the concentration range of serum proteins exceeds 10 orders of magnitude. Although these challenges are daunting, they have stimulated advances in technologies for the analysis of proteins and proteomes. Here we describe a range of mass-spectrometric techniques, discuss their utility for protein analysis, and assess their ability to support or interface with a range of proteomic strategies. [25]

Mass spectrometry was restricted for a long time to small and thermostable compounds because of the lack of effective techniques to softly ionize and transfer the ionized molecules from the condensed phase into the gas phase without excessive fragmentation. The development in the late 1980s of two techniques for the routine and general formation of molecular ions of intact biomolecules—electrospray ionization (ESI) [26] and matrix assisted laser desorption/ionization (MALDI) [27] dramatically changed this situation and made polypeptides accessible to mass spectrometric analysis. This catalyzed the development of new mass analyzers and complex multistage instruments [for instance, hybrid quadrupole time-of-flight (Q-Q-ToF) and tandem time-of-flight (ToF-ToF) instruments designed to tackle the challenges of protein and proteome analysis. [28-29] Mass spectrometers are used either to measure simply the molecular mass of a polypeptide or to determine additional structural features including the amino acid sequence or the site of attachment and type of posttranslational modifications. In the former case, single-stage mass spectrometers are used, acting essentially as balances to weigh molecules.

In the latter case, after the initial mass determination, specific ions are selected and subjected to fragmentation through collision. In such experiments, referred to as tandem mass spectrometry (MS/MS), detailed structural features of the peptides can be inferred from the analysis of the masses of the resulting fragments. The types of mass spectrometers described below are most commonly used to support a range of research strategies in the protein sciences. They differ in their physical principles, their performance standards, their mode of operation, and their ability to support specific analytical strategies.

	IT-LIT	Q-Q-ToF	ToF-ToF	FT-ICR	Q-Q-Q	QQ-LIT
Mass accuracy	Low	Good	Good	Excellent	Medium	Medium
Resolving power	Low	Good	High	Very high	Low	Low
Sensitivity (LOD)	Good		High	Medium	High	High
Dynamic range	Low	Medium	Medium	Medium	High	High
ESI	✓	✓		✓	✓	✓
MALDI	(✓)	(✓)	✓			
MS/MS capabilities	✓	✓	✓	✓	✓	✓
Additional capabilities	Seq. MS/MS			Precursor, Neutral loss, MRM		
Identification	++	++	++	+++	+	+
Quantification	+	+++	++	++	+++	+++
Throughput	+++	++	+++	++	++	++
Detection of modifications	+	+	+	+		+++

Fig 09: Characteristics and performances of commonly used types of mass spectrometers. Check

marks indicate available, check marks in parentheses indicate optional.

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Ion Sources

In the ion sources, the analysed samples are ionized prior to analysis in the mass spectrometer. A variety of ionization techniques are used for mass spectrometry. The most important considerations are the internal energy transferred during the ionization process and the physico-chemical properties of the analyte that can be ionized. Some ionization techniques are very energetic and cause extensive fragmentation. Other techniques are softer and only produce ions of the molecular species. Electron ionization, chemical ionization and field ionization are only suitable for gas-phase ionization and thus their use is limited to compounds sufficiently volatile and thermally stable. However, a large number of compounds are thermally labile or do not have sufficient vapour pressure. Molecules of these compounds must be directly extracted from the condensed to the gas phase. These direct ion sources exist under two types: liquid-phase ion sources and solid-state ion sources. In liquid-phase ion sources the analyte is in solution. This solution is introduced, by nebulisation, as droplets into the source

where ions are produced at atmospheric pressure and focused into the mass spectrometer through some vacuum pumping stages. Electrospray, atmospheric pressure chemical ionization and atmospheric pressure photoionization sources correspond to this type. In solid-state ion sources, the analyte is in a non-volatile deposit. It is obtained by various preparation methods which frequently involve the introduction of a matrix that can be either a solid or a viscous fluid. This deposit is then irradiated by energetic particles or photons that desorb ions near the surface of the deposit. These ions can be extracted by an electric field and focused towards the analyser. Matrix-assisted laser desorption, secondary ion mass spectrometry, plasma desorption and field desorption sources all use this strategy to produce ions. In my Ph.D. experience, I used a nanoESI source, due to its capability of high MW molecules ionization, so I will introduce this kind of techniques more in details.

Electrospray

The success of ESI started when Fenn [26][30] showed that multiply charged ions were obtained from

proteins, allowing their molecular weight to be determined with instruments whose mass range is limited to 2000 Da. At the beginning, ESI was considered as an ionization source dedicated to protein analysis. Later on, its use was extended not only to other polymers and biopolymers, but also to the analysis of small polar molecules. It appeared, indeed, that ESI allows reaching very high sensitivity and is easy to couple to high-performance liquid chromatography HPLC, µHPLC or capillary electrophoresis. ESI [31-36] is produced by applying a strong electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a weak flux (normally 1–10 $\mu l*min^{-1}$). The electric field is obtained by applying a potential difference of 3–6 kV between this capillary and the counter-electrode, separated by 0.3–2 cm, producing electric fields of the order of $106 Vm^{-1}$. This field induces a charge accumulation at the liquid surface located at the end of the capillary, which will break to form highly charged droplets. A gas injected coaxially at a low flow rate allows the dispersion of the spray to be limited in space. These droplets then pass either through a curtain of heated inert gas, most often

nitrogen, or through a heated capillary to remove the last solvent molecules.

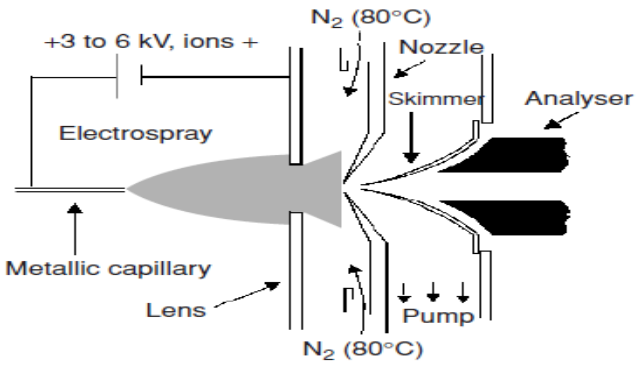

Fig 10: Diagram of electrospray sources, using skimmers for ion focalization and a curtain of heated nitrogen gas for desolvation (top), or with a heated capillary for desolvation (bottom).

Mass spectrometry : principles and applications. – 3rd ed. Edmond de Hoffmann, Vincent Stroobant.

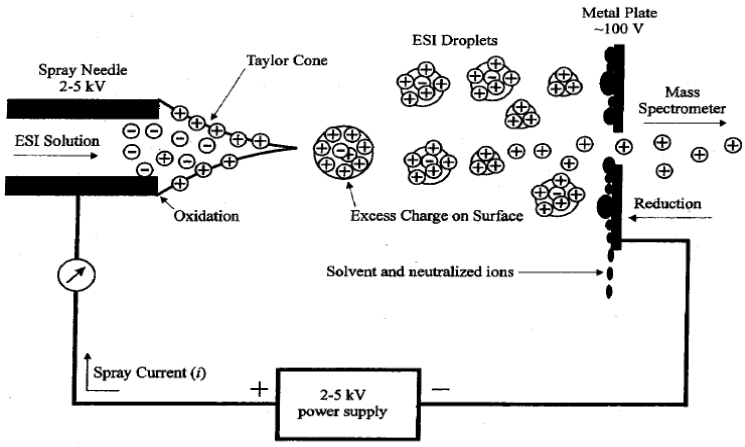

Fig 11: schematic representation of ESI source

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Edmond de Hoffmann, Vincent Stroobant.

Aim of the Project

The work in my project was focused on the quantitative detection of a recombinant protein or its derived peptide in biological matrices like serum and synovial fluid. Due to the fact that this protein is also present in native form, the FDA required us a method that should be orthogonal from ELISA and should be able to distinguish the native form from the recombinant form. These methods should allow us to verify and confirm the validated ELISA method for the detection of this protein.

The research activity on this project was focused on several targets:

- Reaching of the instrumental sensitivity in buffer
- Development of a valid digestion method, if necessary
- Development of a valid purification method

- Ability to distinguish the native from the recombinant form
- Capability of the Internal standard to follow all the analytical procedure
- Ability to validate the process

Materials and Methods

The protein used for quantification is provided internally, already purified in PBS.

All other reagents were bought from commercial font and were used as we received them.

The mass spectrometry analysis was performed on a Thermo Fisher Orbitrap LTQ XL, with an omni-source provided by Prosolia, coupled with an HPLC Ultimate 3000, provided by Dionex.

The second instrument present is a Thermo Fisher Deca-XP coupled with a Surveyor HPLC provided both by thermo.

All the pipelines necessary for connection made by Peak were provided by Upchurch scientific.

The Centrifuge used during our operation is a refrigerated centrifuge 5417R provided by Eppendorf.

All the reactions and the incubations were carried out on the Eppendorf Thermomixer Confort, with 1.5 mL Block.

All the Syringes used for direct MS infusion were Gastight syringe 250 uL volume, provided by Hamilton & Co.

The solid phase digestion was operated by DigesTips Trypsin 100 uL, provided by ProteogenBio.

The regression analyses were performed on Graphad and Curve Expert Basic.

For Whole protein method development was used the following columns

Num.	Model	Phase	Size	Pore	Lenght	I.D.	pH Range
17	BioBasic	C4	5 µm	300 Å	50 mm	0.18 mm	2 - 8
21	BioBasic	C8	5 µm	300 Å	50 mm	0.18 mm	2 - 8
38	Hypersil GOLD	RP	3 µm	175 Å	150 mm	0.18 mm	2 - 8
6	Jupiter	C4	5 µm	300 Å	150 mm	2.0 mm	2 - 8
40	Bio Wide Pore	C5	3 µm	300 Å	100 mm	0.18 mm	2-7.5
43	Jupiter	C4	5 µm	300 Å	50 mm	0.3 mm	2 - 8
44	Jupiter	C4	5 µm	300 Å	50 mm	0.3 mm	2 - 8
45	Jupiter	C4	5 µm	300 Å	150 mm	0.3 mm	2 - 8
9	BioBasic	C18	5 µm	300 Å	50 mm	0.18 mm	2 - 8
11	BioBasic	C18	5 µm	300 Å	150 mm	0.18 mm	2 - 8
52	Jupiter	C4	5 µm	300 Å	50 mm	0.3 mm	2 - 8
11	BioBasic	C18	5 µm	300 Å	150 mm	0.18 mm	2 - 8

For the C-Term method development was used the following columns

Num.	Model	Phase	Size	Pore	Lenght	I.D.	pH Range
51	Ascentis	C8	2.7 µm	90 Å	50 mm	0.2 mm	2 - 9
57	Ascentis Express Peptide	C18	2.7 µm	160 Å	50 mm	0.2 mm	1 - 8

For the N-Term method development was used the following columns

Num.	Model	Phase	Size	Pore	Lenght	I.D.	pH Range
57	Ascentis Express Peptide	C18	2.7 µm	160 Å	50 mm	0.2 mm	1 - 8
1	Magic Acclaim	C18AQ	3 µm	N.A.	50 mm	0.2 mm	
2	PepMap 100	C18	3 µm	100 Å	150 mm	0.3 mm	2.5 - 8
36	Hypersil GOLD	RP	3 µm	175 Å	150 mm	0.18 mm	2 - 8
37	Hypersil GOLD	RP	3 µm	175 Å	150 mm	0.18 mm	2 - 8
59	Luna	C8(2)	3 µm	100 Å	150 mm	0.3 mm	2 - 8
60	Luna	C8(2)	3 µm	100 Å	150 mm	0.3 mm	2 - 8
76	Luna	C8(2)	5 µm	100 Å	250 mm	0.32 mm	2 - 8
77	Luna	C8(2)	5 µm	100 Å	150 mm	0.3 mm	2 - 8
78	Jupiter	C4	5 µm	300 Å	50 mm	0.3 mm	2 - 8
53	Luna	C8(2)	3 µm	100 Å	50 mm	0.3 mm	2 - 8
54	Jupiter	C4	5 µm	300 Å	50 mm	0.3 mm	2 - 8
47	Atlantis	C18	3 µm	100 Å	100 mm	0.075 mm	
33	Hypersil GOLD	RP	3 µm	175 Å	50 mm	0.32 mm	2 - 8
83	Luna	C8(2)	3 µm	100 Å	150 mm	0.3 mm	2 - 8

Acronyms

AA	→ Amino Acids
Ab	→ Antibody
ACN	→ Acetonitrile
BSA	→ Bovine Serum Albumin
CID	→ Collision Induced Dissociation
CMC	→ Critical Micellar Concentration
CV	→ Cross Validation
DOE	→ Design Of Experiment
ELISA	→ Enzyme Linked Immuno-Sorbent Assay
ESI	→ Electro Spray Ionization
ETD	→ Electron Transfert Dissociation
FWHM	→ Full Width at Half Maximum
GPC	→ Gel Permeation Chromatography
HFBA	→ Hepta Fluor Butirric Acid
HPLC	→ High Pressure Liquid Chromatography
HRMS	→ High Resolution Mass Spectrometry
ID	→ Internal Diameter
IP	→ Isoelectrical Point
i-PrOH	→ 2-Propanol
KDa	→ Kilo Dalton
LOQ	→ Limit of Quantification
MeOH	→ Methanol

MIM	→ Multiple Ion Monitoring
mM	→ Milli Molar
MoAb	→ Monoclonal Antibodies
MS	→ Mass Spectrometry
MSn	→Mass Spectrometry major than 1st order
ON	→ OverNight
PBS	→ Phosphate buffered solution
PFPA	→ Penta Fluor Propanoic Acid
PoAb	→ Policlonal Antibodies
PTMs	→Post Translational Modifications
RIC	→ Reconstructed Ion Chromatogram
RT	→ Room Temperature
SCX	→ Strong Cationic Exchange
SDS	→ Sodium Dodecil Sulfate
SRM	→ Single Reaction Monitoring
TDHF	→ Tri Decafluor Heptanoic Acid
TFA	→ Trifluoroacetic Acid
TIC	→Total Ion Current
UV	→ Ultra Violet

Results and Discussion

The Whole Protein Approach

In order to get information of our protein, as first step, some direct infusion of the intact recombinant protein were acquired in order to create and improve the tuning file associated to the molecules. The tuning file is machinery setting that optimizes the signal intensity for the chosen molecule; this procedure is usually done to increase the signal related to the molecule of interest.

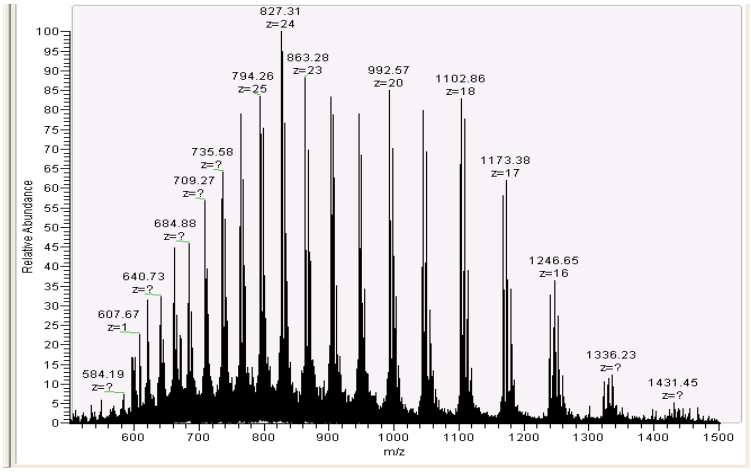


Fig 12: The spectra acquired from the direct infusion

For the intact protein, we obtained several multicharged peaks, because we're using an electrospray source. The ability of recognizing the charge is given us by the high resolution of Orbitrap. Investigating more in details a single charge peak, we can see three different peaks, for a single charge.

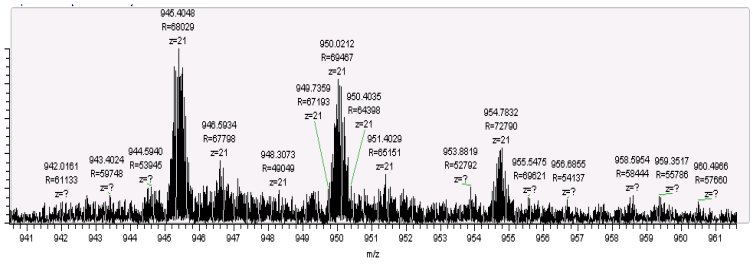


Fig 13: protein peaks with 21 charges

By applying a deconvolution of spectra, the exact difference of masses

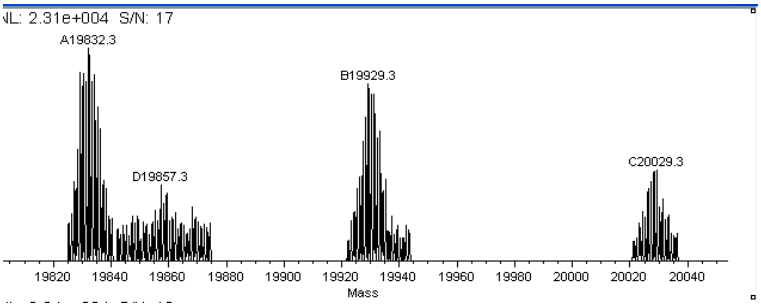


Fig 14: Deconvoluted Infusion of intact protein

Is 97.97Da that perfectly correspond to a phosphate group, so our protein forms some complexes with phosphate groups, due to the fact that the protein is stored at -80°C in a Merck PBS buffer specifically created for this protein created for our protein. After this infusion, the second step of method development was the optimization of chromatography. Unfortunately, due to a breakdown of the HPLC linked to the Orbitrap, the early stage of chromatography development was performed on the Surveyor-LTQ DECA XP system. As first step we took the chromatography developed in production of our recombinant protein and we copied it as it was. The method was performed on a Phenomenex Jupiter C4 column, 150x 2.1mm, 5 µm of particle size and 300 Å of pore size. The flux was set on 200 µl/min and the mobile phase was A= Water +0.1% TFA and B=ACN + 0.1% TFA.

Time (min)	Flow (µl/min)	% of B phase
0	200	27
3	200	27
30	200	45
30.5	200	90

32.5	200	90
33	200	27
40	200	27

Table.01: gradient program for analyses

And in this HPLC system, we have the MS detection, but also the UV detection, so we decided to split the flux and acquire both signals.

As we can see in the spectrum in Fig.16, obtained by a 20 µl injection of a 4.8 mg/mL solution, we obtained a

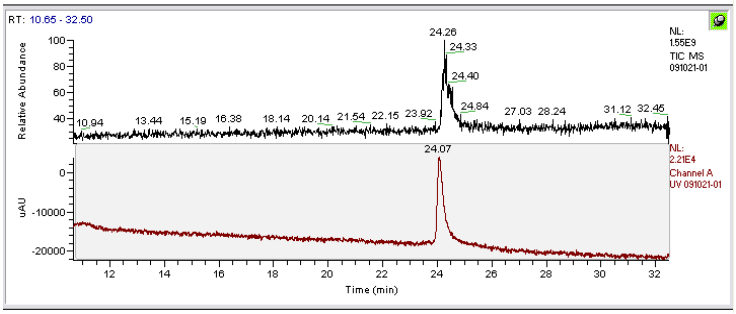


Fig 15: Mass spectrometry and UV spectra

sharp peak around 24 minutes. In the following days we tried to speed up the method, in order to perform more analysis. The UV detection was excluded, because we needed it only for a confirm, but now that the peak has been identified, the MS is a self-sufficient detector. At the end of this development we obtained a shorter

method, whit phase A: Water +5% HCOOH and 0.1% TFA and B= ACN + 5% HCOOH and 0.1% TFA

Time (min)	Flow (µl/min)	% of B phase
0	200	30
5	200	95
6	200	95
6.1	200	30
11	200	30

Table.02: gradient program for analyses

And by comparing 2 replicates of the same injection 20µl of a 4.8 mg/mL solution, the results that we obtained was in acceptance criteria for MS.

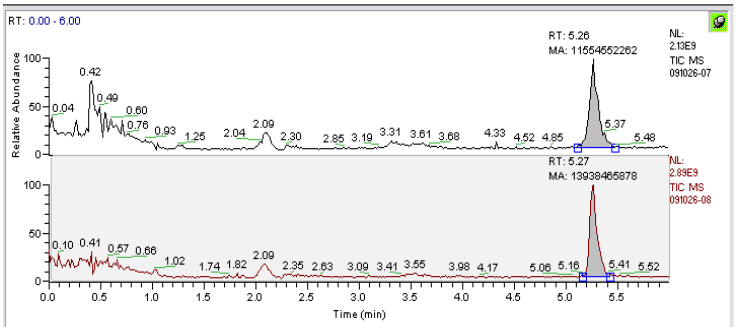


Fig 16: 2 replicates of the same injection solution

Due to the fact that the Ultimate3000-Orbitrap system was finally available, we decided to move the analysis and the chromatography on this system. To replicate the column performance, we used a Thermo-Scientific Biobasic C4 column, 150x0.18mm, 5 µm of particle size and 300 Å of pore size, and with a 4.8µg/mL, we developed the new tuning file, associated with the new mobile phase.

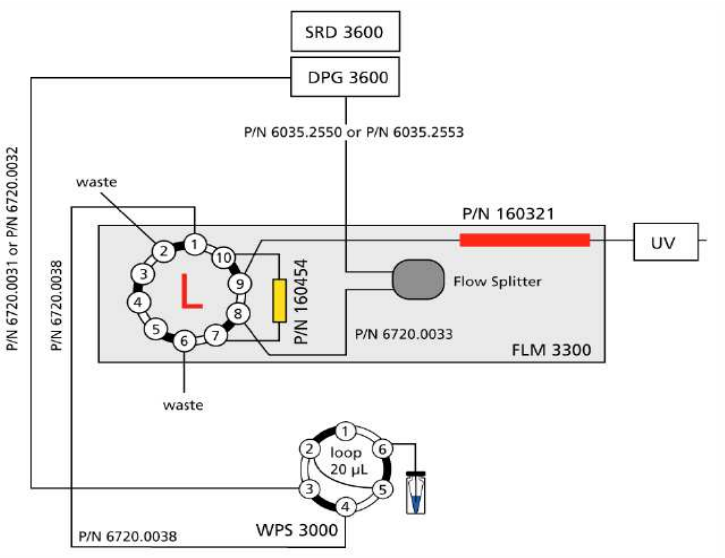


Fig 17: Scheme of internal configuration of Ultimate 3000

As shown by the Fig.17, the ultimate 3000 owns 2 different pumps, one called loading pump and one called micro pump. The system use a splitter, in our case a 1:100 splitter, to provide the micro-flux, for

example, if the flux is 3 µL/min, the pump takes 300 µl/min, and then the splitter throws away the 99% of the solution. The system could be configured with the two pumps connected together or with one pump connected at time. At this early stage of method development, we preferred to use the single pump configuration, working only on micro pump. In Fig.19 after the splitter the flux goes directly in the autosampler valve and then is pushed in the column directly. After the column there is a divert valve and its duty is to manage the column flux, in order to send to waste, the head and the tail of the analysis that very frequently contains several compounds that are non usable with mass spectrometry detection.

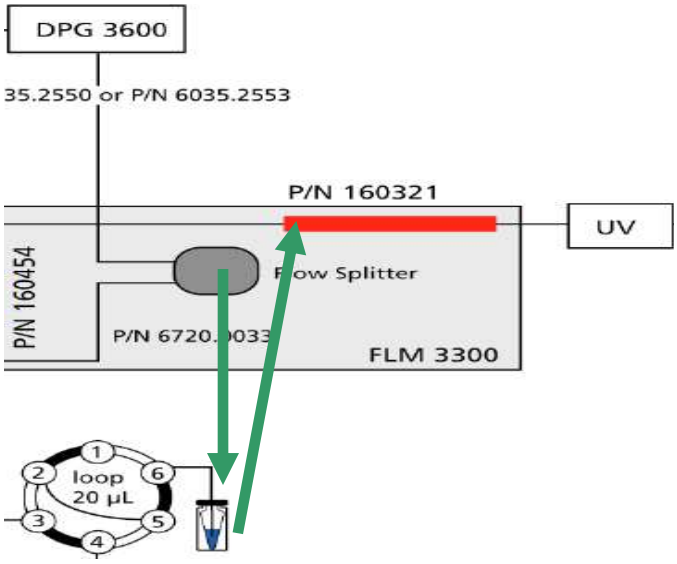


Fig 18: Micro pump alone configuration

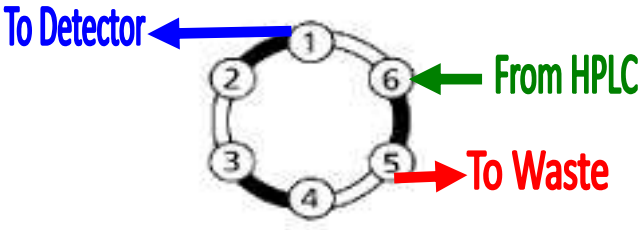


Fig 19: Diver Valve Configuration

Considering that the Orbital trap is 100 times more sensible than the linear trap of the LTQ-Deca-Xp system, we decided to move our trials to a smaller concentration of protein. By decreasing 10 times our concentration, we completely lost our signal in mass spectrometry, and this fact brought us to believe that

we were losing our molecule in the divert valve time, so we connected directly the column to the detector, this was possible because we were operating in buffer, and we changed the injection solution from PBS to HCOOH 0.1% that is fully compatible with mass detection.

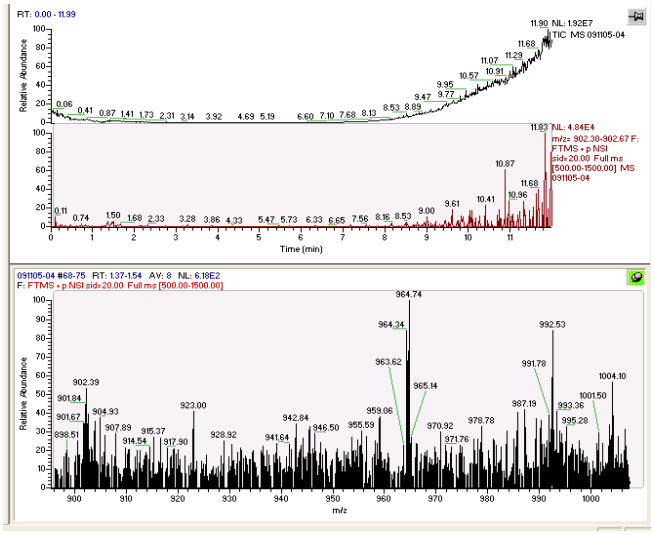


Fig 20: Chromatogram and spectra of no-divert valve analysis.

In this way we obtained a small signal at the beginning of the spectra. In Fact by extraction of our analyte masses (red Chromatogram) we obtained a small peak at 1.5 minutes, so we have a problem of no retention in our column. In order to overcome this problem, we decided to switch the starting % of organic material, to 10%, in order to obtain more interaction between our

protein and the column, what we could observe after this modification is a broad peak at 1.44 minutes and a

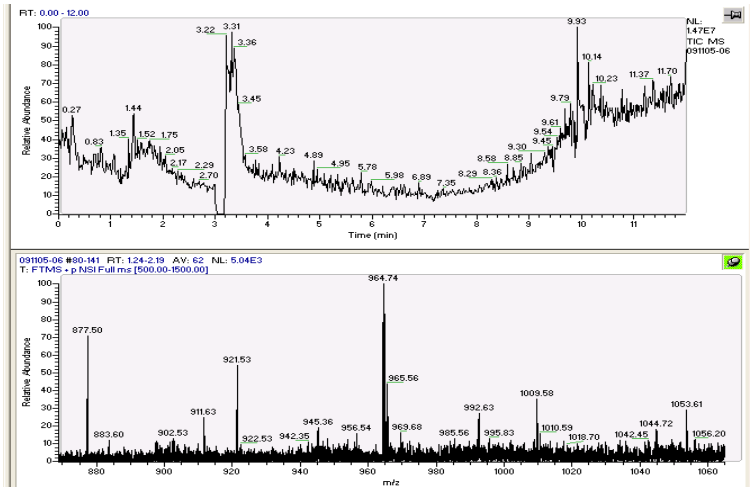


Fig 21: 10% of B phase at the beginning of the analysis

smaller signal in mass spectrometry than expected. In order to be sure to improve the chromatography, the concentration of the injected solution came back to 4.8 µg/mL and this solution was prepared and diluted in 70% of phase A and 30 % of phase B. After this modification, the signal growth to expected, but the chromatogram was still a trouble.

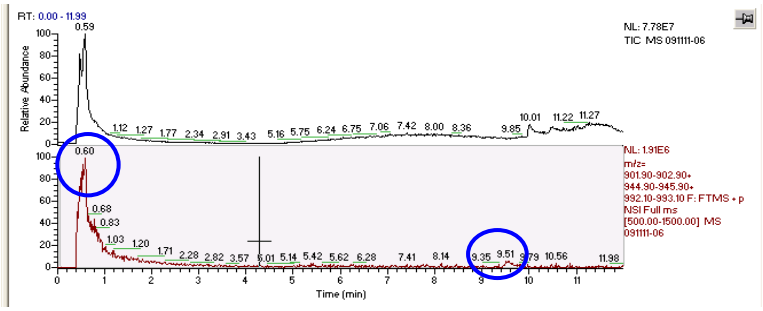
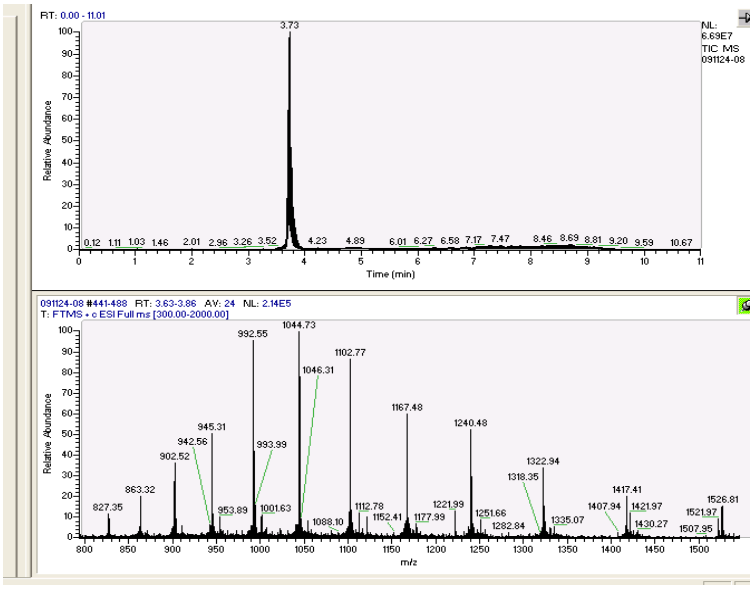


Fig 22: Tic and Ric chromatogram

In fact we had most of our protein that followed the solvent frontline and had a very short retention time and a little amount of our protein that had a good retention time, and a small peak in the RIC chromatogram at 9.51 min. In order to verify if the problem is attributable to the system, to the column or to the analyte, other analyses were performed and the instrument gave good responses, so the system worked perfectly. To bypass this chromatographic failure, we decided to switch our high resolution system in another configuration, from the micro pump alone to the loading pump alone. To perform this change, we had to install on our instrument the ESI source, that substitute the nano-ESI source, in fact, with the micro pump alone configuration, the source was a nano source that perfectly supported the flux of 3 µL/min, but in the new configuration, the flux that perfectly goes with a 2.1

mm ID column is 200 µL/min, and this is only supported by ESI source. So in this way, we used the column that perfectly worked on LTQ-DECA-XP system and we only tried to use the sensitivity increase of the orbital trap. This change in instrumental configuration required the acquisition of a new tuning file, in order to optimize the parameters of this new source configuration. After that, several analyses were acquired to verify the system response. The replicates obtained demonstrated that with this configuration the system is stable and the response is reproducible.



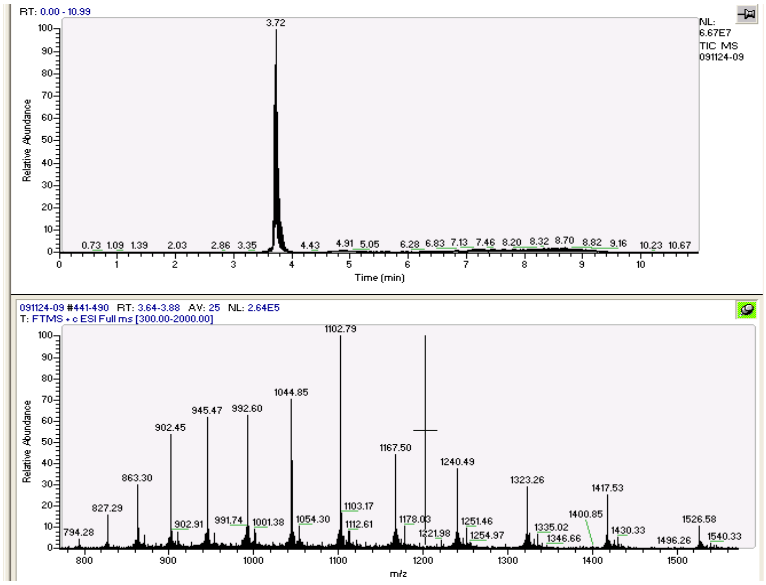


Fig 23: two replicates of protein analysis with ESI source.

After this analysis the cooling system of the Orbitrap-XL broke down and required a long time to be fixed, so we were forced to switch back again to LTQ-DECA-XP system. After this long time, we performed a new tuning optimization and then we verified that this new tuning was really better responsive than the older one. After some analysis on this new system, the LOQ seemed to be around 100 ng/mL and we decided to not perform any improvement in signal, because we were

not in the right LC/MS system, and we decided to start the development of the purification procedure.

Our protein is a very basic protein, with an IP > 9.5 and particularly the 10% of primary sequence are lysines. The procedure that we believe could clean the sample with a great efficacy is the strong cationic exchange. In order to avoid the interference due to the high viscosity of synovial fluid, we decided to use the magnetic beads coated with SCX surface. Magnetic beads are micro iron beads that can be attracted by a magnet. In this way, after the conjugation with the molecule of interest, these beads are immobilized by a magnet and the biological matrix is simply eliminated by pipetting. After the elimination of biological matrix, several wash steps could be performed in order to purify the molecule from other interferences that could bind to the beads. The eluting step could be achieved by increasing pH or ionic strength. As first approach we used the suggested strategy provided by the beads producer. In this case we were using Invitrogen Dynabeads and we prepared a 50mM solution of NaH_2PO_4 1M in NaCl and 1 solution 50mM in NaH_2PO_4 ,10mM in NaCl. The pH of these two solutions was

adjusted at 8. For the procedure we also prepared an acetic acid solution at pH=5, a PBS solution at pH=7 and an eluting solution of ammonium acetate at pH 9.5. We took 40 µL of Dynabeads SCX solution, this solution was placed on a vial and mixed for 2 minutes, and then the solution was placed on the magnet and discarded from the beads. The procedure was repeated 3 times, using the solution at high ionic strength (50mM NaH_2PO_4 1M NaCl), this step is called Pre-Charging wash. After this, the procedure was repeated 3 times again with the low ionic strength solution (50mM in NaH_2PO_4 ,10mM NaCl). At this point the beads were ready to capture our protein, so we put 200 µL, of our protein at 4.8 µg/mL in Buffer solution, in contact with the beads. Then the vial was kept under stirring for 30 minutes, in order to realize the binding. To wash the beads after the charge procedure, 3 more washes at low ionic strength were performed to clean the beads. After this step, we started the eluting procedure, with a pH gradient step; we did an acidic elution, with acetic acid solution, a neutral elution, with PBS and a basic solution with ammonium acetate. Because this was the

first time that we were performing this separation, we collected:

- The charging solution
- The 3 washing solution
- The acidic elution
- The neutral elution
- The basic solution

In these collected samples we would be able to identify where our protein is eluted and we would be able to understand if this procedure is suitable for our protein or needs some modification.

Some standard solutions alone or in ammonium acetate were acquired before the analysis of the purified sample, in order to verify whether the response of the instrument doesn't change after a change of the diluting solution. After the analysis, there was no protein in every acquisition performed, so we thought that the problem could be the pH at which the sample is eluted, because the solution of ammonium acetate is at pH 9.5, that should be enough to elute the protein, but it's still under the IP of the protein, and so the protein is still positively charged.

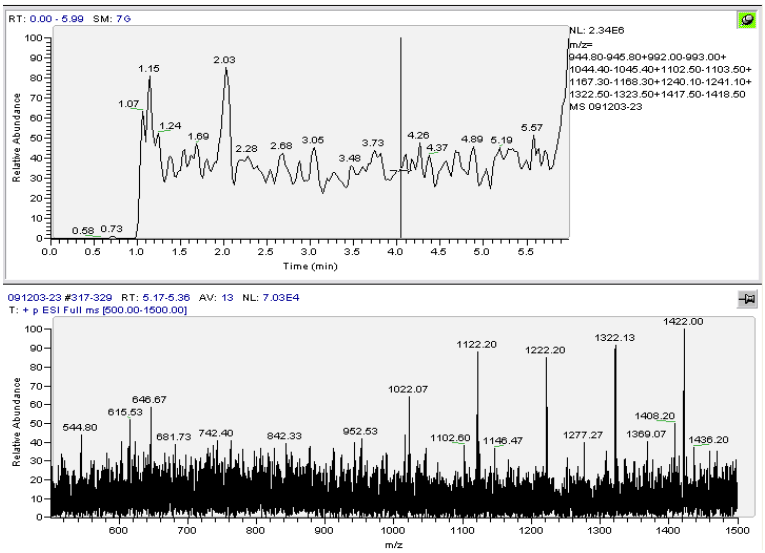


Fig 24: RIC of basic elution, no peaks present in the chromatogram could be associated with our protein

For this reason, we took a 12.5M NaOH solution, and we diluted it 1:10/1:5/1:2/1:1 with methanol, in order to provide also an amount of organic that could help the procedure. We repeated the same procedure performed previously, but the results were the same, so no trace of protein was observed in the elution chromatogram. So as we saw that working on pH gradient did not work, we switched to the second approach suggested by Invitrogen, and so we tried to elute our protein by increasing the ionic strength. We prepared different solutions starting from the 50mM

NaH_2PO_4 1M NaCl, we prepared 500mM, 250mM, 200mM, 150mM and 100mM in NaCl. With this new prepared solution, we performed the purification procedure. We did 3 eluting steps for every solution, and we added a final elution step with 200 µL of 50mM NaH_2PO_4 1M NaCl. All the eluting steps were acquired but even in this case, no signal of protein was present. During this analysis we observed that our protein was not stable at -20°C, and so we needed to store it a -80°C, where this protein is stable at concentration higher than µg/mL. To overcome the difficulties occurred on this method development, we decided to work in harsher condition and we tried to find the critical step in which we lost our protein. So 6 beads units were prepared and on every "column" we operated different purification procedures.

Process Step	Original Protocol pH	Original protocol Ionic	Protocol 1	Protocol 2	Protocol 3	Protocol 4
Pre-Loading 1	3x 100µL with a solution NaH_2PO_4 0.05M + NaCl 1M pH= 8	3x 100µL with a solution NaH_2PO_4 0.05M + NaCl 1M pH= 8	3x 100µL with a solution NaH_2PO_4 0.05M + NaCl 1M pH= 8	Not performed	Not Performed	3x 100µL with a solution NaH_2PO_4 0.05M + NaCl 1M pH= 8
Pre-Loading 2	3x 100µL with a solution NaH_2PO_4 0.05M + NaCl 0.01M pH= 8	3x 100µL with a solution NaH_2PO_4 0.05M + NaCl 0.01M pH= 8	3x 100µL with a solution NaH_2PO_4 0.05M + NaCl 0.01M pH= 8	3x 100µL with a solution NaH_2PO_4 0.05M + NaCl 0.01M pH= 8	3x 100µL with a solution NaH_2PO_4 0.05M + NaCl 0.01M pH= 8	3x 100µL with a solution TRIS*HCL pH = 8.5 + 0.5M NaCl
Sample loading	200µL of sample	200µL of sample	200µL of sample	200µL of sample	200µL of sample	200µL of sample
Wash	3x 100µL with its pre-loading 2	3x 100µL with its pre-loading 2	3x 100µL with its pre-loading 2	Not Perfomed	Not Performed	3x 100µL with its pre-loading 2
Elution	3x 20µL for pH 4.0/7.0/9.0	3x 20µL with a solution NaH_2PO_4 0.05-0.1 0.25M	10 Steps with 200µL 1:1 solution with NaOH 12.5 M + preloading solution	5+5 steps with 200µL with a solution TRIS*HCL pH = 8.5 + 1M/2M NaCl	10 Steps with 200µL 1:1 solution with NaOH 12.5 M + Preloading solution	10 steps with 200µL with a solution TRIS*HCL pH = 8.5 2M NaCl

Table 03: Working instruction for the 6 SCX analyses

As shown in table 03, the first two columns are the conditions suggested by Invitrogen and we replicated every single step. The column 1 had a strong elution solution that combined both strong pH and strong ionic

strength, the column 2 avoided the pre-charging wash at high ionic strength and the wash after the charging procedure, in order to see if this kind of step could affect the purification, so column 2, is the simplest way to perform this purification. In column number 2 the elution was performed with Tris, which maybe is a better buffer than ammonium acetate for our protein. In column 3 we also avoided the pre-charging wash at high ionic strength and the wash after the charging procedure, but we decided to elute operating with pH and ionic strength both, as the column number 1 in column 4, we eliminated the inorganic salts from the procedure, and we did it all with Tris solution. This procedure should clarify us if it's possible to obtain some results from this purification. What we saw is that the column 4 showed a better behaviour in the separation, and so we could think that maybe this phosphate salts are not the best for our procedure. In the charging solution of column 3, we had some trace of our protein

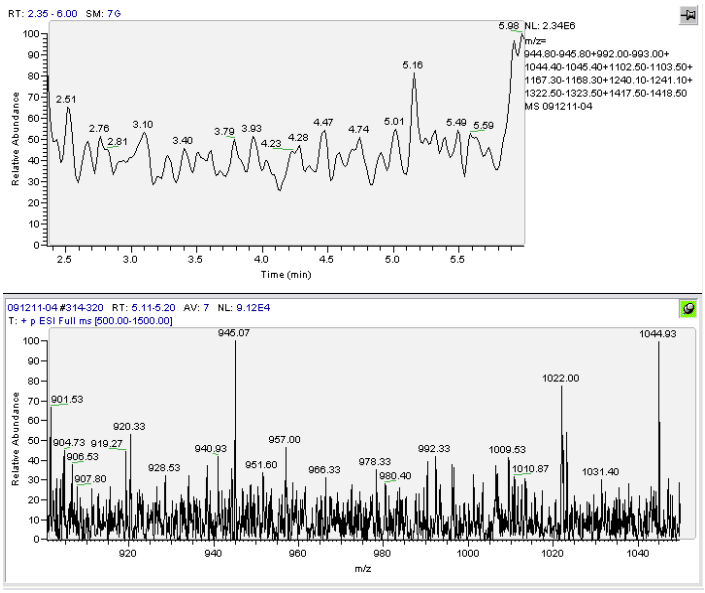


Fig 25: Chromatogram of Charging solution column 3

So this chromatogram clarified us that if the protein is lost somewhere during the procedure, we would be able to target and recognize it. The fact that we didn't see anything before, point us the fact that our protein binds too strongly to the SCX beads, for its high positive charge, so we are not able to break this bond and we can't elute it, so we must move to another purification procedure.

We decided to stay on magnetic beads technology, because we need it for the kind of biological matrix that we're using, so we decided to use the

immunoaffinity procedure to purify our protein from the biological matrix. This procedure wasn't took into account before, because we tried to use a completely orthogonal method from ELISA, in this way we have to use the same Ab from ELISA, but we still also have a different kind of detection, that is physical and the orthogonality is still retained.

This kind of approach is developed by the use of Dynabeads M-280 streptavidin, so we took the monoclonal Ab used for the ELISA assay and we performed a biotinilation of this Ab by EZ-Link Sulfo-NHS Biotinylathion kit, provided by Thermo-Fisher. The steps we followed in order to obtain the final product are:

- Bring MoAb solution to 1 mg/mL concentration by a PBS dilution.
- Prepare the biotin solution with the concentration of 5.5 mg/mL of biotin
- Add 3.5 µL of biotin solution for every200 µL of MoAb solution
- 1h incubation at 22°C with shaking
- Purification on GPC column Zeba Spin MWCO 7500

- 3 washes: centrifugate at 1000g for 5 minutes with 2.5 mL of PBS pH 7.2
- Sample charge: if the volume of sample charged is less than 1mL, you have to add 500 µL of water

The solution obtained in this way still needs to be concentrated for our experiments, so we decided to perform a 1:10 dilution with the same PBS solution used to wash the column.

As suggested by the producer we charged the biotinilated Ab on the streptavidin bead following this procedure:

- Elimination of the starting stock solution of beads
- 3 washes with PBS pH7.2
- 200 µL of beads are charged with 200 µL of biotinilated Ab solution
- The incubation is performed for 30 minutes at room temperature
- 5 Washes with PBS pH 7.2 +0.1% BSA

The charged beads aren't usable directly: they must be used after at least 12 hours, and the optimal window of usage goes from 24 to 48 h.

Our protein is charged to the beads with this procedure:

- The temporary stocking solution is eliminated
- Our protein is charged on beads and incubated for 2h at 37°C
- 3 washes with PBS pH 7.2 in order to clean the sample
- 3-5 Elution with 200 µL of 0.1M citric acid pH 3.0

At the beginning of this trial, all the experiments were performed with our protein in buffer solution. For the first analysis we decided to use a buffered solution of our protein at 4.8 µg/mL. For the first time all the steps were acquired, in order to see if we were losing our protein somewhere during the wash or the charge steps, but these chromatograms were clean, so we were sure that our protein was not lost. As show in Fig.26 in the 1st elution chromatogram, we were able to see a peak around 5 minutes, that is comparable with the one obtained by the injection of a standard

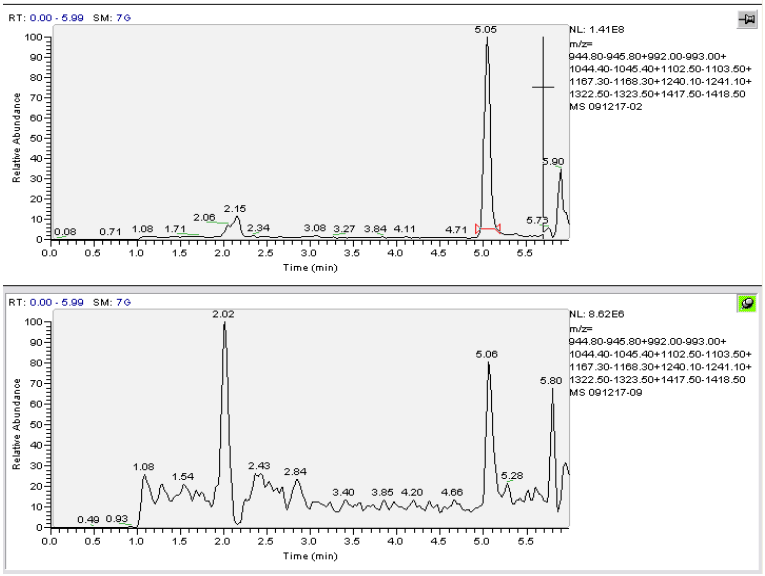
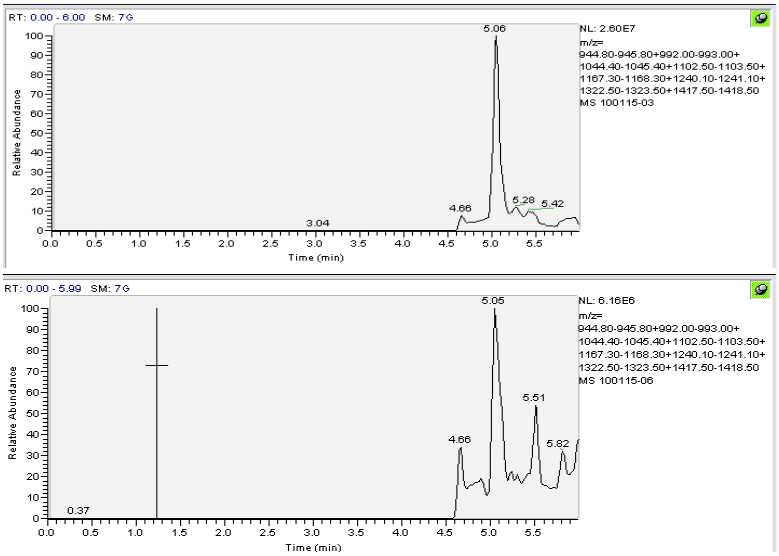


Fig 26: in the 091217-02 file the standard solution chromatogram
in the 091217-09 file the extracted solution chromatogram

solution, the two spectra under this peaks are comparable, and so we're sure that the extraction worked well .In the following 4 others elution steps acquisitions, the peaks of the protein was still present and the area fell near to zero after the third elution step. By a comparison between the injected standard and the peaks obtained with our extraction, the recovery was around 5%, so the procedures needed to be optimized, but we were moving forward comparing to the SCX procedure where we weren't able to obtain

any results. Some tests were carried out to see if the beads could be used for multiple times, but the results were not conclusive and we decided to prepare them fresh for each new extraction. To improve the extraction rate, we decided to increase the acidity of the extracting solution, by adding HCL 37% to the citric acid solution and with this addition the pH was 1.93. To standardize this procedure, we saw that 40 µL of 37% HCl brings 5 mL of 0.1 M citric acid to the desired pH. By this new solution, extractions were repeated, and we had an improvement in results.



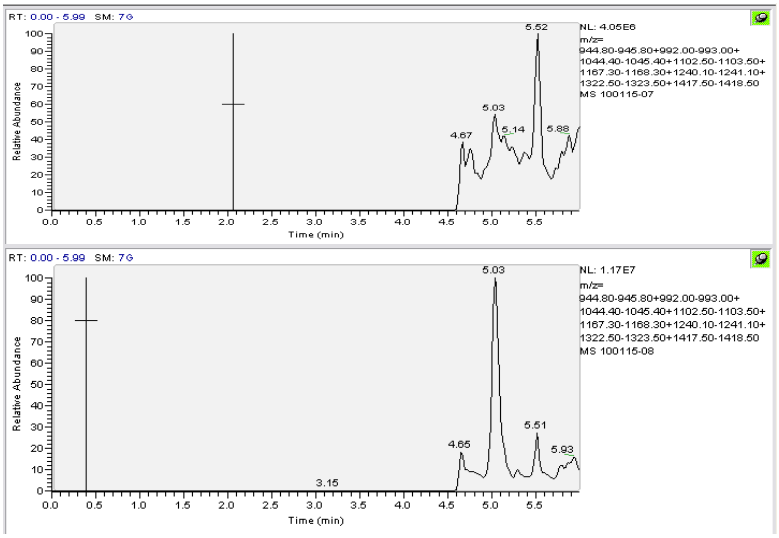


Fig 27: in the 100115-03 file the standard solution chromatogram
in the 100115-06 the first wash step in the 100115-07
the second wash step in the 100115-08 the first elution step

Strangely, this time, we lost some protein in the wash steps, but the 1st chromatogram shows a great improvement in extraction. By a simple calculation we had 23% in the first wash step, 3% in the second wash step, 49% of the 1st elution step and 1.5% in the 2nd elution step, so the total recovery for this procedure is 76%. The more acidic solution provided us a more efficient elution, but we had this strange phenomenon of protein lost in the washes that will need more investigation. After these promising results, we decided

to push on the sensitivity and verify if it's possible to go 10 times down in concentration. To realize this, we newly prepared the beads, but this time, we took 20 µL of protein solution instead of 200 µL but no signal was observed at this concentration. Maybe this was due to the fact that 0.1% of BSA is a too high concentration for this trial and maybe BSA blocks all the sites available for our protein. So our next step was to reduce the amount of BSA from 0.1 % to 0.01%. The procedure of extraction was replicated again, with this new experimental condition, but no trace of protein was observed again. Since this type of approach currently did not allow us to go below 480 ng/mL, we decided to change the linker on the magnetic beads and we tried to use the protein-G as catcher to see if this kind of approach could give us better results. Beads coated with Protein-G are provided by Invitrogen, and the protocol is really similar to the previous:

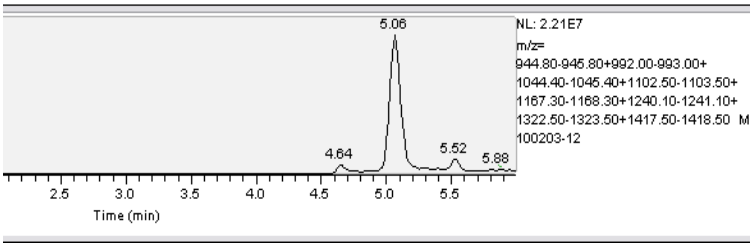
- 200 uL beads are stirred and the stock solution is eliminated
- The Ab is added and incubated 10 min r.t.
- The beads are washed 3 times with PBS pH 7.2 + Tween20 and stored at 4°C

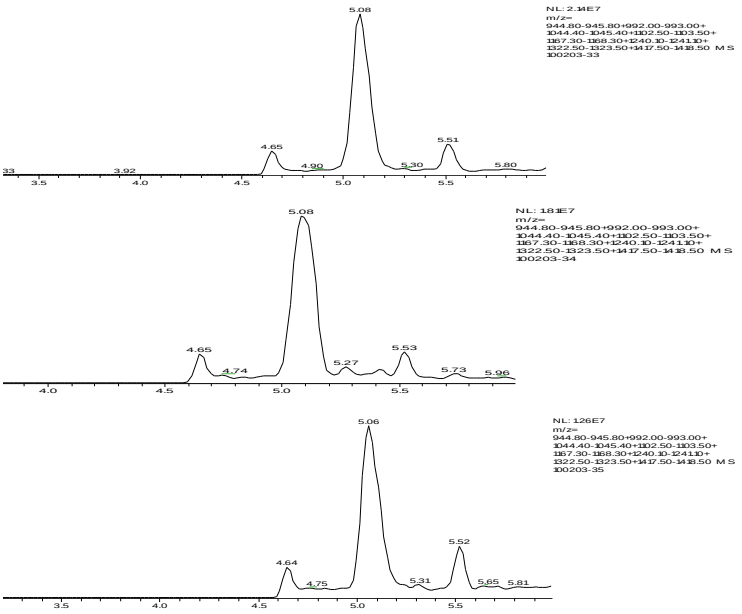
- The protein is added and incubated 2h 37°C
- 3 washes with PBS pH 7.2 in order to clean the sample
- 3 Elution with 200 µL of 0.1M citric acid pH 1.9

To perform the experiment we decided to go back to the previous higher concentration to continue the method development. We bought Streptavidin magnetic beads from another company, Simag Streptavidin, provided by Chemicell. So we decided to compare these two different magnetic beads in order to find the best solution for us. For this new kind of beads the protocol is almost the same, the only change from Dynabeads to Chemicell is the Washing solution used before the Ab binding, that in this case is 10mM TRIS*HCl pH8 150mM in NaCl.

All these samples were extracted and compared to the standard Invitrogen magnetic beads. By the comparison of standard peak versus the peaks obtained with all these elutions, we could easily see that the Chemicell technology seems to be more performing than the Dynabeads coated with either streptavidin or protein-G. In the table 04 is easy to see

that the Chemicell beads gave us around 100% of recovery by means, that is an impressive results, compared with Dynabeads, that gave us around 80% of recovery as best results.





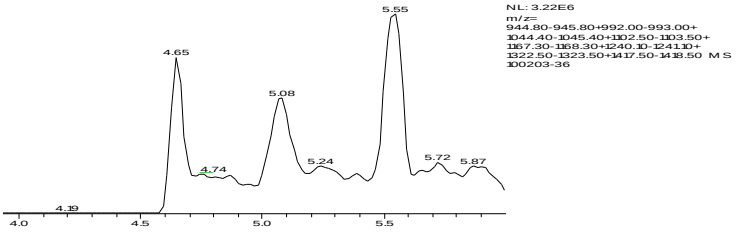


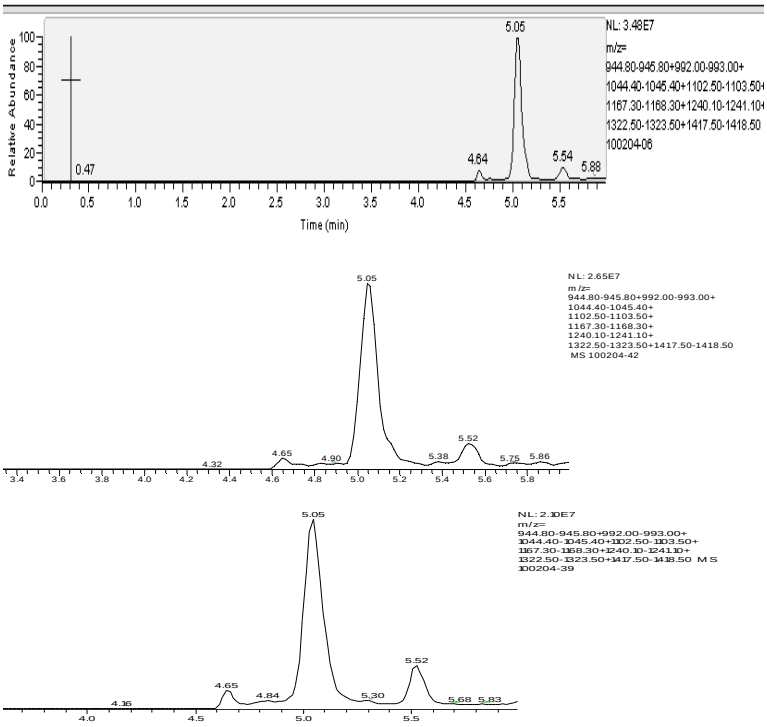
Fig 28: in file number 100203-12 the standard solution injection, in file number 100203-33 1st elution with Chemicell, in file number 100203-34 a replicate of elution with Chemicell, in file number 100203-35 the elution with Dynabeads and in file number 100203-36 the elution obtained by Protein-G technology

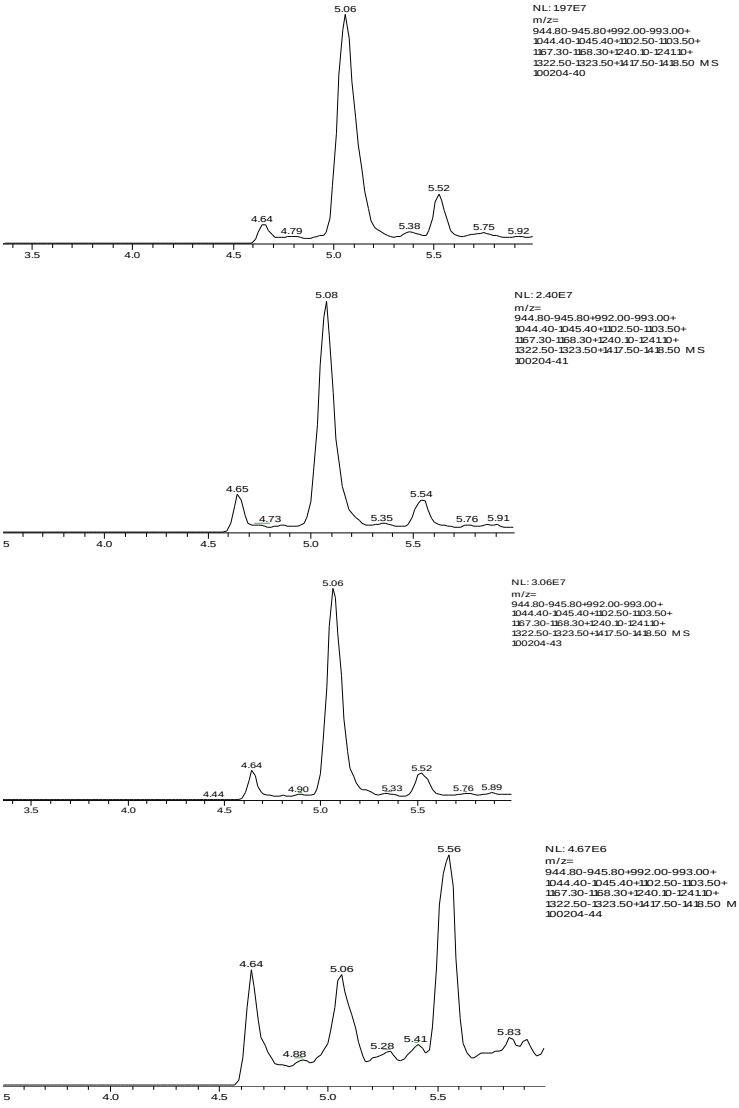
Acquisition	Area	% of Recovery
Standard	123606003	
Chemicell 1	1255999719	101.6%
Chemicell 2	117694653	95.2%
Dynabeads	75762549	61.2%
Protein-G	72446711	5.8%

Tab.04: % of recovery for the acquisition file

In order to verify if these are the real performance of our technologies, the experiment was repeated. In this new experiment 3 replicates of Dynabeads-Streptavidin were used a control of reproducibility, one trial was made with Chemicell, to see if the performance obtained is reproducible and 2 experiments were

performed on Protein-G beads, with two kind of elutions, one with Citric acid, as streptavidin beads and one with 0.5 Glycin pH 2.8 as suggested by the producer. One more experiment was conducted, in fact; we tried to use Dynabeads streptavidin, prepared 72h before the extraction, an experiment that was never tried before.




NL: 1.97E7
m/z=
944.80-945.80+992.00-993.00+
1044.40-1045.40+1102.50-1103.50+
1167.30-1168.30+1240.10-1241.10+
1322.50-1323.50+1417.50-1418.50 MS
100204-40
5.06
5.52
4.64
4.79
5.38
5.75
5.92
3.5
4.0
4.5
5.0
5.5
NL: 2.40E7
m/z=
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1044.40-1045.40+1102.50-1103.50+
1167.30-1168.30+1240.10-1241.10+
1322.50-1323.50+1417.50-1418.50 MS
100204-41
5.08
4.65
4.73
5.35
5.54
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5.91
5
4.0
4.5
5.0
5.5
NL: 3.06E7
m/z=
944.80-945.80+992.00-993.00+
1044.40-1045.40+1102.50-1103.50+
1167.30-1168.30+1240.10-1241.10+
1322.50-1323.50+1417.50-1418.50 MS
100204-43
5.06
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4.44
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5.76
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4.5
5.0
5.5
NL: 4.67E6
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1167.30-1168.30+1240.10-1241.10+
1322.50-1323.50+1417.50-1418.50 MS
100204-44
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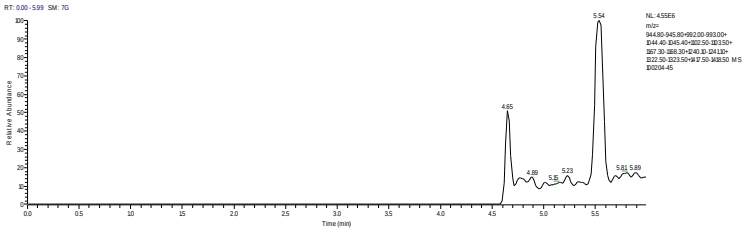


Fig 29: in file number 100204-06 the standard, in file 100204-39/40/41 the Dynabeads streptavidin triplicate, in file 100204-43 the Chemicell elution, in file 100204-42 the 72h Dynabeads streptavidin , in file 100204-44 the Protein-G elution with citric acid and in 100204-45 the protein G elution with Glycin pH 2.8

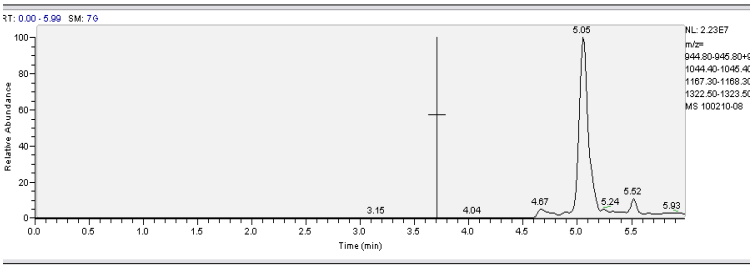
Acquisition	Area	% of Recovery
Standard	192874022	
Invitrogen 1	125142294	64.88%
Invitrogen 2	122644994	63.58%
Invitrogen 3	135478689	70.2%
Chemicell	171571475	88.0%
Invitrogen 72h	153617882	79.5%
Protein-G Glycin	0	0%
Protein-G Citric Acid	10353105	5.36%

Tab 05: % of recovery for the acquisition file

As showed in table 05, we could easily see that Chemicell still retained the better performance, and

Dynabeads streptavidin were very reproducible, in fact, they had a spread area of 10.5 % and a CV of 5%, so a precision of 95%. Protein G clearly appeared as a technology that could not work well in this case, and so it was left.

The major reason for the choice of this technology was the fact that we had to work with synovial fluid that is a very viscous matrix. In order to see if, after this starting screening, these beads were able to work also in our biological matrix, we decided to run a few trials. Due to the fact that at the moment we did not know the volume of biological matrix we would have available to make our extraction, with this analysis we also decided to try a different kind of charge, by adding 1mL of buffer solution instead of 200 µL, but in this case, we kept the total amount of protein constant, so we added 1ml of a 1:5 diluted solution, so we were able to obtain the same recovery and to easily check it.



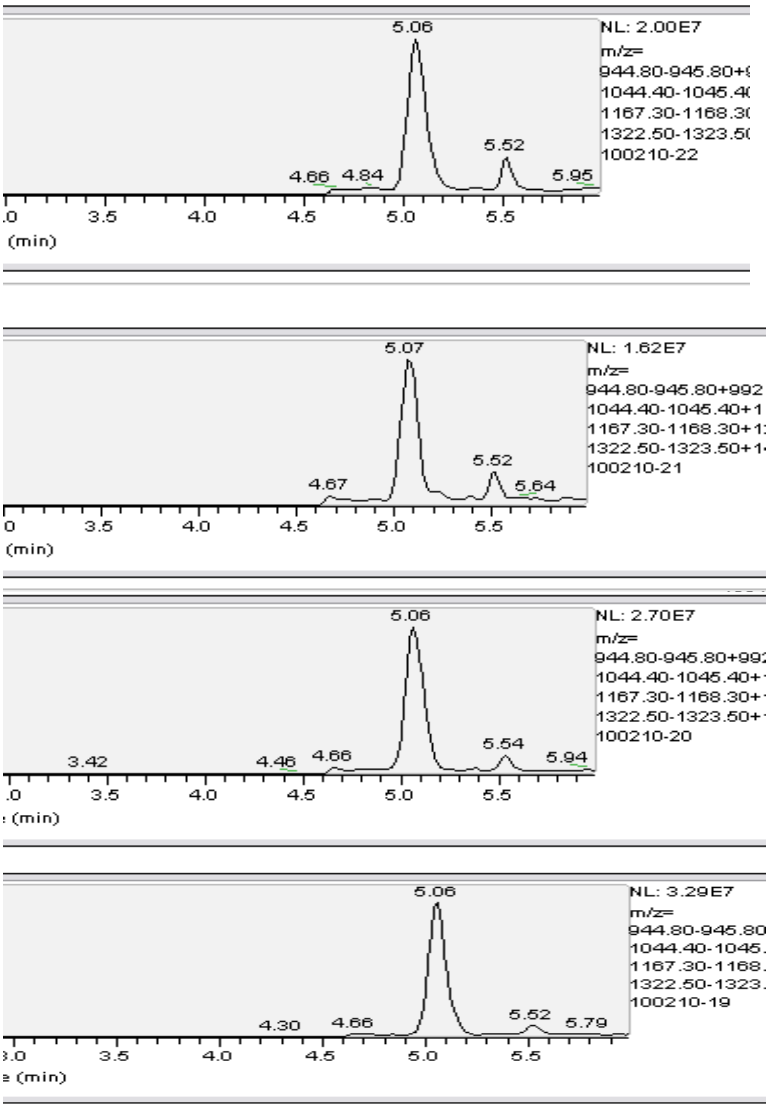


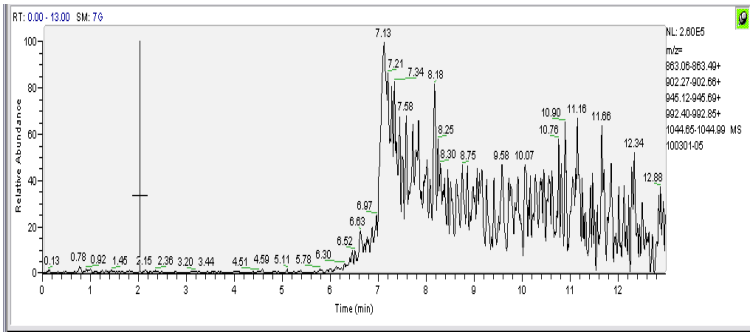
Fig 30: in file number 100210-08 the standard, in file number 100210-21/22 the two capture from diluted solution, in file number 100210-19/20 the two capture from synovial fluid.

Acquisition	Area	% of Recovery
Standard	125234618	
Synovial 1	199650697	159%
Synovial 2	170861692	136%
High Volume 1	98238715	78.3%
High Volume 2	127074694	101.4%

Tab 06: % of recovery for the acquisition file

As seen by Tab.06, working with synovial fluid clearly shows an advantage, in-fact, it seems that there's a positive matrix effect, a phenomenon that happens when the signal obtained from the matrix is higher than the signal obtained by the standard solution in buffer. This could be easily explained by the presence of other proteins which act like scavengers in the vial, and avoid a part of non-specific binding. Considering the high volume capture, it seems that there were no problems, because we had 2 different recoveries, but both were high and one was in line with the previous recovery rate obtained, so this method should be available for several starting amount of biological matrix. We

decided also to verify if the problem in the capture, for Dynabeads, could be the protocol of capture, so we tested some Chemicell beads, prepared with the Dynabeads preparing procedure. The results obtained clearly showed us that we obtained a 20% less when we applied the Dynabeads protocol. When the first amount of biotinilated MoAb was near to the end, we decided to compare it with a new amount, to see if our biotinilation procedure is standardized and it can affect our experiments, but the procedure seemed to be reproducible. After this first stage of development, the Orbitrap system returned to be available and so we moved again on the high resolution mass spectrometer. At the beginning we optimized the molecule by direct infusion, in order to obtain a signal improvement, and then, with this new tuning file, we tried to screen 2 columns, HRMS-17 and HRMS-40 in order to replicate the optimal chromatography that we had in Deca-XP system. In these acquisitions, Fig.31, is easy to see that the starting condition appeared really worse



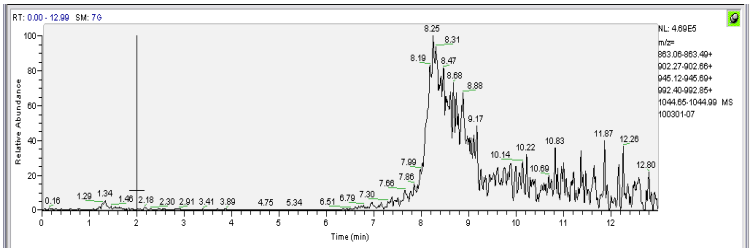
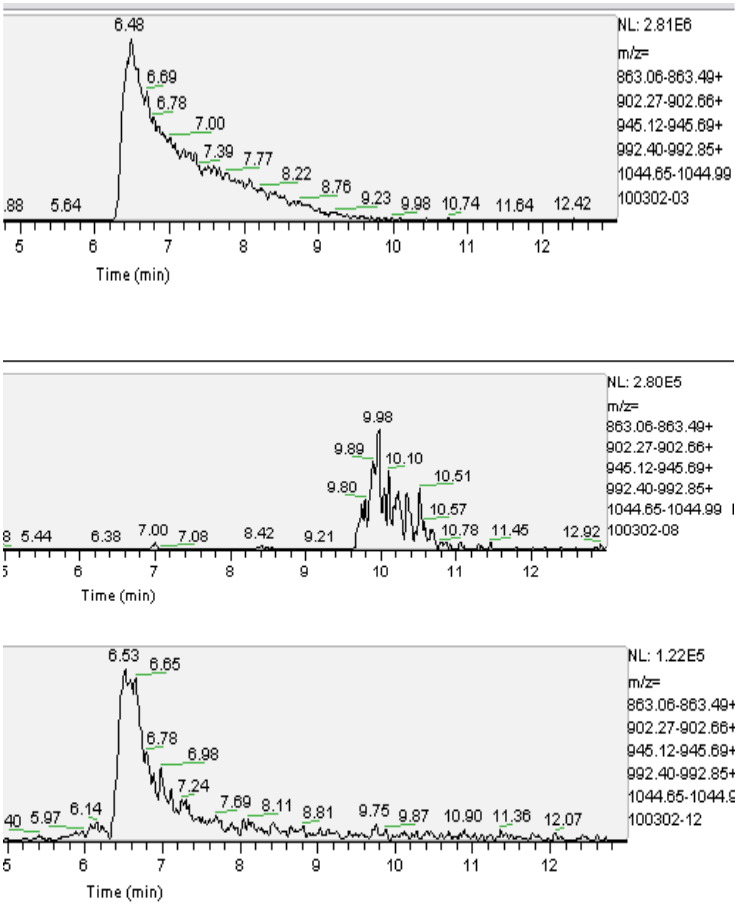


Fig 31: in file number 100301-05 the Peak obtained with HRMS-17 in the second the one obtained with HRMS-40

The spectra obtained for the two peaks clearly showed the presence of the protein of our interest, but at the moment, the chromatography was really bad, due to the shape and the length of the peaks obtained. There was clearly a huge problem when we switched from the normal chromatography to the micro chromatography. The mobile phase developed in the

previous chromatographic experience was not as good as in normal chromatography, so we decided to start the mobile phase development again. We tested the two columns with 0.1% of HCOOH and TFA separately in order to find the best starting condition.



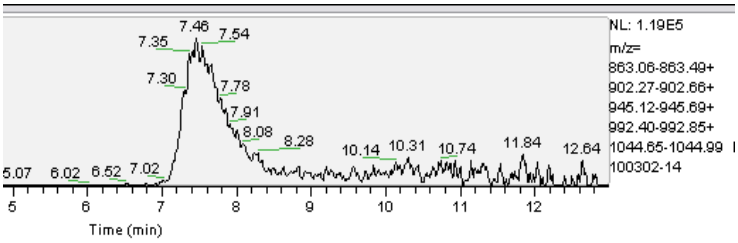


Fig 32: 100302-03 HRMS-40+0.1% HCOOH, 100302-08 HRMS-17 +0.1% HCOOH, 100302-12 HRMS-17 +0.1% TFA, 100302-14 HRMS-40+0.1% TFA

Actually, we could see that with the TFA, the chromatography peaks resulted in a better shape, so the possible cause could be the ionic couple, a different kind of chromatography in which a molecule binds by electrostatic interaction the analyte and the chromatography and the retention time is given by the molecules bound and not from the starting one. The addition of this kind of acid gave to the chromatography a better peak shape, but also a reduction of area intensity. This kind of reduction is modulated by the length of the carbon backbone, more length-less reduction could be an easy explanation of this phenomenon. According to this rule, we decided to take a longer backbone and see, with the same chromatographic condition, if there was any improvement in signal. The test was still conducted on

the two column elected for the analysis. After the first trials on HRMS-40, we had some column pressure problems, so we decided to work only with HRMS-17, and after a starting % of 0.1%, we found a better intensity of peaks by reducing 10 times the percentage of this acid in the mobile phase.

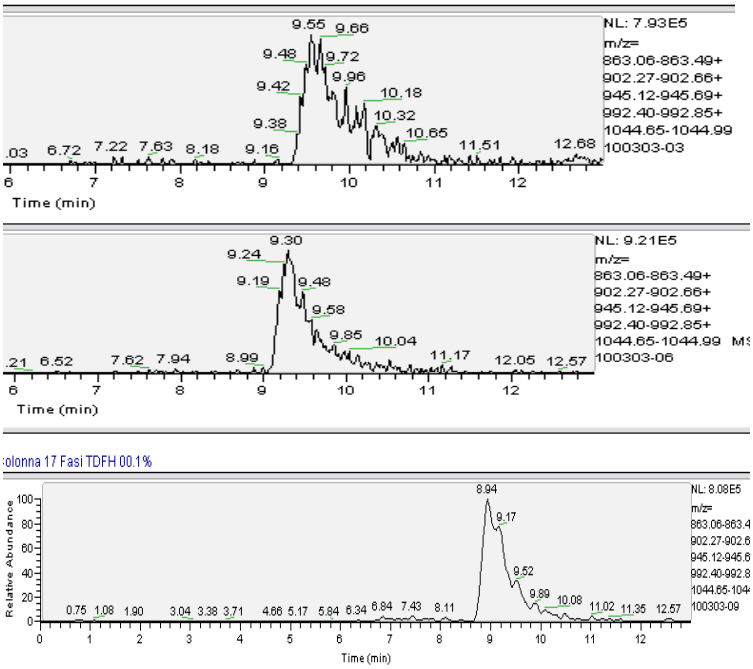
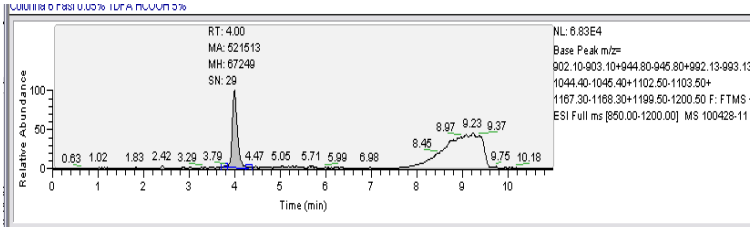


Fig 33: in file 100303-03 HRMS-40 with 0.1% of TDFH, in file 100303-06 HRMS-17 with 0.1% of TDFH and in 100303-09 HRMS-17 with 0.01% of TDFH

In order to find the optimal condition and to have a great improvement in signal, we tried to apply a design

of experiment on HRMS-17, 20 experiments were performed but the peaks were so broad and not constant that we had to switch the HPLC system on the solo loading configuration. We took the column that we were using in the Deca-Xp system and we moved it on the Orbitrap. At the beginning we had 4 different ionic couplers to try, in order to find the best condition. The four acids were the TFA, PFPA, HFBA, TDFA. We made several injections in the same chromatographic condition, in order to verify which acid give us the best condition. To have a clear signal and to operate quite far from our actual limit of detection, we decided to use a solution at 9.6 µg/mL of our protein. The 4 acids were tested at the same percentage, coupled with the 5% of HCOOH that were the optimal condition on the previous system.



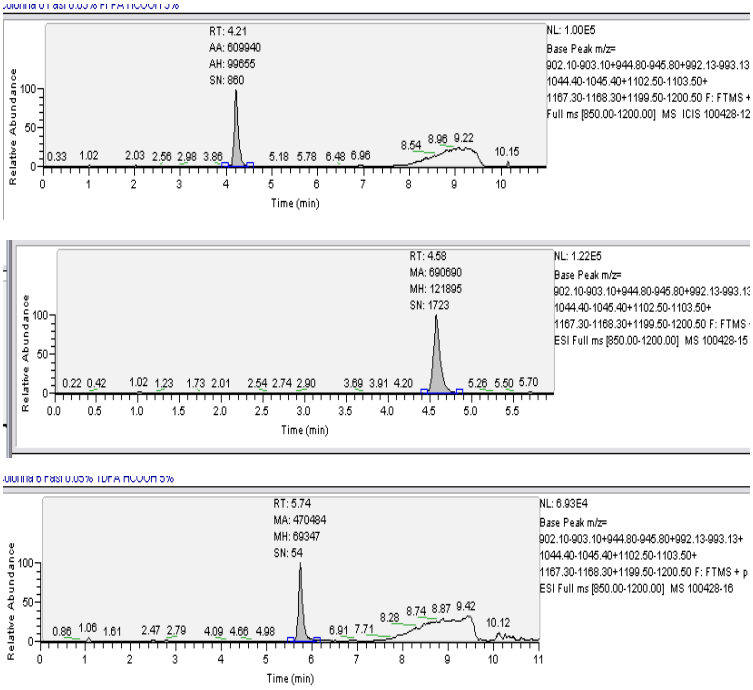


Fig 34: in file 100428-11 mobile phase with TFA, in 100428-12 mobile phase with PFPA, in file 100428-15 mobile phase with HFBA, in file 100428-16 mobile phase with TDFA

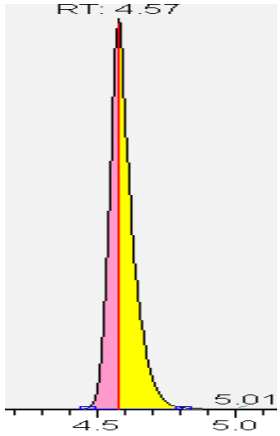
As suggested by the acquisition Fig.34, the HFBA seemed to be the more proficient ionic coupler for our scope, so we set another design of experiment, based on this new acquisition. On the data acquired the previous day we decided to set a 2^K Experimental design, based on classic multivariate approach.

Exp Name	Run Order	Incl/Excl	%acido formico	%Hfba
N6	10	Incl	3	0.03
N7	11	Incl	3	0.03
N8	8	Incl	5	0.01
N11	4	Incl	1	0.01
N2	7	Incl	5	0.01
N5	9	Incl	3	0.03
N1	3	Incl	1	0.01
N3	1	Incl	1	0.05
N10	2	Incl	1	0.05
N12	6	Incl	5	0.05
N4	5	Incl	5	0.05

Fig 35: proposed experimental design and randomization.

We set the limits in this way, the formic acid from 1% to 5% and the HFBA from 0.01% to 0.05%, because we saw that was the condition that we expected as the best experimental design region. The 2^k experimental design must be executed by making experiments at the edge points and 3 replicates at the centre point of experimental domain in order to obtain the standard deviation associated to our experiment. The responses that we decided to observe are different: as first approach, Area and Highness of the peak, that are the simplest value to check, because higher peak means higher sensibility. Then we decided also to evaluate the peak shape, the length is measured in minutes. To

evaluate also the shape of the peaks obtained we decided to set this parameter by our own. We took the apical point of the peak, and then we went orthogonally straight down till the base of the peak. This operation set two new zones, called head and tail. Then we looked at the elution time of these two new zones.



1	3	4	5	6	7	8	9	10	11	12
Exp No	Run Order	Incl/Excl	%acido formico	%Hfba	area picco	Altezza picco	Lunghezza Picco	Forma Picco	S/N	Intensità Media MS picco
3	1	Incl	1	0.05	987567	151999	0.38	0.1	515	39600
10	2	Incl	1	0.05	1.06e+006	149655	0.43	0.17	559	36800
1	3	Incl	1	0.01	1.43e+006	168317	0.57	0.27	1249	45400
11	4	Incl	1	0.01	2.006e+006	220938	0.46	0.2	1158	68000
5	9	Incl	3	0.03	913925	214882	0.38	0.18	1000	42900
6	10	Incl	3	0.03	1.005e+006	211580	0.41	0.13	1066	44700
7	11	Incl	3	0.03	1.37e+006	283896	0.46	0.16	1325	51400
4	5	Incl	5	0.05	983913	215685	0.4	0.08	971	40600
12	6	Incl	5	0.05	1.3e+006	272693	0.42	0.2	1191	50600
2	7	Incl	5	0.01	1.85e+006	380476	0.54	0.1	1481	64600
8	8	Incl	5	0.01	1.42e+006	288400	0.51	0.15	1071	47900

Fig 36: Peak shape explanation and data collected in DOE software

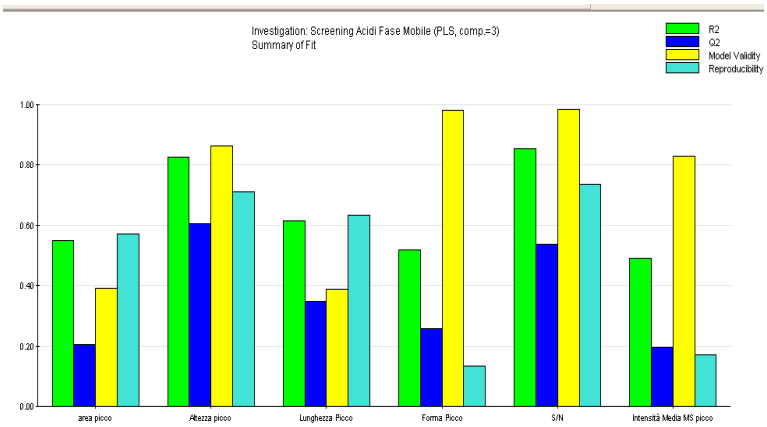


Fig 37: Model Obtained by DOE

If the peak is perfectly symmetrical the difference should be zero. If there's any difference in the zone, we obtain a result that we call peak shape. So the numbers collected are the difference of these two areas in each peak acquired. Our knowledge before performing the experimental design was that formic acid could help improving the signal and HFBA improving the peak shape and the peak length, but it gave us also signal suppression, so we had to find a perfect compromise with these two acids in order to have a great improvement. As showed by Figure 37, we obtained a strong result in model validity for all the investigated responses, even if our Q2 and our reproducibility were

not so good in every response. For the construction of this model we decided to exclude 1 of the 3 replicates at centre point, because it showed clearly some strange parameters, particularly in peak area, maybe due to a theoretical carryover. We decided to exclude it because the model fits better without this point in the consideration.

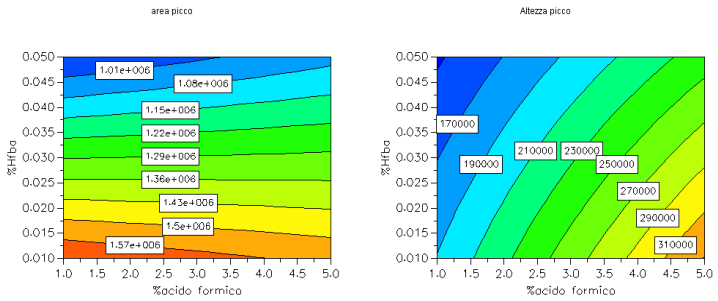


Fig 38: Responses observed for Peak area and peak height

As showed by Fig.38, for peak area, we saw that the effect is mainly obtained and directed by HFBA, because lines are more or less parallel to the X-Axis. The peak height is more complex and clearly shows a different, but equal in strength, behaviour by the two acids, in fact, as showed in Fig.38 the formic acid strongly increases this response, and the HFBA decrease the height.

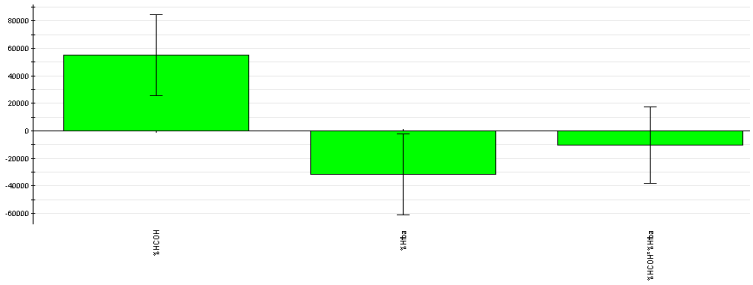


Fig 39: Effect of acid for peak height

The interaction has the standard deviation that goes trough zero, so is not significant by a statistical and a practical point of view. In both cases, a high percentage of formic acid is suggested by the design to increase the sensitivity. By following our discovery process to the best optimize mobile phase, if we take in account the peak shape and the peak length, the optimum seems to be slightly different. For the peak length, we see the complete dependence of this response from

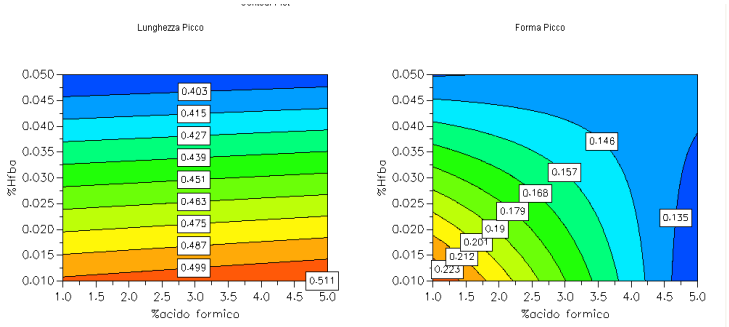


Fig .40: Peak length and peak shape

the HFBA, in this case, higher level of HFBA improves the peak shape, in fact, the peak length is less. For the peak shape the results are quite different, because it seems more complex. What we took by the experimental domain is that at high level of HFBA there isn't any interaction and the HFBA role is dominant.

The last two effects that we considered are the signal to noise ratio, that is clearly improved at high level of formic acid and the intensity of mass spectrometry signal, that is completely controlled by HFBA. Our results perfectly match with our previous knowledge

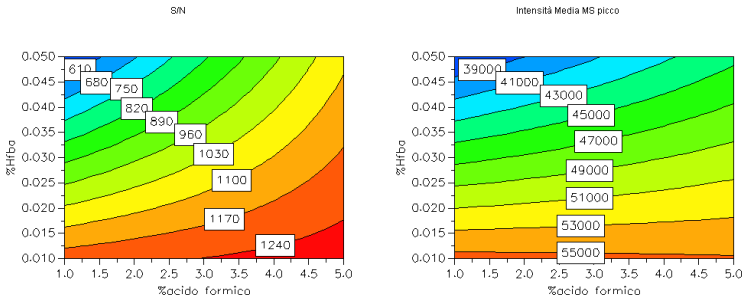


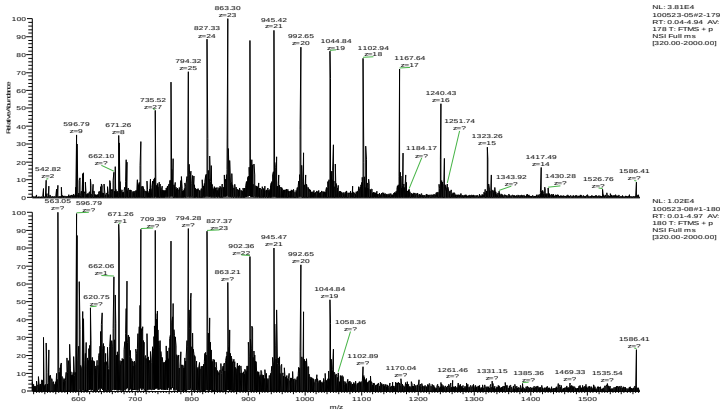
Fig 41: Signal To noise and Medium MS peaks intensity

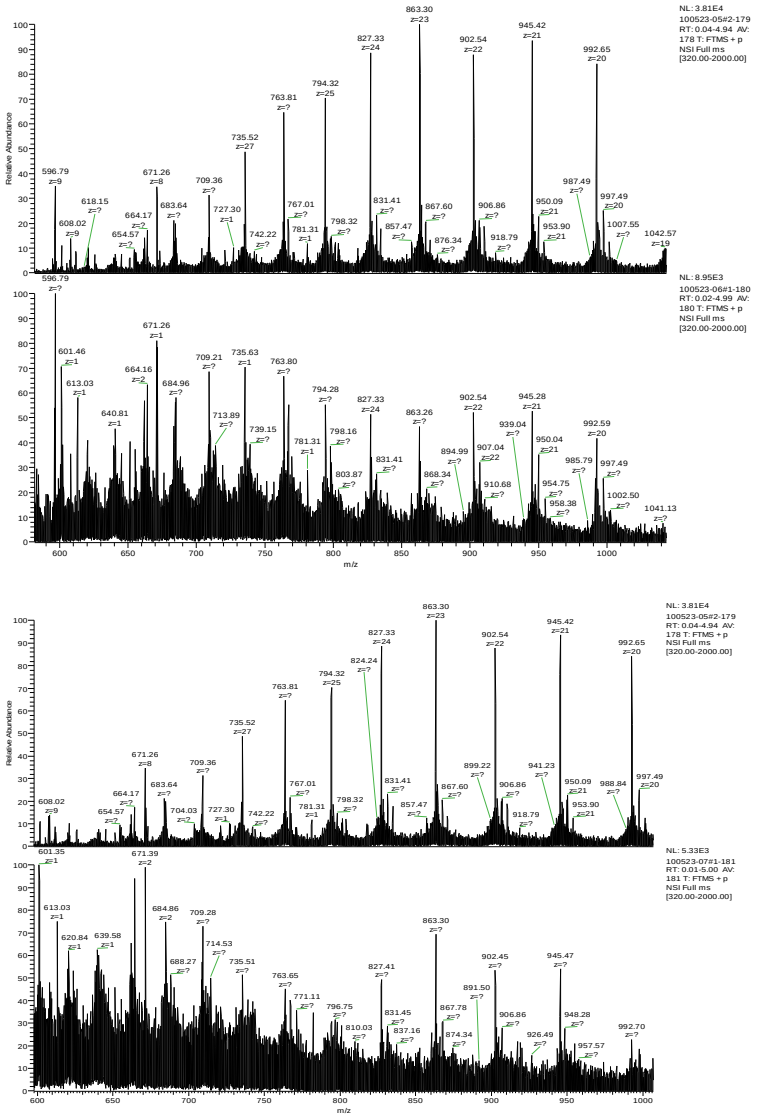
and what we chose for the following steps are the 5% of formic acid and the 0.01% of HFBA in our mobile phase. We performed some experiments on this new mobile phase, after DOE, but they seemed to be affected by a large carryover. Since we decided to

operate with a new mobile phase, a new tuning file was necessary to improve the signal. Due to the fact that our mass spectrometer could operate with different peak resolution, we decided to try different resolutions, in order to obtain the maximum performance. Normally the quantitative analysis is conducted not in full scan, but in 2 other ways: the Ion monitoring and the Reaction Monitoring. These two technologies can be combined in single or multiple ways. In the Ion monitoring, the mass spectrometer analyzes only 1 precise mass value. In this case, for our protein, we have different m/z value, based on the different ionization of the molecule, and so a multiple ion monitoring (MIM) is more usable than a Single Ion Monitoring (SRM). This technology is not as precise as reaction monitoring. In reaction monitoring one or more m/z are selected and then, fragmented with CID/ETD technology. The fragments produced are then analyzed. Usually, for quantitative analysis, at least 2 reactions are necessary to recognize and clearly identify the molecule. In fact the most abundant fragment is quantified and the others fragments produced are monitored to have a clear identification

of the starting molecule; in fact, every kind of molecule produces different fragments based on its molecular structure. With Ion monitoring or reaction monitoring the resolution is not a fundamental parameter, we decided to use also the low resolution, in fact, high resolution requires more time for a single scanning analysis, and so, low resolution should provide better shape for chromatographic peaks. Several methods were created with different revelation methods. According to the results obtained, that are poor in reproducibility, the most reliable and sensitive method is the one we used before, and so a full scan with a resolution of 30.000 FHWM. This is quite easy to explain, because in the reaction monitoring experiment, we saw that the molecule is too big and does not show a quantitative reaction in fragmentation. For the ion monitoring, the full scan is more complete and gave us a lower noise, so it was optimal for us. Due to this result, we also tried to lower the resolution: so 15.000/10.000 and 7.500 to improve the chromatographic peaks shape but with these settings the mass spectrometry ions peaks obtained were too broad and unfittable for our analysis. In the

process of methods development, we tried to use additive to organic mobile phase, called supercharger, in this particular case we used 2 substances: sulfolan and m-Nitrobenzilalchool. These substances are able to increase the charge of the molecules in analysis without modifying the pH of the solution. We tried to use these substances because we wanted to improve the specific charge state of the protein. If this approach is feasible, theoretically we should be able to improve our sensitivity. To test this hypothesis we made a direct infusion with our protein dissolved in 50% of mobile phase A and 50% of mobile phase B plus supercharger addiction at different concentration.

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NSI Full ms
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180 T: FTMS + p
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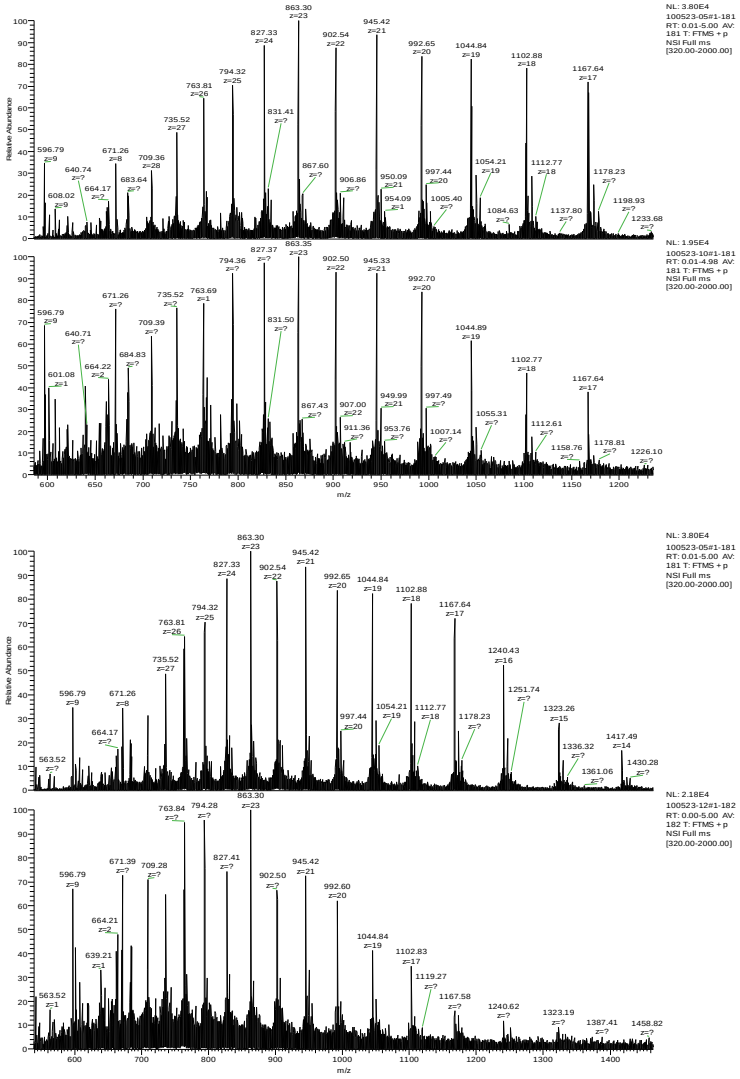
Relative Abundance
m/z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m/z

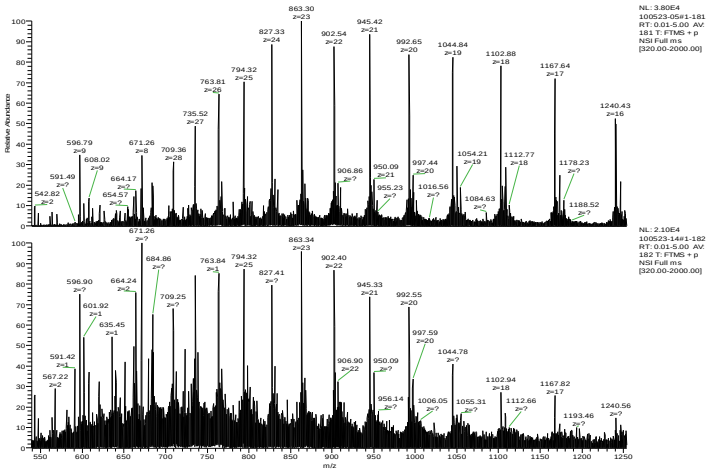
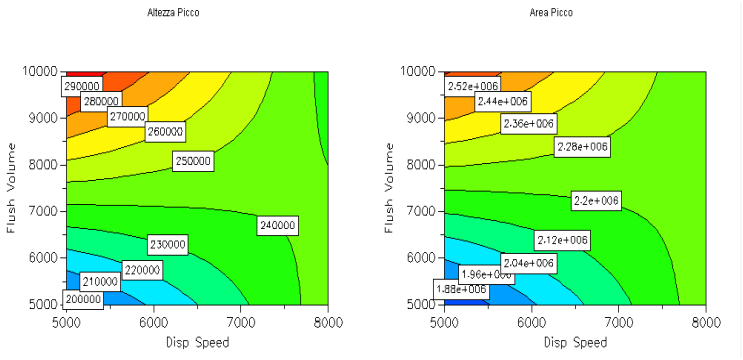


Fig 42: comparison between normal infusion scan and supercharged infusion scan

These spectra in Fig.42 underline that the effect of the supercharger was continuous and concentration dependent, so it was not possible focus the effect on a single charge state. In addition, the higher charge state falls in a more noised zone, so we lose sensibility. Because of these two facts, we decided not to use the supercharger for our further development. According to the extraction development, we knew that the sample would be injected in citric acid 0.1M pH=1.9, we tested the possibility to add the HFBA to this solution, in order to form the ionic couple from the beginning and also to avoid the non specific binding. According to

our mobile phase, we added the 0.01% of HFBA. From the chromatogram obtained, this addition seems to be effective in the improvement of the peak shape and of the peak intensity, maybe due to a higher time for ionic couple formation. After mobile phase selection, we decided to improve the chromatographic part, by modifying the starting percentage of organic phase. These analyses highlighted that the method is very sensible, because an alteration of initial organic phase from 25% to 30% makes the retention time of the protein change in a significant way. We saw that the optimal starting percentage of organic phase is 27.5 %, because a higher value let the molecule exit at the dead time. According to other studies we decided to leave this kind of column with inner diameter of 0.018 mm, because the tailing was a persistent phenomenon and it was impossible to enhance sensibility with this phenomenon. According to what we saw before, we switched to a broader column, with internal diameter of 0.3mm. The column that we chose is a Jupiter C_4, coded HRMS-43. At the beginning of this new development step, we decided to fix the previous mobile phase and proceed to another method

development. A fundamental part of the HPLC analysis is the autosampler parameters. We found that these parameters that regulate the speed of sampling, dispensing and loop conditioning can have a strong influence on the final results, and so we decided to investigate this matter more in details. The two parameters that were tested on DOE were the dispense speed that regulates the injection of the sample and the flush volume, that regulates how many sample will be used to condition the loop before the sampling. The dispense speed was tested from 5000 to 8000 nL/min, because these are the limit of the instrument. Otherwise in first approximation, the flush volume was tested from 5 to 10 µL that are 1 time and 2 times volume of the loop.



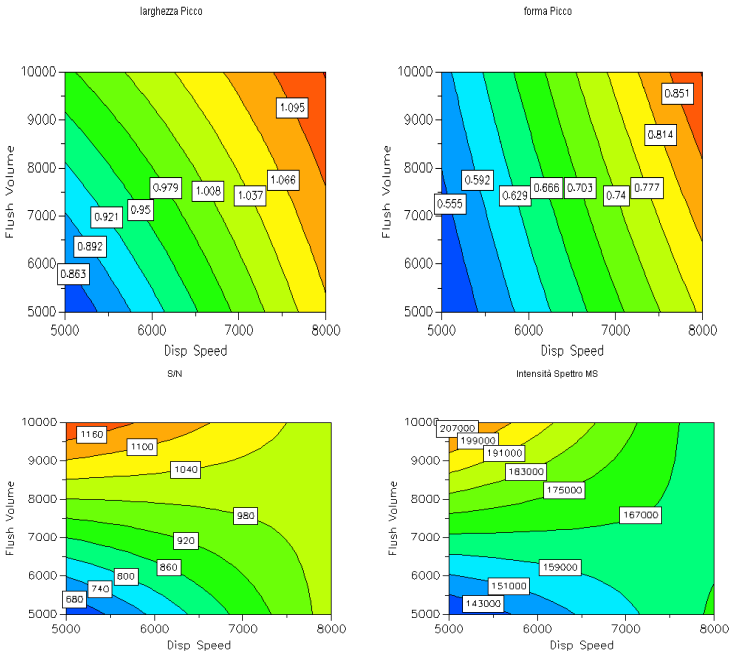
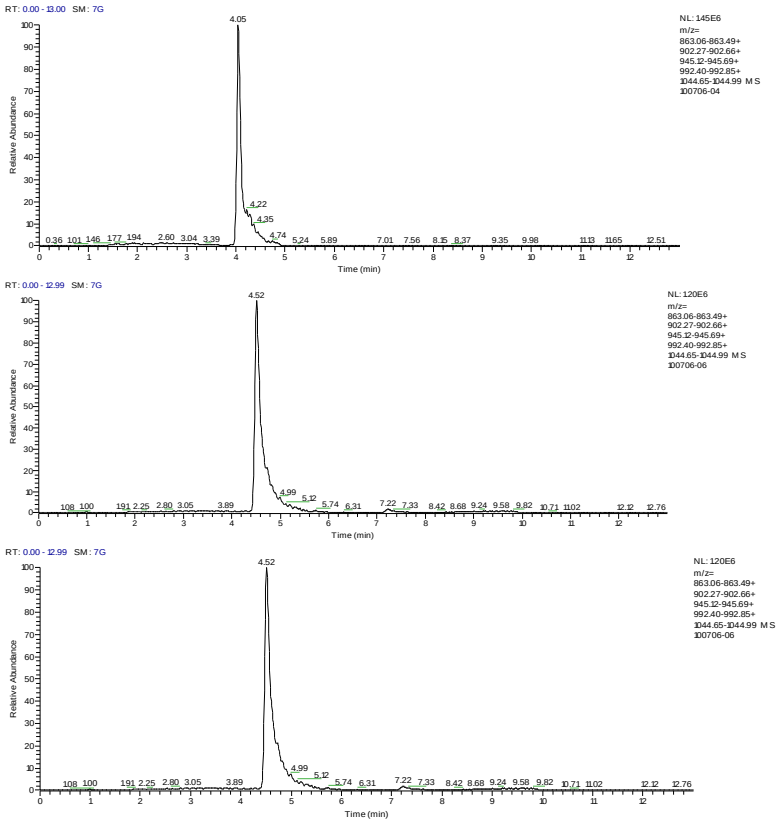


Fig 43: Contour plot of DOE results on autosampler parameters

The clear information that came out from this test was that a higher flush volume is better in every single tested parameter. The dispense speed was not as much influent as flush volume and we decided to keep it at our previous settings. Other test were performed to extend the experimental domain of dispense speed, but results were worse than obtained before and so the dispense speed remained unchanged. According to fluid-dynamics, the column should have a flux of mobile phase around 5-6 µL/min, but at this velocity

the column shows poor peaks shape. According to the limit that we had for our source around 10 µL/min, we tested all the flux speeds between 5 and 10.



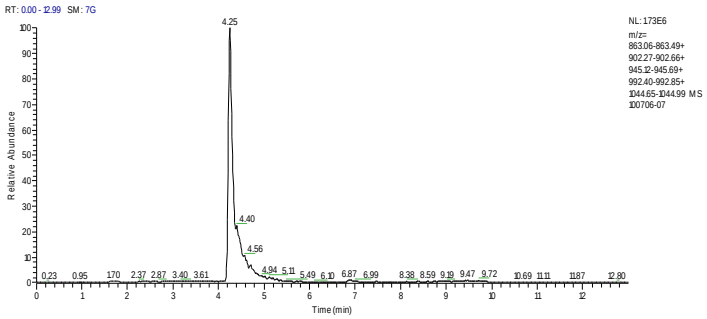
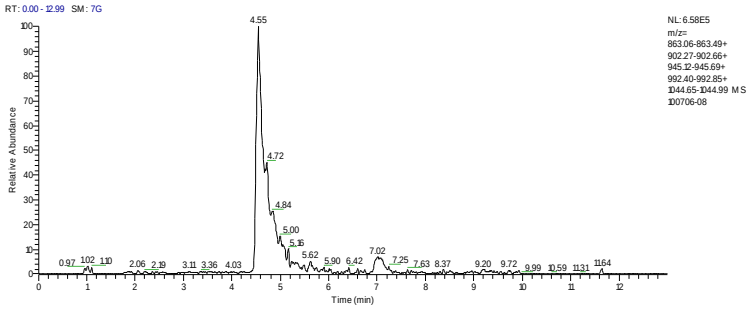


Fig 44: in file 100706-05 the 7 µL/min flux, in file 100706-06 the 8 µL/min, in file 100706-04 the 10 µL/min and in file 100706-07 the 9 µL/min

According to area value and tailing, the 8 µL/min was the chosen flux, according also to other problematics, like pressure and source clogging that are lower at lower flux. In order to reduce the column pressure and to improve peak shape we tested all the temperature allowed by column from 30°C to 60°C.



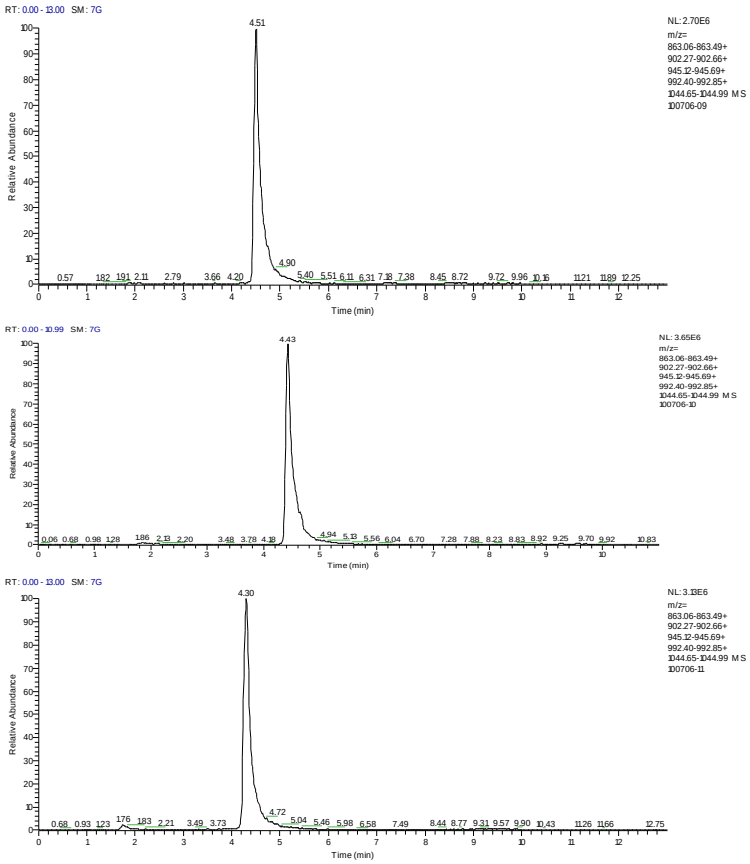


Fig 45: in file 100706-08 the 30°C experiment, in file 100706-09 the 40°C experiment, In file 100706-10 the 50°C experiment and in file 100706-11 the 60°C experiment.

According to our previous knowledge, increasing the temperature brings to a decrease of the retention time and to an improvement of the peak shape. Due to the fact that 60°C is an extreme temperature for both column and oven, we decided to use 50°C because the

difference in terms of peak between the 2 temperatures is not relevant. Fixed these fundamental points to obtain a good chromatography, a new experimental design was done in order to see what could the best mobile phase for these columns and conditions.

The parameters evaluated were the same as the previous experimental design.

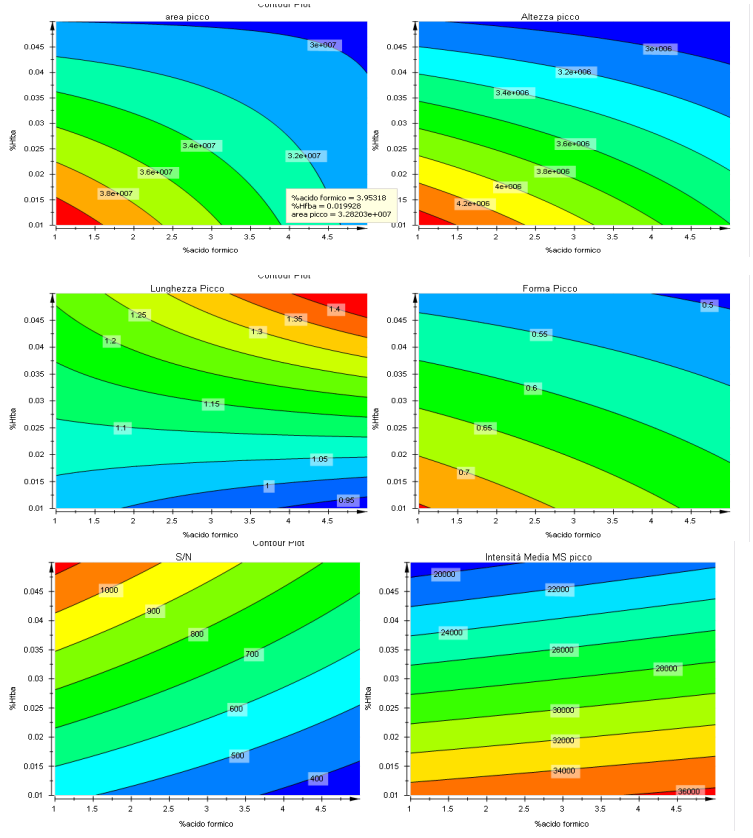


Fig 46: Experimental domains obtained by DOE

Oppositely to what we obtained for the previous DOE on mobile phase, this time, what we obtained did not show a clear and precise indication of which should be the best mobile phase. This time every factor pointed on a different direction. We decided, at the end, to keep the same mobile phase as before, because the mass spectrum intensity and the peak length were at the optimum in these conditions. To understand the reason why we lost sensibility during our previous experiments, we decided to test on this new column, a new approach. Simulated lower concentrations were obtained by reducing the volume of injection and comparing the simulated low concentrations with the real low concentration. By comparing the results, it was clear that the non specific binding phenomenon was the real problem, because in simulated injection we obtained a peak for our protein; otherwise in real injection we didn't obtain anything. In order to try to reduce the non-specific binding, we looked for other approaches to keep the molecules in solution. Other groups in this company worked on the same protein, and for their experiments, they used a specific formulated buffer, created with a detergent (Lutrol

P68) and the BSA. We didn't use this solution before, because we were considering the ionic suppression. The amount of charge for any droplets formed by ESI or nano ESI is not unlimited, so the more substances you add to the solution, the less molecules of protein will be charged and so analyzable. But, at this point, using additives is the only solution to proceed with intact protein, because, clearly, our limit at the moment is only at the level of μg/mL.

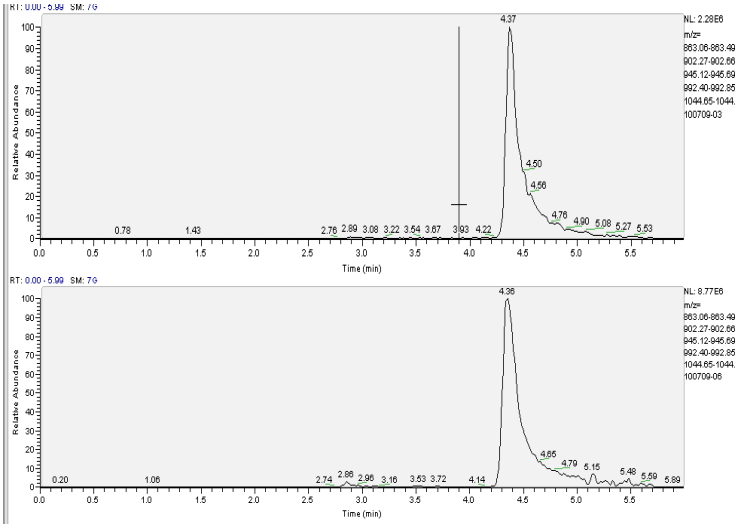
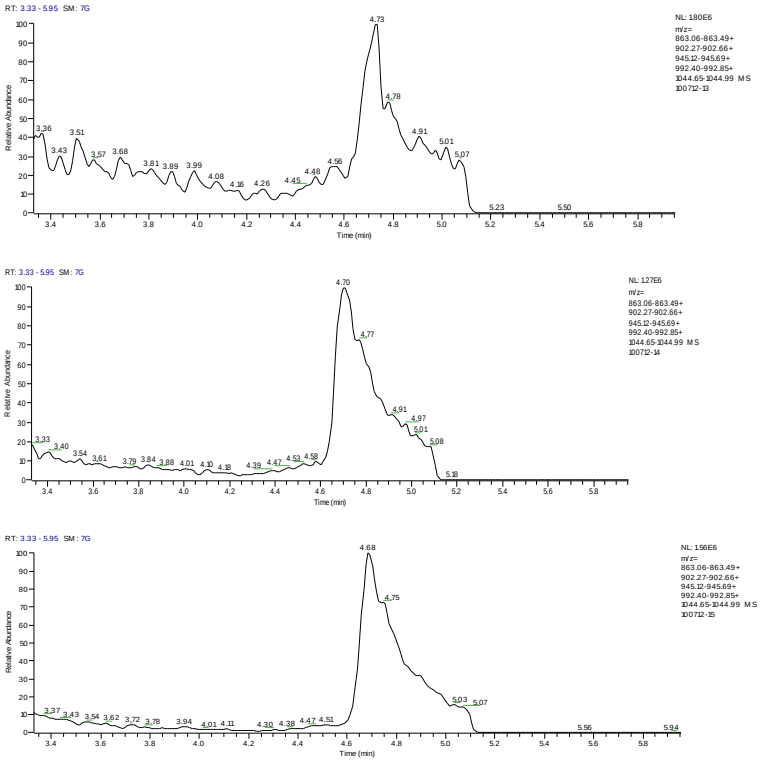


Fig 47: in file 100709-03 the normal injection condition,
in file 100709-06 the injection with additives

Just looking at the chromatograms, and the peak height on the appropriate layout in Fig.47, it was easy to see

that the peak obtained with the additive had a higher area and so we were able to increase sensitivity. This was made to avoid specific binding along the instrumentation pattern. The positive effect of additives seemed to be higher than the ionic suppression that they brought. The method development was followed by an optimization of the BSA percentage.



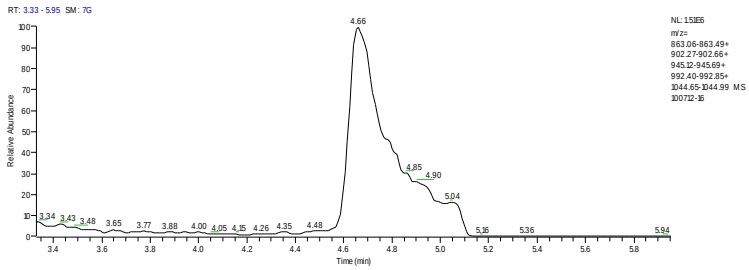


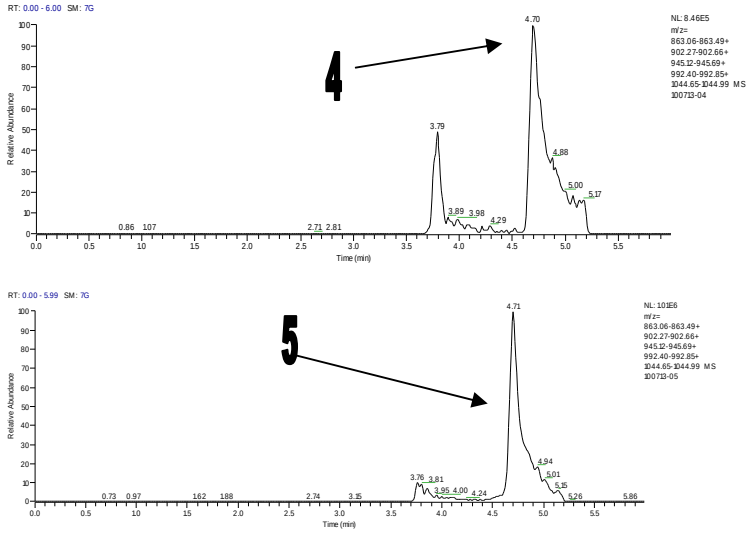
Fig 48: in file 100712-13 analysis made with 0.005% of BSA, in file 100712-14 analysis made with 0.01% BSA, in file 100712-15 analysis made with 0.025% of BSA and in file 100712-16 analysis made with 0.05% BSA

According to our analysis, the increasing of BSA percentage showed an improvement of chromatographic performance. A higher percentage of BSA could reduce the non specific binding, According to this theory; we tried to evolve our solution by using some scavengers that are normally used for displacing chromatography. Displacement chromatography is a chromatography technique in which a sample is placed onto the head of the column and is then displaced by a solute that is more strongly sorbed than the components of the original mixture. The result is that the components are resolved into consecutive zones of highly concentrated pure substances rather than solvent-separated peaks. It is primarily a preparative technique; higher product concentration, higher purity,

and increased throughput may be obtained compared to other modes of chromatography. The basic principle of displacement chromatography is: there are only a finite number of binding sites for solutes on the matrix (the stationary phase), and if a site is occupied by one molecule, it is unavailable to others. As in any chromatography, equilibrium is established between molecules of a given kind bound to the matrix and those of the same kind free in solution. Because the number of binding sites is finite, when the concentration of molecules free in solution is too large respect to the dissociation constant for the sites, those sites will mostly be filled. This results in a downward-curvature in the plot of bound versus free solute, in the simplest case giving a Langmuir isotherm. A molecule with a high affinity for the matrix (the displacer) will compete more effectively for binding sites, leaving the mobile phase enriched in the lower-affinity solute. Flow of mobile phase through the column preferentially carries off the lower-affinity solute and thus at high concentration the higher-affinity solute will eventually displace all molecules with lesser affinities. The molecules that could operate in a positive scavenger

mode are nitrogen rich molecules. So what we did is to try different solution to see what is the optimal. What we compared was:

1. 0.05% Lutrol F68 and 0.01% BSA
2. 0.05% Lutrol and 0.035% BSA
3. 0.05% Lutrol, 0.01% of BSA and 0.01% Tetraethyl pentammine
4. 0.05% Lutrol, 0.01% of BSA and 0.01% 2-Guanidinium-imidazole
5. 0.05% Lutrol, 0.01% of BSA and 0.01% Polyimmine Mw 1200



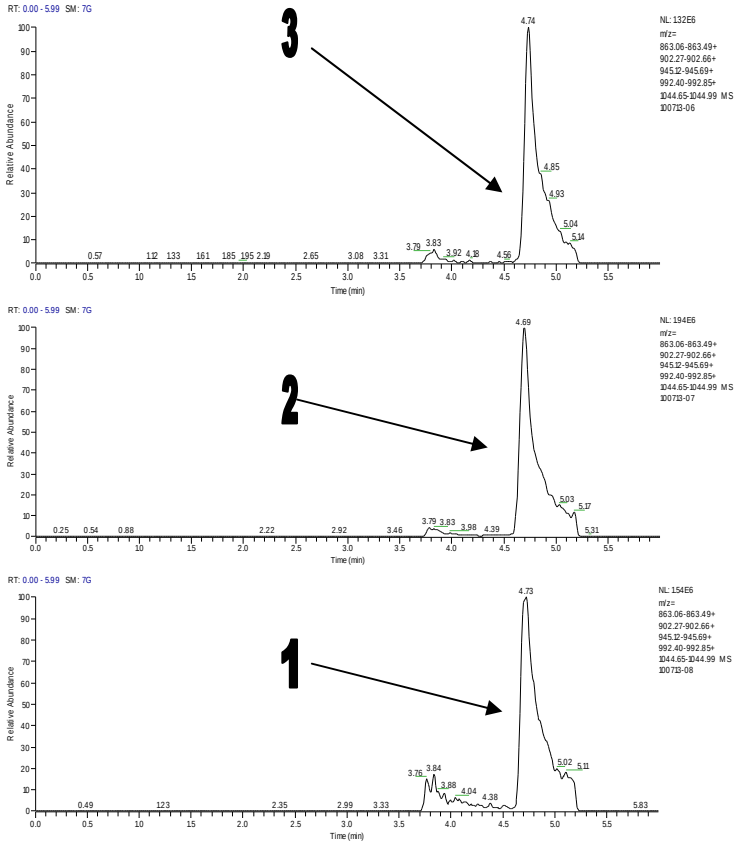


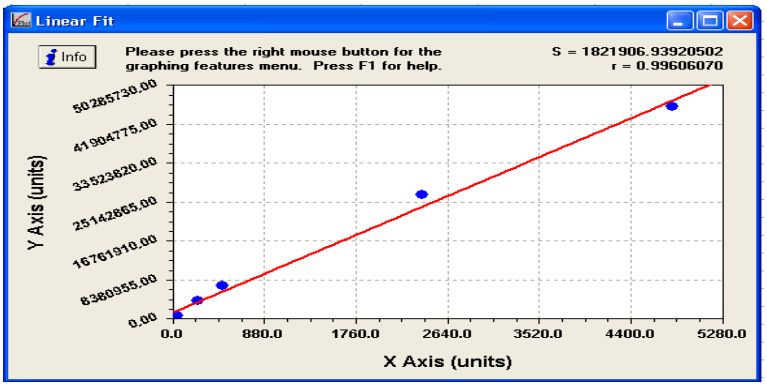
Fig 49: Chromatograms obtained with additive and displacers

Analyzing the data obtained by this study, Fig.49, the best results were obtained when the BSA was increased and in the presence of Pentammine. The polyimmine is a viscous solution and it's difficult to mix with other components, so it was not considered for the next screening. The additives were tested again,

but with an increased percentage of BSA in solution. The results were analogue to what we obtained before, confirming that the pentammine was the best signal enhancer. Based on the latest results, we decided to see what the actual instrumental limit of quantification with this new injecting solution was. Two curves were tried:

1. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.035% BSA
2. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.035% BSA +0.01 % Pentammine

The curves were constructed starting from 12 ng/mL to the final point is 4800 ng/mL.



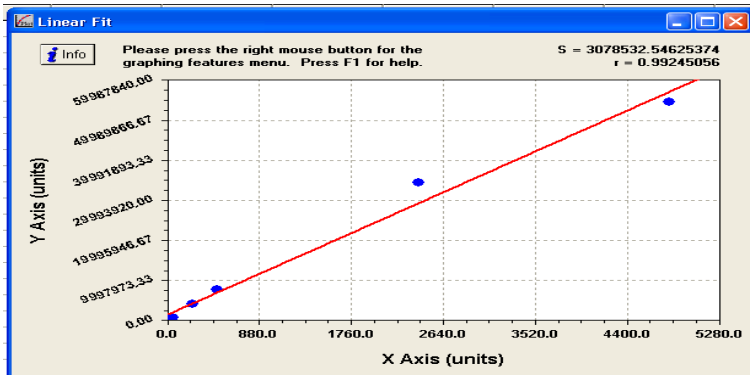


Fig 50: in the upper picture, the curve obtained with Displacers, in the lower picture the curve obtained without displacers

The obtained results showed that the lower visible and quantifiable concentration at the moment is 48 ng/mL in both conditions. The curve obtained with cationic displacers shows a better linearity, even if the absolute signal is lower than the others. By comparing the chromatograms obtained, the curve with pentammine showed a better signal to noise ratio. On the previous analysis we took the detergent as it was, but after this development on the other condition, we decided to test different detergents, in concentrations higher and lower than the CMC, to see what fit best for our analysis. The solutions prepared for this new kind of study are:

1. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.0495% BSA +Tween 20 $1.21*10^{-6}$ M
2. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.0453% BSA +SDS $3.46*10^{-4}$ M
3. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.035% BSA
4. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.035% BSA +0.01% pentammine
5. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.0495% BSA +Tween 20 $1.21*10^{-3}$ M
6. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.0453% BSA +SDS $3.46*10^{-2}$ M
7. Citric acid pH 1.9 +0.01% HFBA +0.5% Lutrol F68+0.035% BSA

The solutions from 1 to 4 were all below the CMC, and the solutions from 5 to 7 were all above the CMC. The different % of BSA present in the solutions were due to the different conditions of preparation, but are ininfluent to the final results, because we saw that from 0.025% to 0.05 % the effect of BSA in solution is constant. Every single condition was tested at 12-24-48-120 ng/mL 4 times. Even in these experiments, the

LOQ is 48 ng/mL. What we saw is that the concentration higher than the CMC showed bad results for every surfactant. In particular, SDS showed bad results even below the CMC. The analysis showed a good behaviour of Tween-20 that offered great results, comparable to Lutrol, when the concentration was under the CMC. For this reason, new analyses were performed, by modifying the final concentration of Tween and testing these 2 new concentrations:

1. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.0495% BSA +Tween 20 $1.21*10^{-5}$ M
2. Citric acid pH 1.9 +0.01% HFBA +0.05% Lutrol F68+0.0495% BSA +Tween 20 $1.21*10^{-4}$ M

The solution numbers 1 gave us the best chromatographic results and let us see a signal, clearly different to the noise, for the 24 ng/mL concentration. The improvement related to the chemical part of the work, now appears meaningless, because we tested all the possible modifications according to the purification process developed. The following step necessary to increase our performance was to switch the system in purge and trap configuration. For this purpose the

connection in the instrument needed to be changed, because the loading pump needed to be connected to the loop. Following the Fig.51, you can see that the loading pump brings the flux in the loop and then transport the sample to the pre-column, where the pre-concentration happens. During this operation, the micropump conditionates the column at the starting conditions.

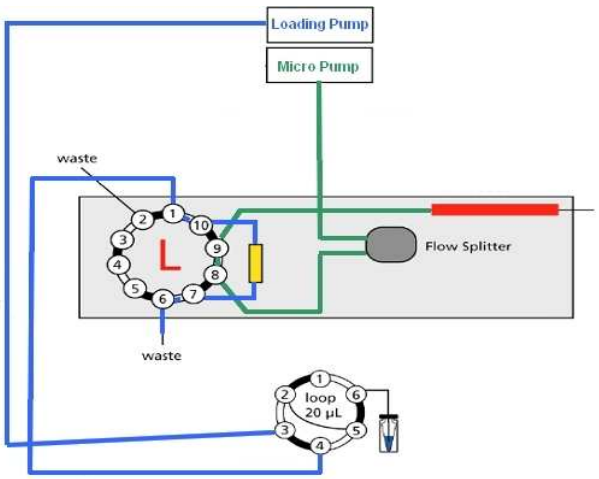


Fig 51: Injection and pre-concentration configuration.

When the sample is loaded in the pre-column, and cleaned by other substances that do not bind the stationary phase, the 10 outs valve switch from white configuration to black configuration. When the switch

is completed, the loading pump is switched off, because it brings its flux directly to the waste. The micro pump goes in the pre column

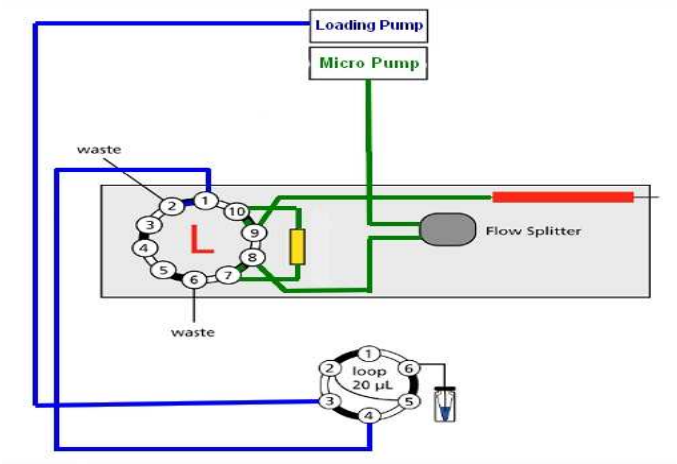


Fig 52: Analysis configuration of Ultimate 3000

counterflux respect to the loading pump and extract the substance from the cartridge. The analyses after this step proceed normally as well as before. To have a better comprehension of what is the behaviour of the cartridge, we used it alone, as a normal column. The methods were set up, with several injections of a 4.8 µg/mL protein solution. The peak obtained from the pre column was worse than expected, because it was broad and took a couple of minutes to completely exit from the pre-column.

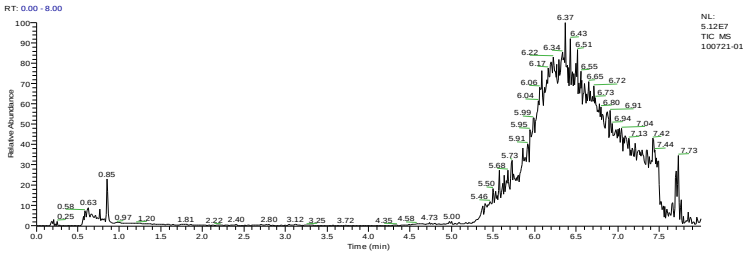


Fig 53: Peak obtained from the pre-column

According to this data, we set the complete method to have a long charging phase, and allowed the protein to fully enter into the analytical column. We performed several experiments, but the cartridge had a strange behaviour. In fact the pre column seemed to charge some injection of the protein, and to release them all together, so we had 3 o 4 injection blanks, and then a sharp and high peak related to our protein. Due to the fact that in this way we kept the injecting solution constant, but we injected 10 times more substance in HPLC system, we thought that maybe the total amount of BSA could be too much. Even with this expedient, the results of analysis remained inconstant. Several analytical column were tried after the cartridge, but none of them gave us what we expected, so we decided to use HRMS-44, that is equal to HRMS-43, as a

pre column cartridge, because the total pressure of the system allows us to have 2 column on the same pathway. The 2 analytical columns system gave us a sharp peak for the µg/mL concentrations, but then, when we tried the ng/mL concentrations the peak completely disappeared.

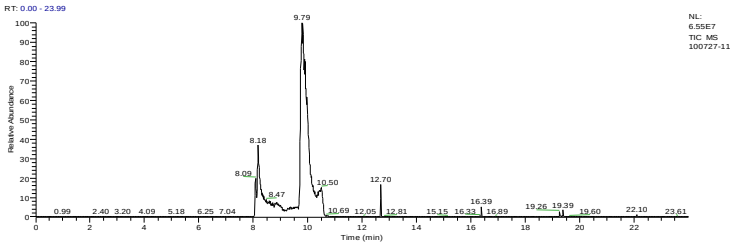


Fig 54: 1200 ng/mL injected in the 2 column purge and trap system.

According to these latest results, we hypothesized that our protein that highly suffers of non specific binding, was not fit for this kind of system, because the major extension of the pathway inside the instrument enlarged the non specific binding phenomenon and so, for concentrations under the µg/mL we completely lost our signal. Even for higher concentration this system was not fit for our protein, because after 10 times volume injection we obtained only a 2 times gain in signal. In order to improve the signal, the system was set again in solo micro pump configuration and a new

column was tested. This column was a new generation column, with fused core technology.

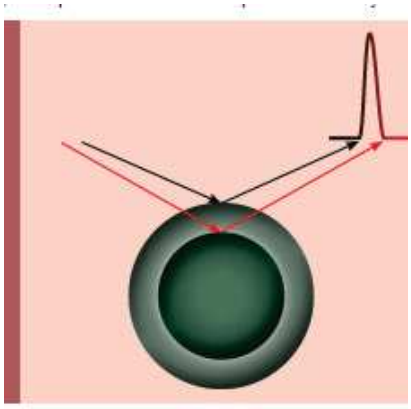


Fig 55: Fused Core particles.

Provided by Sigma Aldrich

In the fused core technology, the active part of stationary phase is only a crown that surrounds a fused solid core that is not penetrable. This results in a sharper peak, due to a minor contact time and a reduction of multiple pathways in the particle. Even if the particle size is similar to the other columns, the real performance of this kind of column is comparable to less sized particles. Even this column doesn't show better results in terms of sensibility. In our experience in this method development, we saw that cationic displacers had a potential effect and could improve our

sensibility. As a last trial, we decided to perform a last SCX extraction from the magnetic beads, using the cationic displacers in the elution phase to see if this new procedure could be better than immunoaffinity. We charged the same amount of protein on different kind of Dynabeads:

1. standard immunoaffinity purification on Chemicell beads
2. Dynabeads SCX
3. Chemicell magnetic beads with solfonic stationary phase
4. Chemicell magnetic beads with phosphate stationary phase

It's easy to compare these results and verify that the immunoaffinity extraction is still the best. Comparing the SCX extraction to what was performed at the beginning of method development shows that we made an improvement with displacers, because at the moment we had a little recovery instead of no recovery without the displacers.

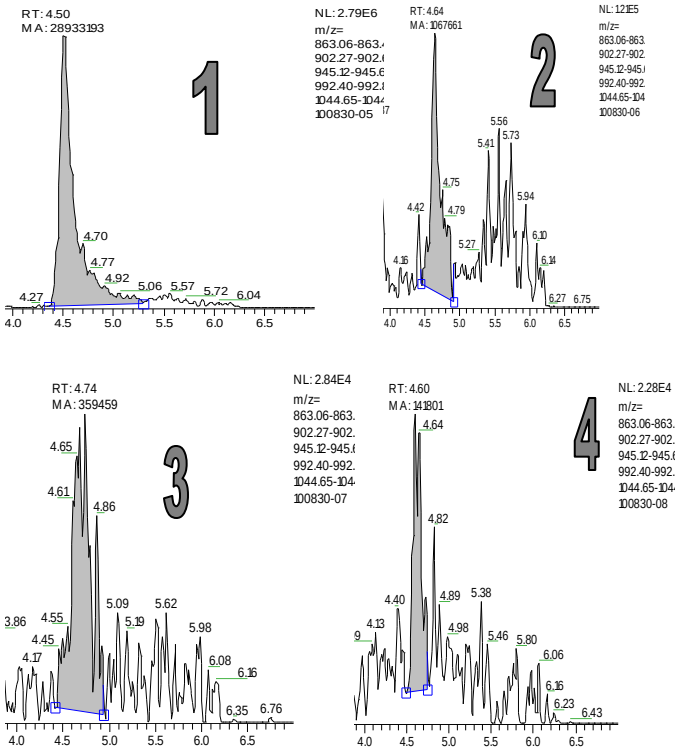


Fig 56: chromatogram obtained from purification

In order to avoid the non-specific binding we decided to test if the amount of magnetic beads used for separation could have some effect. We tested 3 different volumes of beads, 10-100-200 µL of beads with the same amount of protein (200 µL at 4.8 µg/mL) to see the best condition.

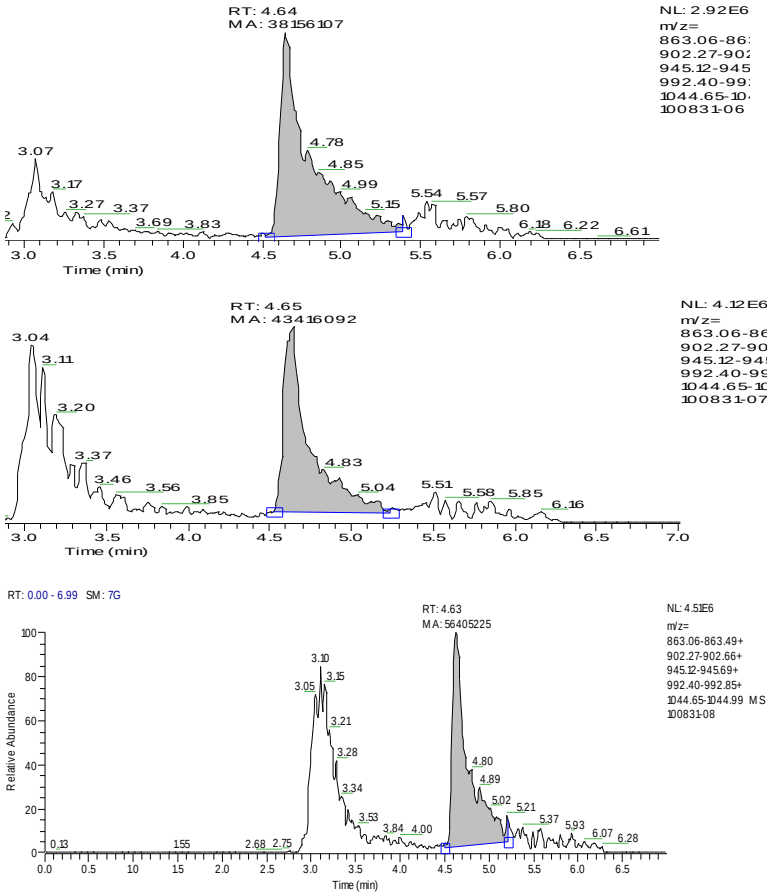
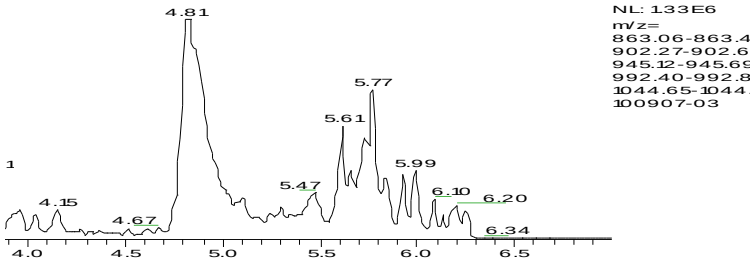


Fig 57: in file 100831-06 the 10 µL beads extraction ,in file 100831-07 the 100 µL beads extraction and in file 100831-08 the 200 µL beads extraction.

According to these results, the amount of beads that we were actually using gave us the best results.

Addition Method

To explore other possibilities, because at the moment we had a very bad LOQ, we decided to move forward to the addition methods, to see if it was possible to have this back up method. 200 µL of a 120 ng/mL solution of the drug was prepared and used as unknown sample. Starting from this solution, the other solutions were prepared by adding 200 µL at these concentrations: 480/1200/2400/4800 ng/mL and then these samples were analyzed in order to find if we could have a linear response and to see if it was possible to recalculate the real concentration of the unknown sample.



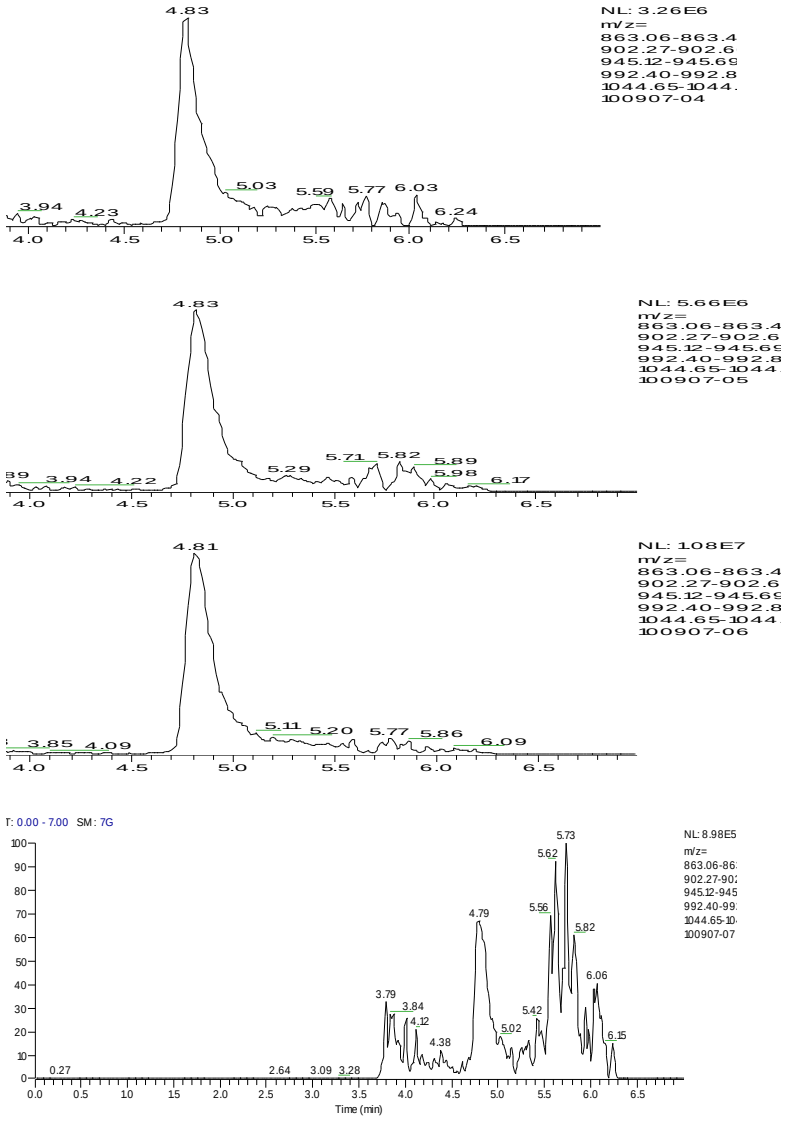


Fig 58: chromatogram from the addition methods

Added concentration	Area
480 ng/mL	$1.22*10^{7}$
1200 ng/mL	$2.90*10^{7}$
2400 ng/mL	$5.34*10^{7}$
4800 ng/mL	$1.06*10^{8}$
Unknown (120 ng/mL)	$5.85*10^{6}$

Tab 07: Area obtained for addition method

After the analysis, the data obtained gave a very strong result, with a good linearity.

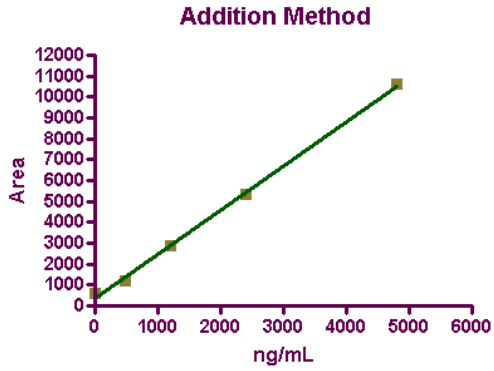


Fig 59: Linear curve obtained from data

The equation obtained from the data is $Y=2.6451*10^{6}+2.1455*10^{4}X$, and the recalculated concentration for unknown sample is equal to 123

ng/mL, with an error of 2.5%. Considering that the tolerated error in accuracy for mass spectrometry methods is plus or minus 15%, and considering the strong linearity obtained, this could be a good and useful backup method for sample analysis.

Internal Standard Production

At the end of this method development, we still miss the internal standard. An internal standard in analytical chemistry is a chemical substance that is added in a constant amount to samples, the blank and calibration standards in a chemical analysis. This is done to correct for the loss of analyte during sample preparation or sample inlet. The internal standard is a compound that must be very similar, but not identical to the chemical species of interest in the samples, because the effects of sample preparation should be, relative to the amount of each species, the same for the signal from the internal standard as for the signal(s) from the

species of interest (in the ideal case). According to the definition of internal standard and operating with a mass spectrometer, we thought 2 strategies to develop internally our internal standard. Usually internal standards for mass spectrometry are the same molecule in analysis, but isotopically marked. In this case, we used different strategies to develop internally our internal standard. The first strategy is based on disulfide bridges; in fact our protein has only 2 cysteines, bonded together. By opening this bridge in reducing conditions and then, label the two cysteines with iodoacetamide, we should obtain a different molecule for mass spectrometry. The second strategy is based on the lysines that are very abundant in this protein: we decided to add biotin to our protein, and see if it could be possible to obtain few species that could be used as internal standard. A first synthesis was performed, by following the same procedure adopted for other proteins, but after the Iodoacetamide addition and GPC purification no product was obtained. A second synthesis was set up and two different concentrations of guanidinium were added to the

solution, 0.3M and 0.15M. With this second approach, made to stabilize the unfolded protein after the GPC column, we obtained the desired product.

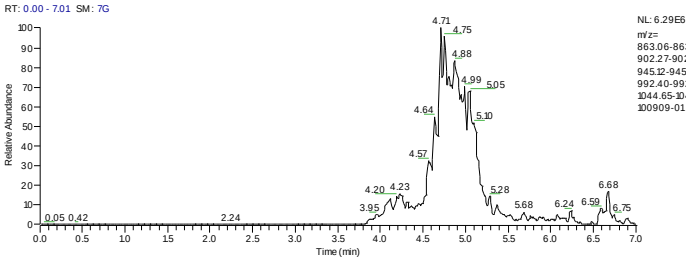


Fig 60a: chromatogram of iodoacetylated protein.

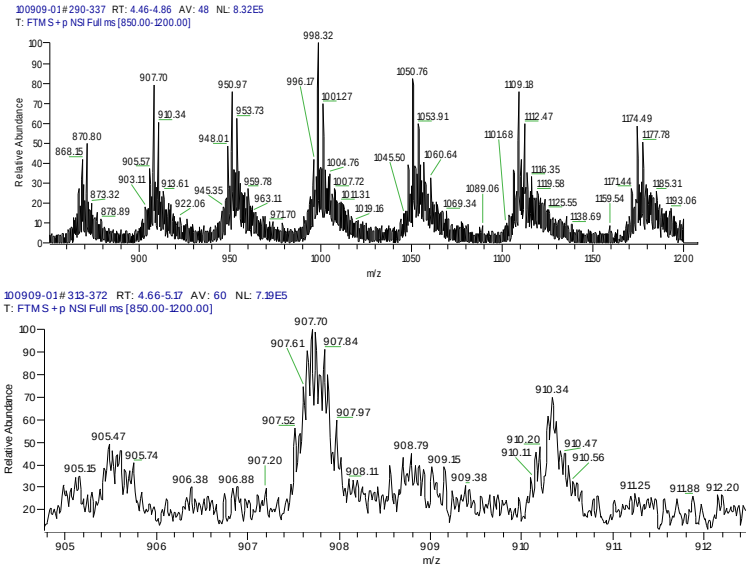


Fig 60b: mass spectrum of iodoacetilated protein.

At a closer look it's easy to see 3 near peaks. After deconvolution three species were identified: our protein plus one, two or three molecules of imminobiotin added. No signals about the intact folded protein are present in our spectra. We presume that for the third substitution the iodoacetamide goes on some amino acid that contains a hydroxyl group. The synthesis is very clean, as the final product after the purification, but it has strong carryover effect because the open protein is stickier than the folded one. This phenomenon is due to the fact that all the lysines that are masked on the hydrophobic core when the protein is folded are exposed after the breakage of disulfide bond. To see if this protein was fittable as our internal standard, we extracted it on magnetic beads, as our analyte protein. We ran this experiment in double, but no results were obtained by this procedure.

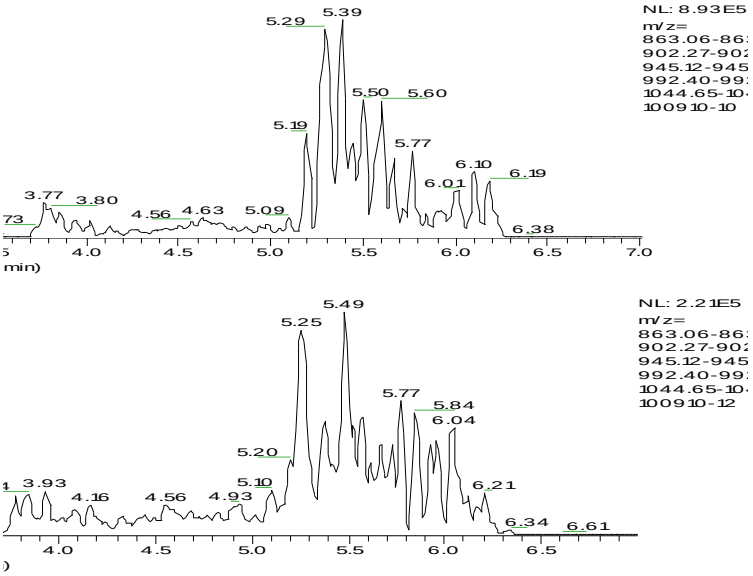


Fig 61: no signal of extracted protein around 4.5 min

The second procedure for internal standard production was the addition of immiobiotin to our protein. In the first trials imminobiotin was added to protein in DMF and then the reaction went on for 4h at 4°C. Unfortunately this procedure didn't give us any product after purification. The reaction was performed a second time, but in this case, we prepared a concentrated solution of immiobiotin in DMF, and then we added this solution to our protein. This time the procedure brought us several products.

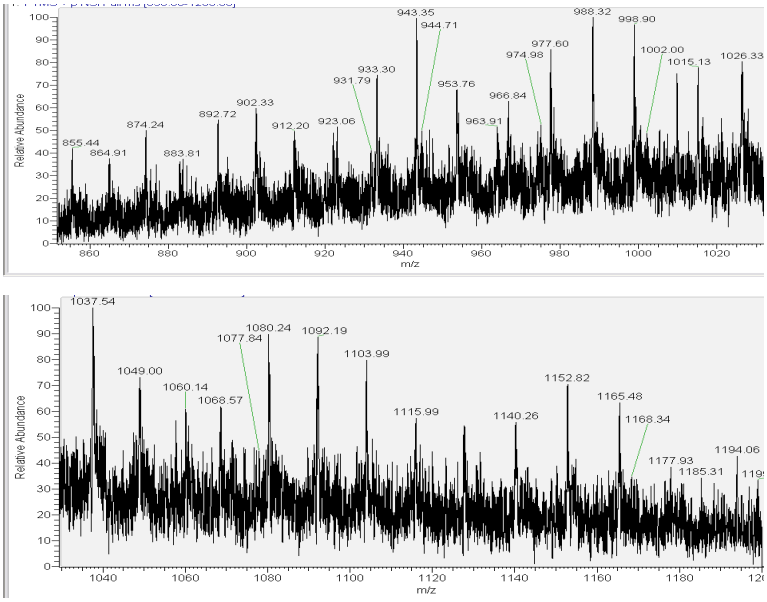


Fig 62: mass spectrum of imminobiotinilated protein.

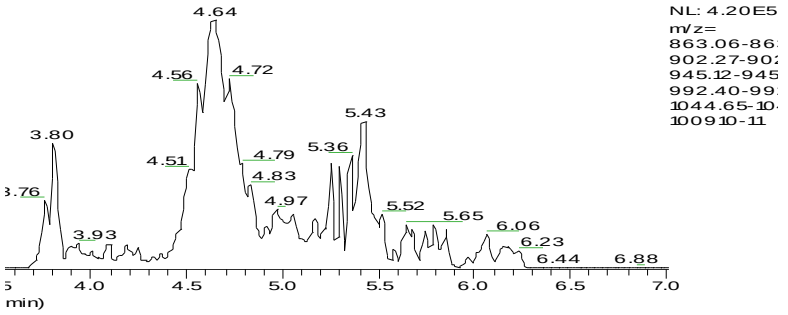
By analyzing the principal peaks obtained in the spectra, Fig.62, we could see that all the principal peaks were related to imminobiotin substitution.

M/Z	Charge	Sub.	M/Z	Charge	Sub
855.44	24	3	1015.13	20	2
864.91	24	4	1026.33	20	3
874.24	24	5	1037.54	20	4
883.03	24	6	1049.00	20	5
892.72	23	3	1060.14	20	6
902.33	23	4	1068.56	19	2
912.20	23	5	1080.24	19	3

923.06	22	2	1092.19	19	4
933.16	22	3	1103.99	19	5
943.35	22	4	1115.99	18	1
953.53	22	5	1127.68	18	2
966.84	21	2	1140.26	18	3
977.60	21	3	1152.82	18	4
988.32	21	4	1165.48	18	5
998.90	21	5	1177.94	18	6
1009.80	21	6	1194.06	17	2

Tab 08: Spectra interpretation of imminobiotin analysis, substitutions assigned

To test these new substances as internal standards, we put them on beads and then we started with extraction and purification from magnetic beads. Even in this case the experiments were conducted in double.



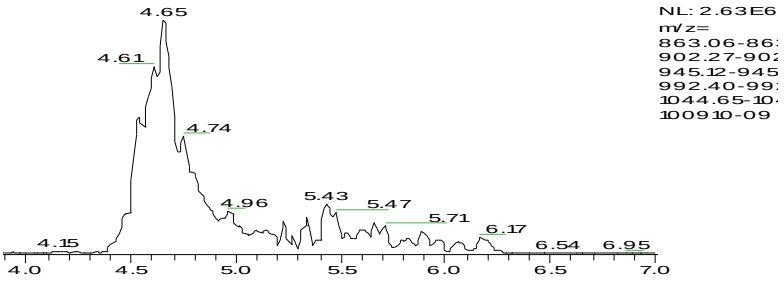
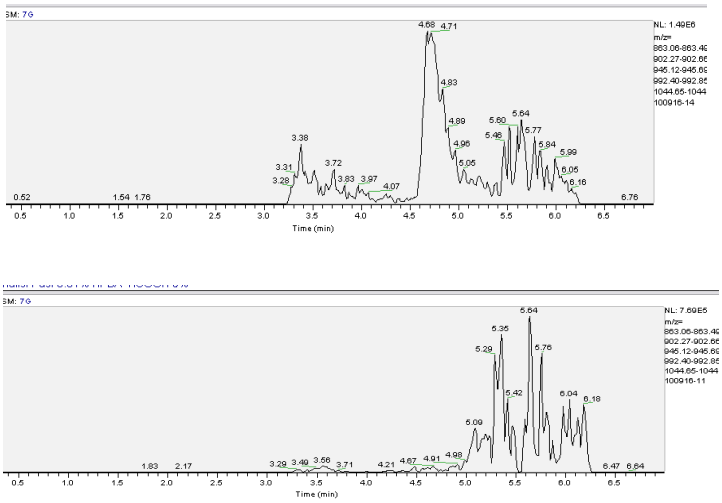


Fig 63: chromatogram from extracted imminobiotinilated protein.

By the data obtained, the imminobiotin seemed to have no effect on the capture performed by the monoclonal Ab, so we could theoretically use this substance as internal standard. Due to high noise on the spectra, we still have some doubts regarding its possible use, because we cannot exclude the presence of some starting protein that we are not able to detect. According to what we obtained in this method development, we set the last array of experiments in order to find the LOQ for the whole method. Different samples of spiked protein added to 200 µL of synovial fluid were created, in order to obtain the following final concentrations: 4.8/48/480/4800 ng/mL. These concentrations were tested in double, with the internal standard, added in a fixed concentration of 1200

ng/mL. All these concentrations were extracted following the procedure, with the extracting solution prepared with the best condition to avoid non specific binding: Citric Acid 0.1M pH=1.9 + 0.035% BSA+ 0.01% Lutrol F68 +0.01% HFBA + 0.01% Pentammine. An other solution, at 4800 ng/mL was extracted by following the old protocol, using only citric acid 0.1M pH=1.9 as a control solution. Unfortunately none of the extracted sample with the new extracting solution gave us any signal in chromatography about our protein, instead of our control solution that was perfectly extracted from synovial fluid.



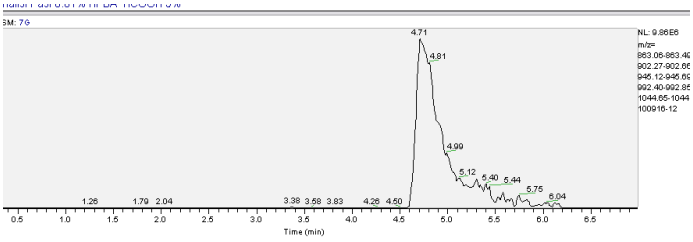


Fig 64: in file 100916-14 the control extraction, in file 100916-11 the 480 ng/mL extraction with additives, in file 100916-12 4800 ng/mL extraction with additives

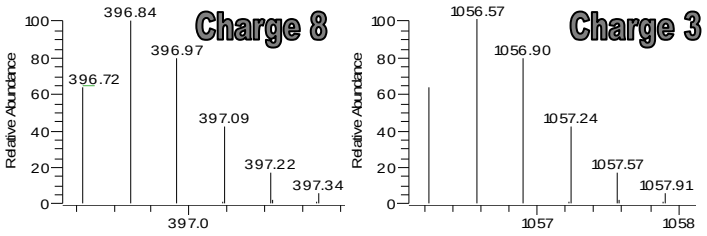
Looking at these results clearly appears that we won't be able to make any extraction at concentration under µg/mL, because the extracting solution is not able to extract the substance and keep it in solution at this concentration. Maybe this phenomenon is also caused by non-specific binding on the magnetic beads, so this way to reach the lowest detectable concentration doesn't bring us too far. In conclusion, what we can say about this methods, is that we were able to develop a solid extraction, as intact protein, by applying an immunoaffinity protocol, on magnetic beads, and our LOQ is in the range of µg/mL, because every solution tested to lower it, gave us strong phenomenon of non

specific binding, that made unexpectedly disappear our protein.

The C-Term Approach

Since we were not able to improve our LOQ under the range of µg/mL, we were forced to explore other possibilities to have a strong and sensible analytical method. According to other general approaches in analytical chemistry, we decided to use an enzymatic cleavage to obtain a peptide that should be easier to analyze. The first stage of this new approach was a theoretical study, to highlight the differences between our recombinant protein and the naturally present native protein. This fact forced us to choose a peptide that is unique, that is relative only to our protein in order to avoid mismatch and false positive. But unfortunately the unicity is not enough, because we must choose a peptide that is different in the sequences of the 2 proteins. By matching these two conditions, the C-Terminal of our protein is a zone that perfectly matches with these descriptions. In fact the sequence of the recombinant protein is longer than the

native one. After some evaluation, related to cleavage point, pH and temperature we chose the Asp-N; Endoproteinase. AspN (flavastacin) is a zinc metalloendopeptidase which selectively cleaves peptide bonds N-terminal to aspartic acid residues; this kind of cleavage gave us a 26AA residue with a MW of 3165.6 KDa. With the Mass spectrometer software, we simulated the results of what we should obtain in mass spectrometry. After this simulation, we set up the first digestion, using 100 µg of our protein, following the digestion protocol reported on the sigma Aldrich datasheet. The digested solution was then injected using a new analytical method with a 70 minutes gradient, keeping the same mobile phase as the intact protein, because the IP of this peptide is similar to the intact protein, in fact is around 9.19, due to the high number of lysine.



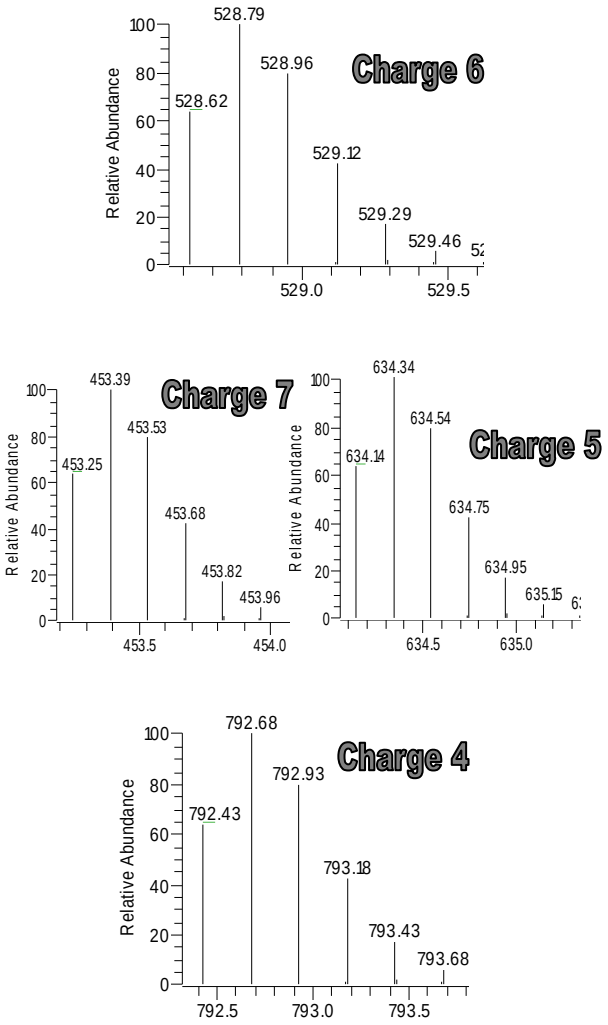


Fig 65: High resolution simulation of different charge state for the C-term peptide.

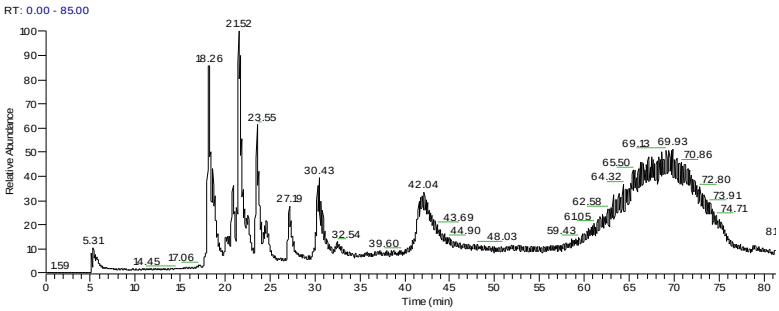


Fig 66: peptide mapping analysis

The chromatogram shows a peak around 21.5 minutes, and this peak is relative to our peptide. From this point, we go on talking about concentration, referred to intact protein equivalent concentration, and not referred to the real peptide concentration present in the sample.

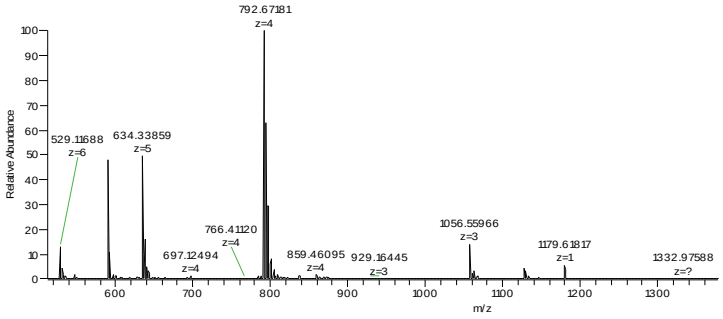
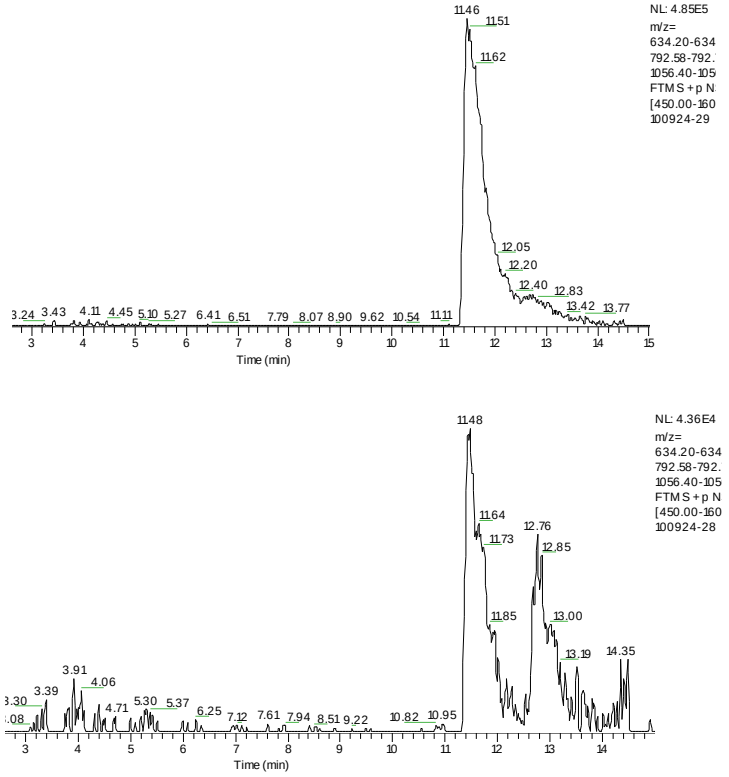


Fig 67: mass spectra of 21.5 minutes peak

The following step was the development of an appropriate tuning file for this molecule, with an

infusion of a 1 µg/mL solution of our peptide. With this new tuning file and with the addition of pentammine, a curve was tested from a concentration of 0.48 ng/mL to a concentration of 4800 ng/mL. The results highlighted that the lower visible concentration, actually, is 48 ng/mL.



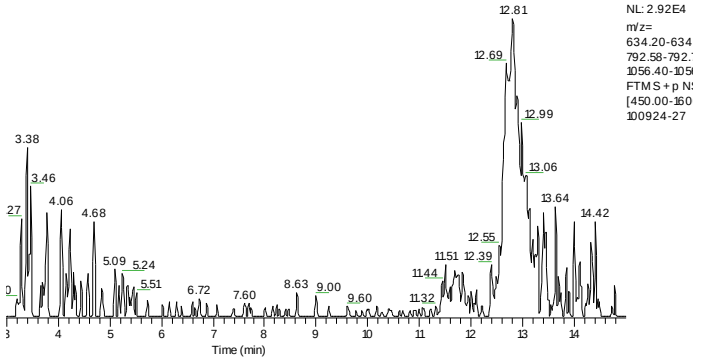


Fig 68: in file 100924-27 the 48 ng/mL concentration, in file 100924-28 the 480 ng/mL concentration and in file 100924-29 the 4800 ng/mL concentration.

To increase the instrumental sensibility we decided to switch to the purge and trap configuration, because we hoped that the peptide was not affected by non-specific binding as the whole protein. According to fundamental principles of reverse phase chromatography, a c8 cartridge was chosen as pre-column, because a C18 was chosen as analytical column. In the first analysis, where the unlock from the pre column was performed by a gradient, no signals of our peptide was seen in the final chromatogram. This was caused by the high percentage of organic necessary to detach the peptide with the gradient program. A new accurate study was conducted to test

the possibility to detach the peptide with an isocratic low organic condition. The pre column was tested alone, with a variable percentage of organic from 10 % to 20 %.

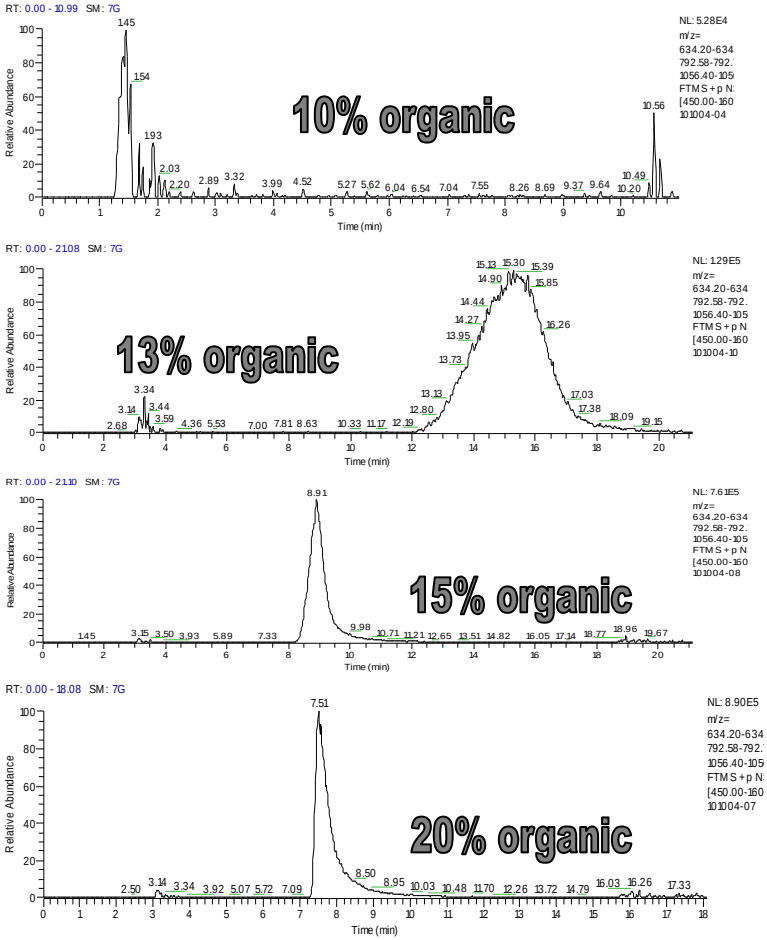


Fig 69: Chromatograms of different starting organic percentage

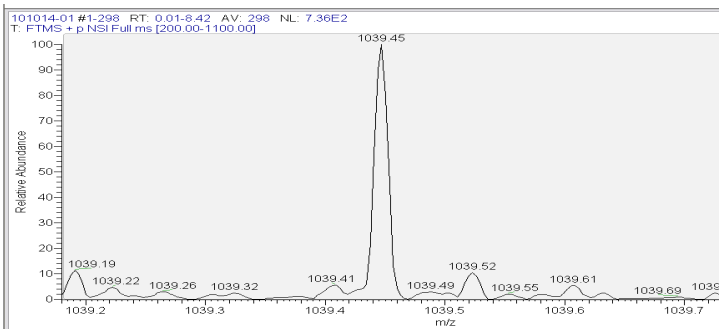
At 10 % of organic, the peptide was retained by the cartridge, a too small organic percentage to have the unlock from the stationary phase. To have a better conditioning of the analytical column, unless the peak is very broad, we decided to go on with 13% of organic mobile phase, because a smaller percentage could help the second column retaining all the molecules of analyte. Several injections of c-term peptide were performed, to explore the LOQ, but the limit obtained was 480 ng/mL, maybe because, as like as the whole protein, the higher length of the pipeline increases exponentially the non specific binding. In this case the solo micro system seems to be more performing than the purge and trap solution.

The N-Term Approach

The chromatographic development

Verifying the protein structure, one of the few points where the isoelectrical point is under 7, is the N-term. According to what we said before, the N-term has

some sequence differences, so it's usable to differentiate the recombinant form from the native form. After an enzyme analysis, the three possible enzyme candidates are: lysine-C, Trypsin and Arginine-C. With lysine-C we obtain a 24 AA peptide instead of 8AA peptide obtained with the other two enzymes. We performed the three digestion procedures with a larger amount of protein, in order not to have sensibility problem, and then, we checked them by direct infusion in mass spectrometry. Unfortunately, lysine-C didn't give us any desiderate peptide, maybe due to the length of this peptide. The other two enzymes digested our protein and then we compared their performances.



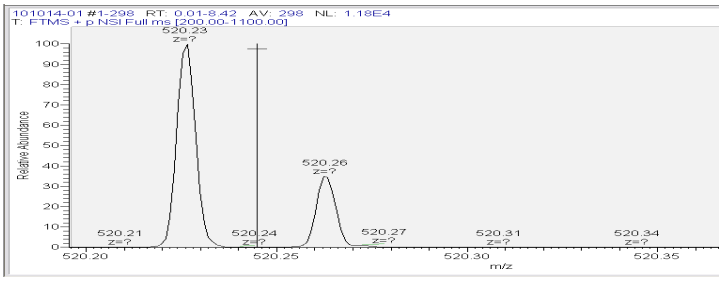


Fig 70: charge state 1 and charge state 2 for the new peptide, Arg-C digestion

By comparing the two enzymes that digested the same amount of protein, Trypsin seems to be more performing, and including that fact that is cheaper, Trypsin has been chosen.

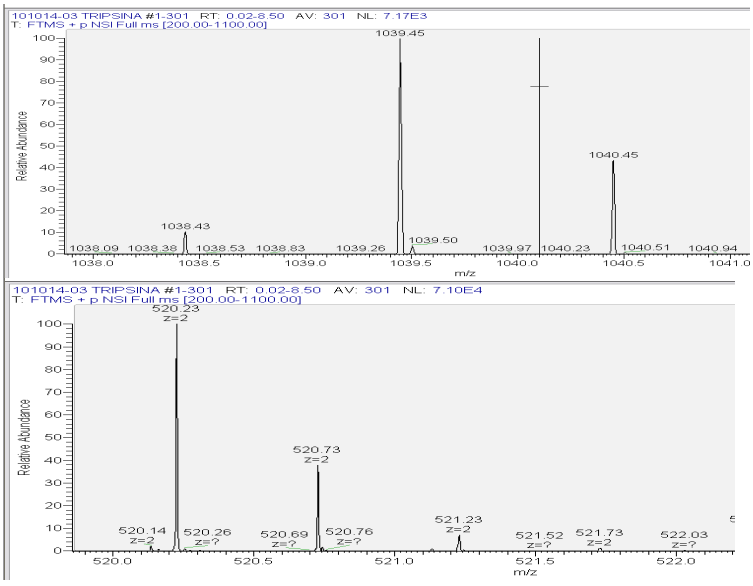
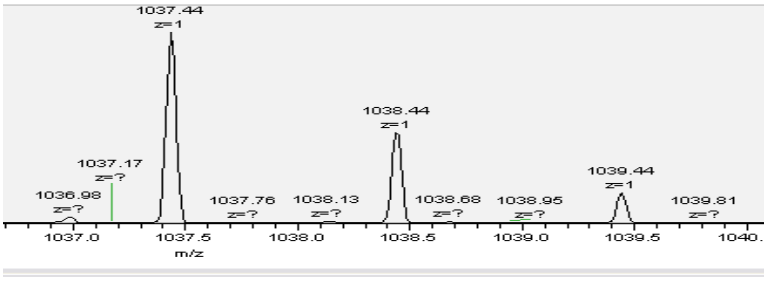


Fig 71: charge state 1 and charge state 2 for the new peptide, Trypsin digestion

According to the isoelectrical point of this new peptide, that is 4.2, the molecule has some chance that must be seen in negative mode. Negative mode usually gave less signal, because the ionization it's more difficult to do. This means also that fewer substances are present in the chromatogram and so there's usually a better signal to noise ratio.



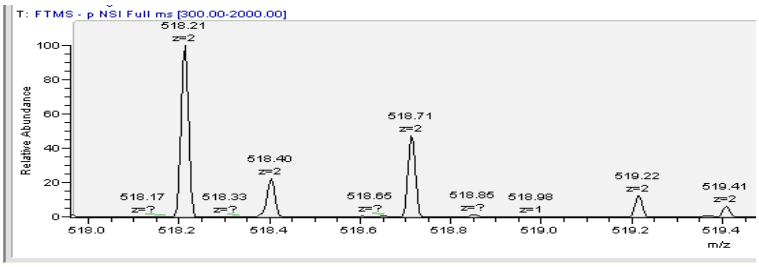


Fig 72: negative acquisition of digested peptide.

As a first approach of the new peptide analysis, we decided to find the optimal column for this analysis,

because we had a different set of parameters that could be chosen including different inner diameter and different stationary phase. As a first approach we tried to use 2 different temperatures, 25 and 40 °C and we saw that there was no difference and the master system pressure was low, so we decided to operate at r.t. to give less stress to the entire system. The starting mobile phases used for this study were Water and ACN, plus 0.1% of formic acid for both phases. After some screening, we decided to use HRMS-55 that gave us the best performance in term of intensity of the signal and reproducibility. A first curve was set up in order to test the sensibility for this new column. The result was encouraging, because without a specific tuning file, the actual sensitivity was 1.9 ng/mL, considering protein concentration equivalent. The results were affected by a little carryover phenomenon, because in the replicate injection for every point, the area value was higher.

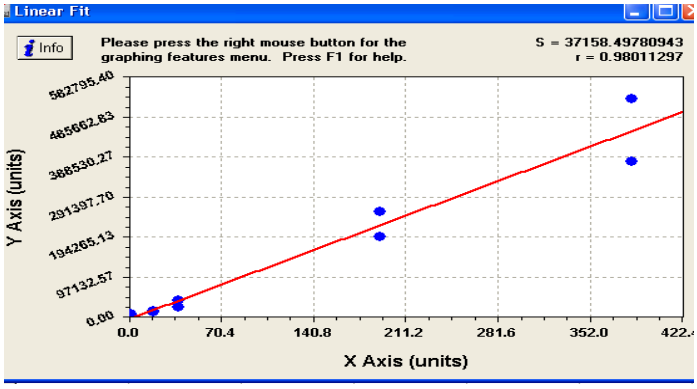


Fig 73: Calibration curve of peptide.

On figure 73 clearly appears that the carryover phenomenon was present when the concentration was upper than 10 ng/mL and huge when the concentration was upper than 100 ng/mL. Despite the carryover, the R^2 value was still good. The analyses performed were made with a general tuning file. 2 tuning files, one for each charge state, were developed in order to move all the ions produced in only one charge state to improve the signal. The new tuning files were tested with two different concentrations, 1.9 and 3.8 ng/mL, to find which was able to give us the best sensitivity.

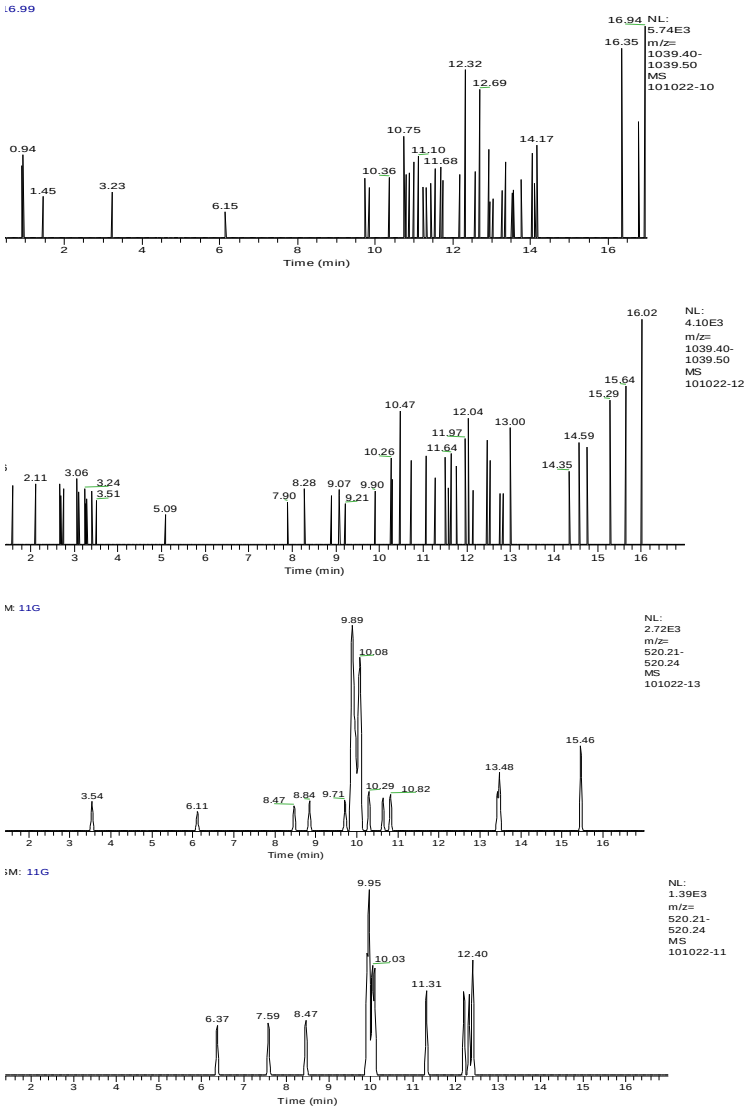
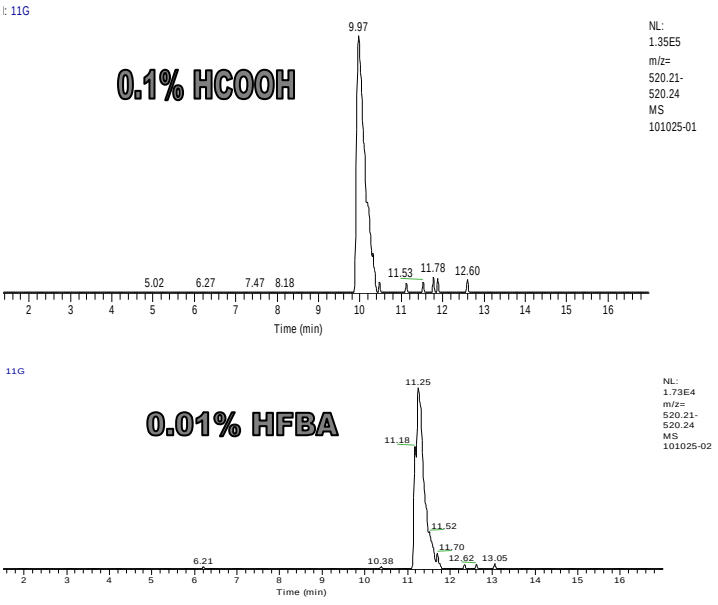


Fig 74: in file 101022-10 1.9 ng/mL with charge 1 tuning file, in file 101022-11 1.9 ng/mL with charge 2 tuning file, in file 101022-12 3.8 ng/mL with charge 1 tuning file, in file 101022-13 3.8 ng/mL with charge 2 tuning file

The chromatogram highlighted that the tuning for charge 2 is more performing than the other one, because we obtained a peak for our peptide. After this decision, a short mobile phase set up was performed to test several conditions for chromatographic analysis. 4 conditions were tested in triplicate, the starting condition was: HCOOH 0.1%, the whole protein condition was: 5% HCOOH and 0.01% HFBA, the negative condition was: 0.1 % NH_3 and a fourth condition with only the 0.01% of HFBA.



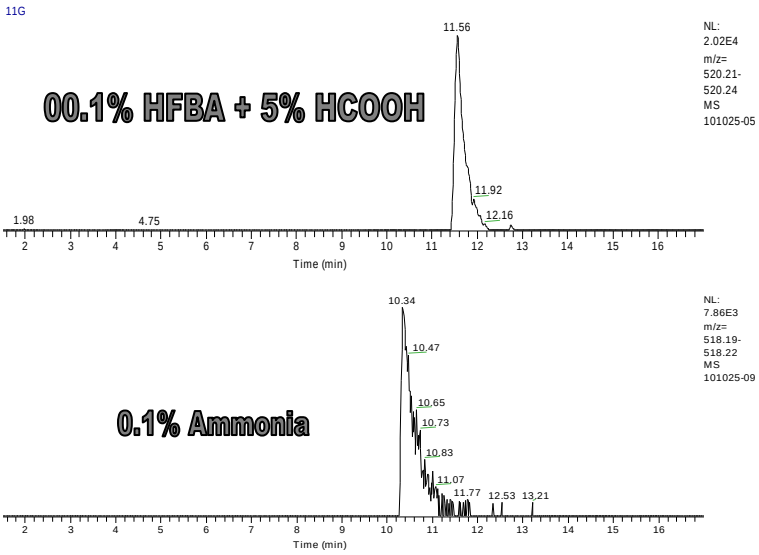


Fig 75a: Mobile phase screening chromatograms.

The data obtained by the chromatography underlined that the starting condition was the best condition, so we decided to keep going on with this mobile phase. Whit this new tuning file and the formic acid mobile phases, we tested the new instrumental sensitivity and we had an huge improvement, because move the LOQ down to the pg/mL

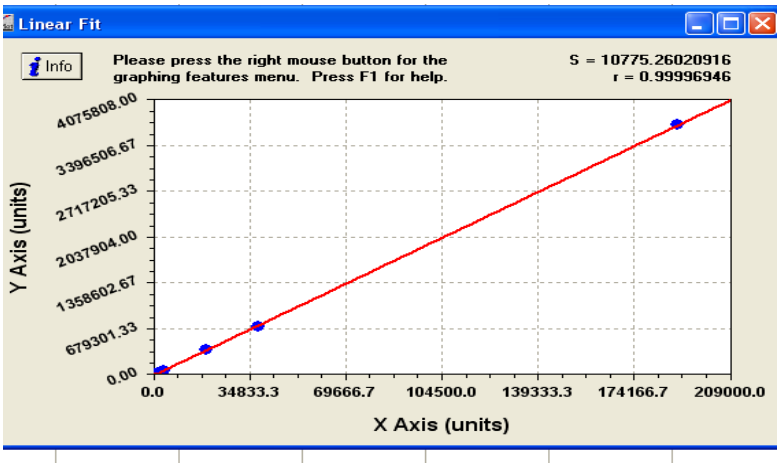


Fig 75b: Calibration curve for peptide charged $^{+2}$

As we can see by Fig.75b we obtained a strong linearity for this peptide from around 300 pg/mL to 190 ng/mL. After this kind of strong data replicated different times, we decided to move on whole methods, starting from the extraction development.

The extraction development

Once pointed the chromatographic condition, we moved to the purification procedure. As first approach we decided to try an SPE. The first stationary phase was a Strata-X polymeric, provided by Phenomenex; the protocol followed was the standard one provided with

the plate. We decided to use both MeOH and ACN as organic solvent, and different % of organic solvent in elution solution were tested (2/5/10/20/50/100). This screening was made in order to find the best solution for the cleaning of the samples. All the steps involving the loading procedure and the washing procedure were checked in mass spectrometry, to see if some peptide molecules were lost during the extraction procedure. Casually, 2 loading solutions, once for every organic solvent were checked to see if they were clean or they contained some traces of peptide.

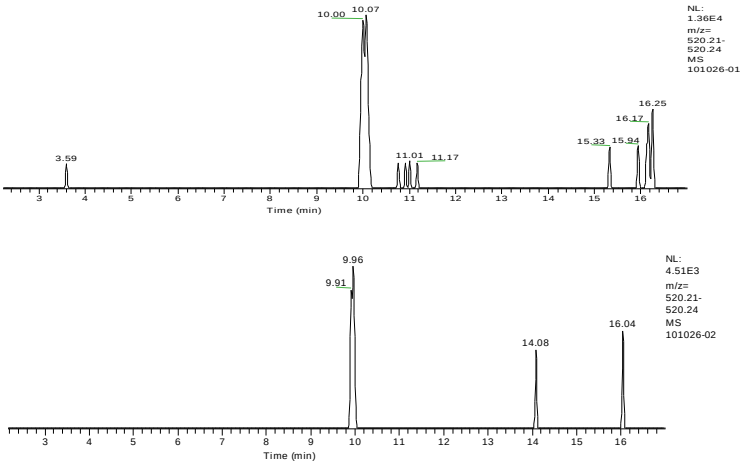
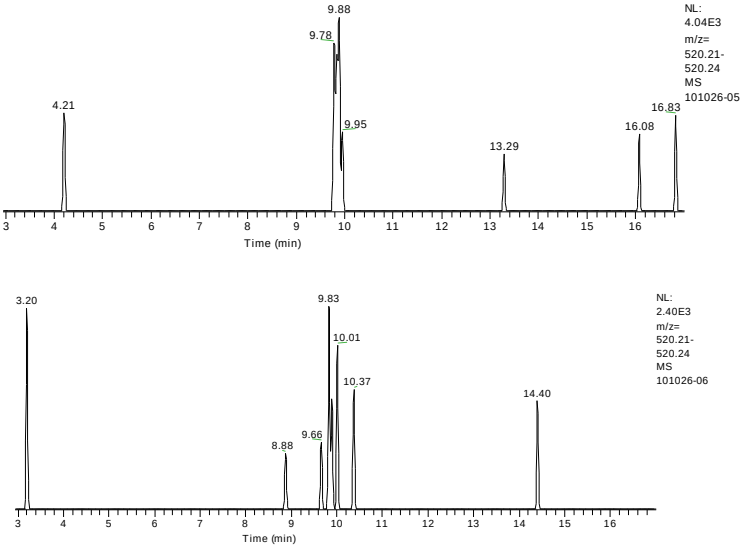


Fig 76: in file 101026-01 the ACN loading solution,
in file 101026-02 the Methanol loading solution

Both chromatograms showed the presence of our peptide. We thought that the peptide was not bonding the stationary phase, because the charging solution was basic (the peptide is dissolved in basic PBS for digestion) so, as confirm of that hypothesis, we charged a new pit with the same amount of peptide, but acidifying the solution before the charge. Three possible degrees of acidification were tested: an equal volume of HCOOH 0.1%, 98A/2B % initial mobile phase, HCl 100 mM were added to the loading solution.



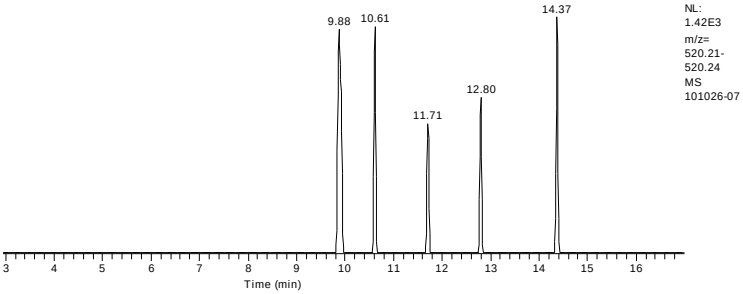
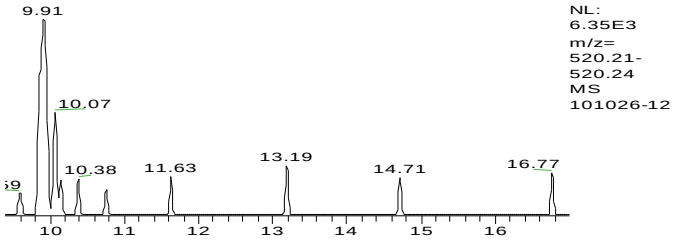
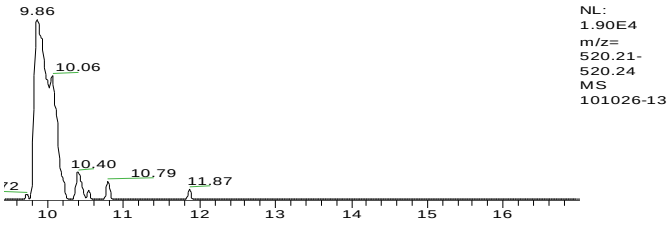


Fig 77: in file 101026-05 the formic acid addition, in file 101026-06 the mobile phase addition, in file 101026-07 the HCl addition

According to the chromatogram the mild acidification didn't work, because we still saw the presence of the peptide peak. In the last chromatogram, we didn't have the presence of the peak: that means that the charge was completely adsorbed by the stationary phase. Despite the partial sample lost during the loading stage, we decided to go ahead with the analysis of eluting solutions.

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10.40
10.79
11.87
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m/z=
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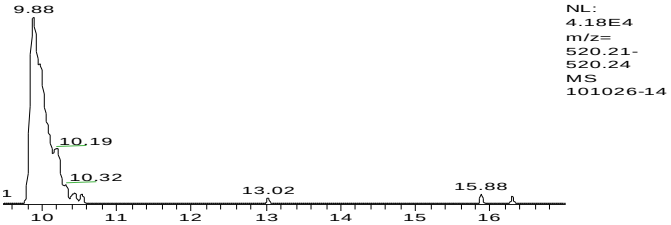
520.24
MS
101026-13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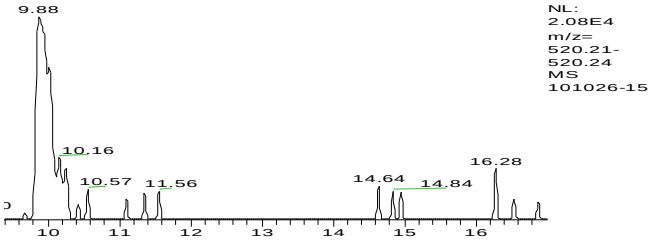
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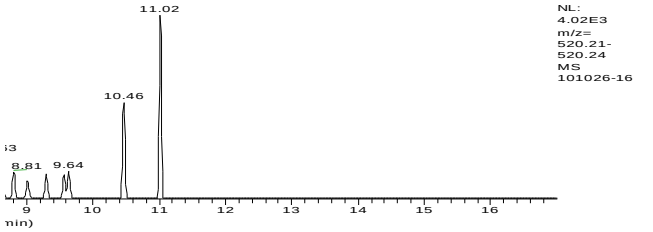
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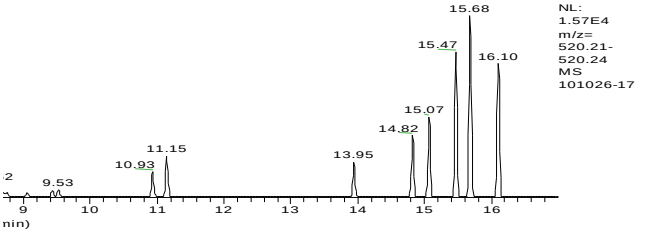
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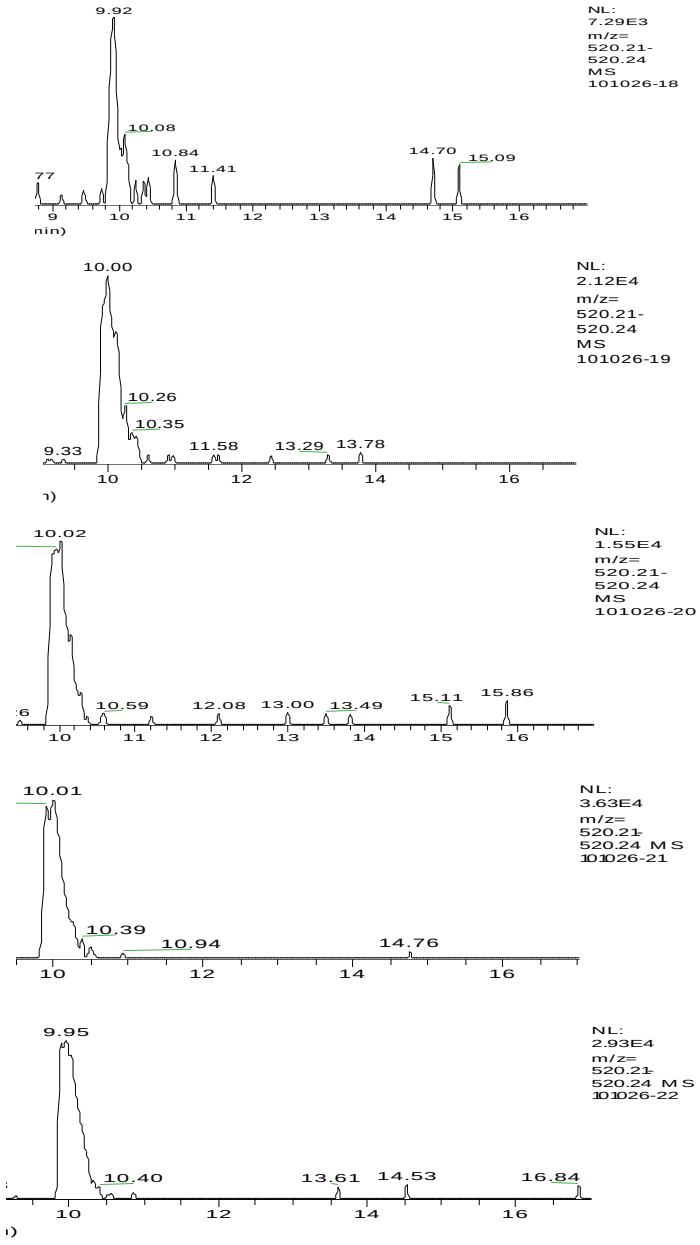


Fig 78: eluting chromatogram from SPE extraction

File	Eluent	%
101026-12	ACN	2
101026-13	ACN	5
101026-14	ACN	10
101026-15	ACN	20
101026-16	ACN	50
101026-17	ACN	100
101026-18	MeOH	2
101026-19	MeOH	5
101026-20	MeOH	10
101026-21	MeOH	20
101026-22	MeOH	50
101026-23	MeOH	100

Tab 09: table of extraction analysis.

The analysis highlighted that methanol was able to extract the sample in all the conditions, but ACN lack of extraction for percentage major or equal than fifty. Since we had a peak even for 2% of organic, the washing procedure before the elution should be made by 100% of aqueous solution. According to the results obtained, no best solvent were found in the screening, so we decided to go ahead. We set up a DOE on

extracting mobile phase. We decided to implement the sample charge with chloridric acid, as developed before.

	Exp No	Exp Name	Run Order	Incl/Excl	Tipo di organico	Acidificazione	Quantità di organico
1	1	N1	9	Incl	AcN	0	10
2	3	N3	7	Incl	AcN	1	10
3	5	N5	11	Incl	AcN	0	20
4	7	N7	2	Incl	AcN	1	20
5	9	N9	16	Incl	AcN	0	50
6	11	N11	17	Incl	AcN	1	50
7	13	N13	1	Incl	AcN	0	100
8	15	N15	6	Incl	AcN	1	100
9	17	N17	15	Incl	AcN	0.5	10
10	18	N18	3	Incl	AcN	0.5	10
11	19	N19	19	Incl	AcN	0.5	10
12	2	N2	13	Incl	MeOH	0	10
13	4	N4	10	Incl	MeOH	1	10
14	6	N6	4	Incl	MeOH	0	20
15	8	N8	5	Incl	MeOH	1	20
16	10	N10	12	Incl	MeOH	0	50
17	12	N12	14	Incl	MeOH	1	50
18	14	N14	8	Incl	MeOH	0	100
19	16	N16	18	Incl	MeOH	1	100

Fig 79: Experimental Design of SPE

By the fact that chloridric acid helps in loading the sample, we decided put it also in the eluting solution, we defined this parameter as “acidificazione”, in which the value 1 is 10 µl of 1M acid solution and 0.5 is 5 µL. Unfortunately, during this test, some sample were switched in elution step. In fact experiments 5/6 and also 9/10 were loaded with the correct organic wash, but then were eluted with the other organic solvent. Unfortunately, the lack of results for very highly organic experiments weren’t compatible with experimental

design. What we saw, by the way, confirmed what we obtained before, so high percentage of organic solvent in elution step gave us no results. By tabular the results obtained the picture of extraction appeared clearer.

	Standard	Filename	Area	% of Recovery
3	Digested protein 38.4 ng/mL	101027-02	534117	
4	Digested protein 192 ng/mL	101027-03	2724979	
5	Digested protein 384 ng/mL	101027-04	7756813	
6				
7	**ACN**			
8	AcN 10%	101027-12	1295678	**16.70**
9	AcN 20%	101027-14	506200	**6.53**
10	AcN 50%	101027-16	801540	**10.33**
11	AcN 100%	101027-18	26452	**0.34**
12				
13	**MeOH**			
14	MeOH 10%	101027-23	432360	**5.57**
15	MeOH 20%	101027-25	931107	**12.00**
16	MeOH 50%	101027-27	8428	**0.11**
17	MeOH 100%	101027-29	1619781	**20.88**
18				
19	**ACN 1/2 Acid**			
20	AcN 10% 1/2 acid	101027-20	1105705	**14.25**
21	AcN 10% 1/2 acid	101027-21	1598382	**20.61**
22	AcN 10% 1/2 acid	101027-22	1404678	**18.11**
23				
24	**Acid ACN**			
25	AcN 10% Acid	101027-13	666714	**8.60**
26	AcN 20%, Acid	101027-15	262401	**3.38**
27	AcN 50% Acid	101027-17	1902182	**24.52**
28	AcN 100% Acid	101027-19	5718	**0.07**
29				
30	**Acid MeOH**			
31	MeOH 10% acid	101027-24	186494	**2.40**
32	MeOH 20% acid	101027-26	933896	**12.04**
33	MeOH 50% acid	101027-28	2791806	**35.99**
34	MeOH 100% acid	101027-30	934217	**12.04**

Fig 80: table of extraction, in the final column the % of recovery compared with a standard.
File in row 9/10/15/16 are the extracted with the other solvent.

According to this data, we could easily see that the 100% CAN is not usable, because we didn't obtain any

recovery. The acid usage, in particular conditions seems to be effective in improving the recovery. Actually the best condition obtained is with 50% of MeOH, full acid solution that gave us 36% of recovery. Based on this data, we decided to abandon the ACN and in order to have a better and more detailed investigation with methanol. Due to the fact that experiments with 100% of ACN were not effective in eluting our peptide, we thought that maybe a non protic organic solvent wasn't functional for detaching the peptide. We decided to try if this was true, by setting up a new experiment with a double wash procedure. Two experiments underwent first a formic acid wash and then a second wash with 100% of ACN. By the data reported in Fig.81, it's shown that the general recovery is lower than what we obtained the day before. This could be attributed to a possible clogging of heater capillary. The results obtained highlighted that the best elution condition is around 50 to 80 % of organic solvent in elution solution. The double wash procedure, eluted with methanol or acetonitrile, gave us bad results lower than what we obtained with a single wash step.

39	190 ng/mL standard	101029-01	3966757	
40	20% MeOH acid	101029-05	964265	12.43
41	30% MeOH acid	101029-06	30625	0.39
42	40% MeOH acid	101029-07	437446	5.64
43	50% MeOH acid	101029-08	736214	9.49
44	60% MeOH acid	101029-09	781634	10.08
45	70% MeOH acid	101029-10	690236	8.90
46	80% MeOH acid	101029-11	629300	8.11
47	90% MeOH acid	101029-12	333819	4.30
48	100% MeOH acid	101029-13	274363	3.54
49	50% MeOH non acid	101029-14	1080126	13.92
50	50% MeOH acid, double wash	101029-15	292670	3.77
51	50% AcN acid double wash	101029-16	173924	2.24

Fig 81: table of extraction with different % of MeOH

Based on these results, we decided to explore more in details the experimental domain, we include iPrOH in the experiments and we tried different acid for eluting the sample, like TFA, HFBA.

Nome File	Condition	Area
101105-09	MeOH 50% non acid	689864
101105-10	MeOH 50%, acid	769728
101105-11	MeOH 50% ½ acid	17010
101105-12	MeOH 50% ¼ acid	24607
101105-13	MeOH 50% +0.1% HFBA	0
101105-14	MeOH 50% +0.1% TFA	263839
101105-15	iPrOH 10% acid	23878
101105-16	iPrOH 20% acid	18237
101105-17	iPrOH 50% acid	85225
101105-18	iPrOH 80% acid	77128
101105-19	MeOH 50% non acid	139946
101105-20	MeOH 50%, acid	229548

Tab 10: Peak area obtained for extraction experiments

The best result obtained was still the MeOH 50%, both acid or non acid gave us the highest area. The other acid or the iPrOH didn't reach the same performance of these conditions. 2 replicates were analyzed after all the others samples, in order to see if the clogging phenomenon was affecting the final results, but we saw that the 2 final experiments were worse than the first analyzed, but still better than the others, so we could easily state that 50% of methanol was the best extracting condition by the moment.

Autosampler Optimization

The reconstitution of sample is one problem that could affect our sensitivity results. Because more volume for reconstitution means more sample to inject and more replicates, but also means lower concentration. Finding the equilibrium is a necessary step, in order to find the best condition to develop the entire method. We decided to set up an accurate experimental design; according to what the instrumentation set up allows us to do. The two major players of this DOE are the Buffer tubing and the loop volume. Since the Buffer tubing is

50 µL and the loop is 5 µL, the total injectable volume is 54 µL. At the end we decided to screen two factors, the injection volume, and the flush volume, already tested in past experimental designs. What we took as responses were the area obtained and also the volume consumed.

	1	2	3	4	5	6	7	8
	Exp No	Exp Name	Run Order	Incl/Excl	Volume Iniezione	Flush Volume	Area Picco	Consumo Reagente
1	1	N1	9	Incl	3	2.4	35731	5.4
2	7	N7	7	Excl	4	2.4	244170	6.4
3	2	N2	4	Incl	5	2.4	97685	7.4
4	12	N12	12	Incl	3.5	11.2	192700	14.7
5	14	N14	14	Incl	4.5	11.2	278783	15.7
6	5	N5	11	Incl	3	21.2	402390	24.2
7	9	N9	2	Incl	4	21.2	465653	25.2
8	10	N10	8	Incl	4	21.2	298904	25.4
9	11	N11	3	Incl	4	21.2	209395	25.4
10	6	N6	5	Incl	5	21.2	412201	26.2
11	13	N13	13	Incl	3.5	31.2	492649	34.7
12	15	N15	15	Incl	4.5	31.2	400827	35.7
13	3	N3	1	Incl	3	40	463014	43
14	8	N8	10	Incl	4	40	464485	44
15	4	N4	6	Incl	5	40	533032	45

Fig 82: Table and results of experimental design.

The experiments were set up in order to fit a CCF design, a quadratic model that could explain all the possible effects present in the autosampler. The trial number 7 was excluded by the experiment because it was clearly affected by carryover as it was preceded by a larger sample injection. The model obtained after this exclusion was very good, in fact we had an excellent

model validity supported by two strong parameters like R^2 and Q^2.

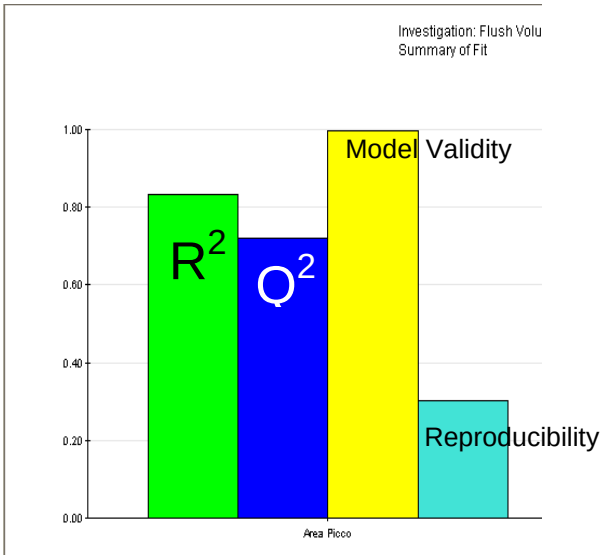


Fig 83: Model parameters

A clear picture of what happens with autosampler parameters variation is given by the contour plot of the model.

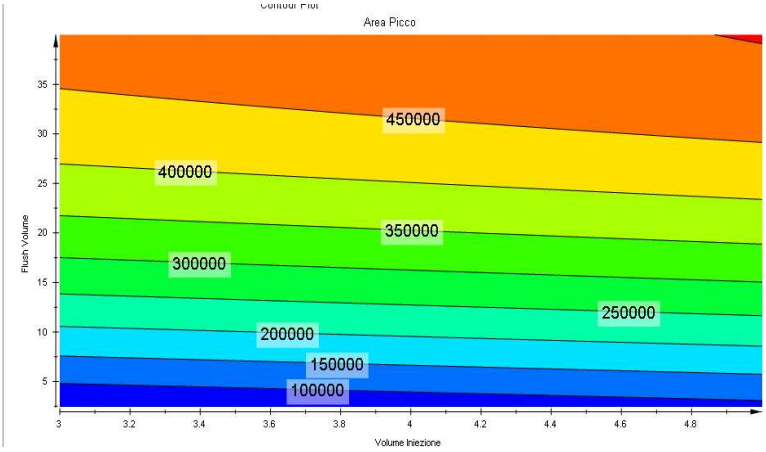


Fig 84: Contour plot of peak area

We saw that the flush volume was the real important parameter that really helped in improving sensibility, due to the fact that the volume variation for these parameters is higher. The injection volume still played a role, in fact, gave a little help to our signal. According to this model, we decided to verify which could be the optimal condition for us, so we set for response the maximum for peak area and the minimum for reagent usage.

1	2	3	4	5	6
Volume Iniezione	Flush Volume	Area Picco	Consumo Reagente	iter	log(D)
3.0042	3.8843	82374.3	6.9648	60	1.6622
5	36.5209	491504	41.4433	129	1.6696
3.0302	3.88	82720.8	6.984	68	1.6616
5	37.3693	494637	42.2855	93	1.6879
5	22.6361	392378	27.61	79	1.3701

Fig 85: Optimizer result.

A similar condition was still present in our DOE: a central point that used 21.2 µl as flush volume. We decided to keep this condition as our best condition and go ahead with the development.

According to the previous indication obtained by the peptide extraction, we decided to set up a new experiment, with methanol, in order to decide the final % of methanol present in the extracting solution.

Two experiments for each condition were performed in order to find the best eluting solution from 50 to 70 % of MeOH.

71	50% MeOH acido	101129-05	1426670	29.41
72	50% MeOH acido	101129-06	1519207	31.32
73	55% MeOH acido	101129-07	1086369	22.40
74	55% MeOH acido	101129-08	1829984	37.73
75	60% MeOH acido	101129-09	1493278	30.78
76	60% MeOH acido	101129-10	1622044	33.44
77	65% MeOH acido	101129-11	1901746	39.21
78	65% MeOH acido	101129-12	2195283	45.26
79	70% MeOH acido	101129-13	2489499	51.32
80	70% MeOH acido	101129-14	2219630	45.76
81	Standard 190 ng/ml	101129-15	4850739	

Fig 86: table of % Recovery for peptide

The first complete method

The results clearly showed that the best conditions for peptide extraction were between 65 and 70 %, with a

little preference for 70 % in term of net results. By the way, more organic means detaching more substances, and so, we chose 65% in order to obtain almost the same recovery but with the possibility to detach less unwanted molecules from the solid phase.

The whole extraction and analysis method was performed the following day. The samples were tested in serum, and checked in parallel in buffer, to test if there were some clear differences between the matrix and the buffer. The final concentrations tested in serum were 6.4/9.6/48/480/4800 ng/mL in protein equivalent. The final concentrations for buffer sample were 0.24/0.48/2.4/24/240 ng/mL. To have a complete picture we decided also to extract two blanks. A sample of our peptide was digested in buffer and then added to serum, or kept in buffer. These two samples were extracted and their concentration was 3840 ng/mL. The extractions results showed clearly the digestion problem. The two spiked samples added to the blank, both serum and buffer were extracted as expected, and the two chromatograms were reproducible, so the matrix didn’t interfere with the extraction procedure.

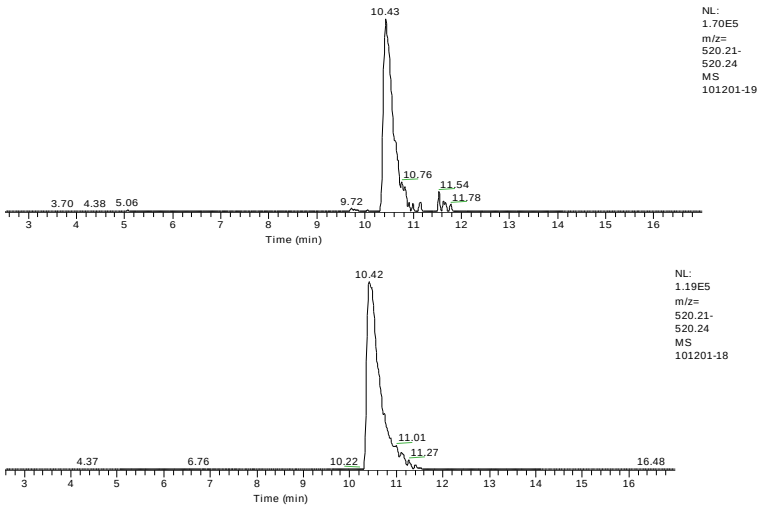


Fig 87: in file 101201-19 the serum spiked blank
and in file 101201-18 the buffer spiked blank

The other extraction files were completely blank, we didn't think about digestion troubles and we switched our attention to the stickiness of the protein, because all the concentrations were prepared in buffer and only the last dilution was performed in matrix for serum extractions. According to the obtained results we decided to perform all the diluting steps in serum, in order to have a large amount of proteins that should shield the protein from adhesion. The peptide concentrations in serum for the digestion and

extraction procedure were 2.4/24/48/261/480 ng/mL. We coupled these analyses with pre digested peptide spiked in the same matrix. In this case the digestion was performed in buffer and we eliminated the digestion problem. The concentrations used for this procedure were 3.8/18/38/180/380 ng/mL. The results obtained gave us a brighter picture of the situation, in fact, for diluted and extracted sample we obtained a LOQ of 18 ng/mL.

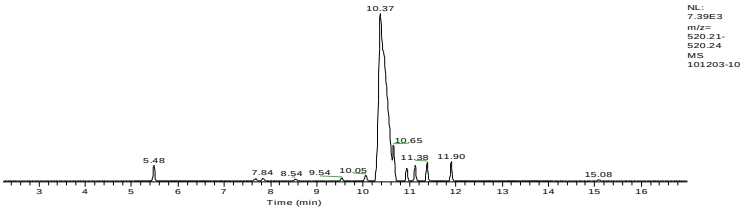


Fig 88: 18 ng/mL diluted and extracted

Moreover, all the digested and extracted samples didn't show any signs about our peptide. This results moved the attention to the digestion procedure, in fact, this results highlighted that we were not able to obtain a complete digestion of our protein. For this reason we needed a new approach for sample digestion. The novel approach for protein digestion was the use of Digestips. Digestips are pipette tips coated

on top with immobilized Trypsin. This kind of tips works well in ammonium formate buffer, that is perfect for a mass spectrometry analysis. According to the instructions given us by the provider, we can digest the solution just pulling and pushing off the solution from the tip. In order to have the best functionality of the tip, the immobilized tripsin should be washed before the digestion with ammonium formate. We decided to digest a 480 ng/mL solution, which after some dilutions and some other steps reached a final injected concentration of 109 ng/mL. This approach was studied in order to reduce the time that the proteins stays at r.t. or higher temperature, and to have a faster production of peptide that is less sensible in term of non specific binding.

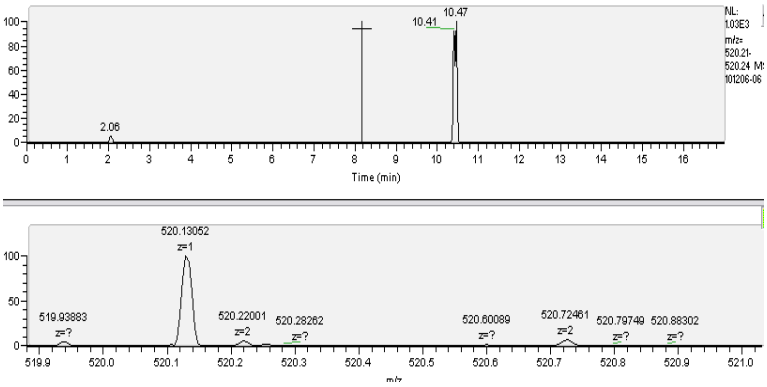


Fig 89: the peak obtained with Digestips digestion.

According to the chromatogram and the mass spectrometry data, Fig.89, we obtained a peak, so this approach appears useful. There were some troubles regarding the area of the peak, because it appeared clearly lower than what we obtained by in solution buffer digestion, but this method is for sure a shorter approach for protein digestion. To improve this first result, two possible pathways were opened to improve the result. The first and more immediate was to improve the digestion time, for this reason, we made one experiment that took 5 minutes instead of 1 in which the digestion is performed. The second and more fascinating was the use of Rapdigest. Rapdigest, provided by Waters, is an unknown mixture of surfactants that should help the digestion by opening the protein by unfolding process, so the enzyme has an easier access to the cleavage site. Both of these strategies were successful, compared with the previous result.

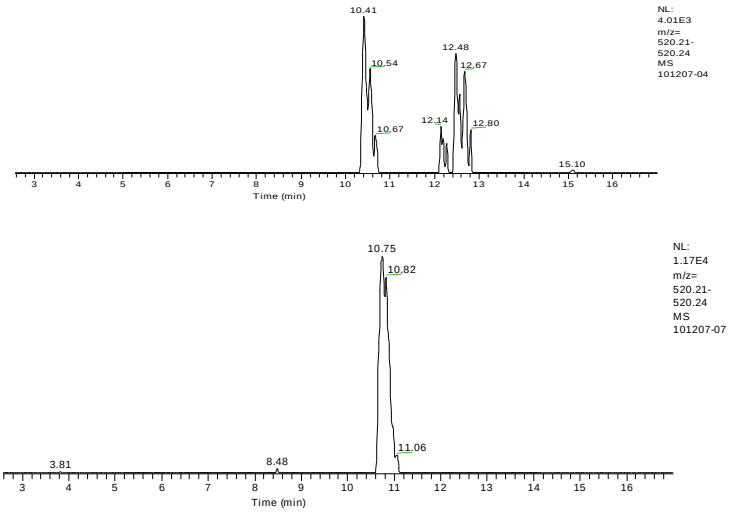
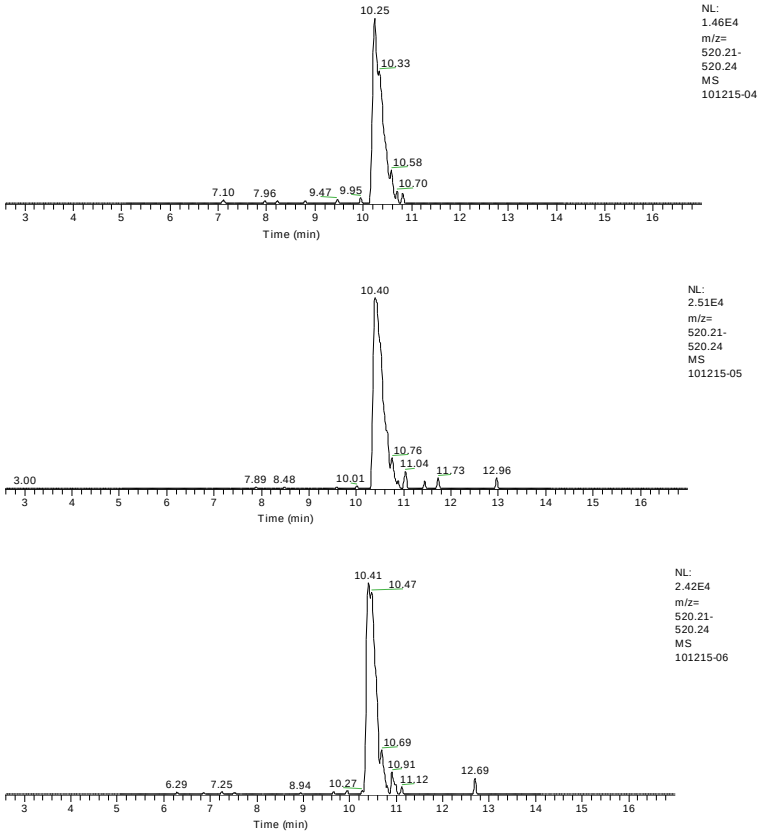


Fig 90: in file 101207-04 the five minutes digestion,

in file 101207-07 the Rapidgest digestion

As showed by chromatogram in Fig.90 Rapdigest is more effective in increasing peptide signal than in increasing the time. For this reason, we decided to explore the possibility to use more concentrated Rapdigest, to see if the improvement is linear. One last set of experiments was performed in order to try if the classic in solution digestion was completely not working. A huge amount of trypsin was added, since we reached a 10 times higher concentration than the one used in the previous experiments. Also Rapdigest were added to the solution, to see if this surfactant could

prove some improvement even for this kind of digestion, but none of these new conditions worked. After these results, we got back to solid phase digestion, and we tested if the concentration of Rapdigest was correct to obtain the digestion improvement. Some experiments were performed, but there wasn't a real improvement in terms of sensibility.



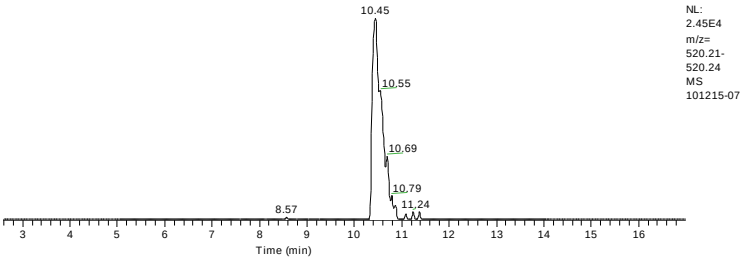
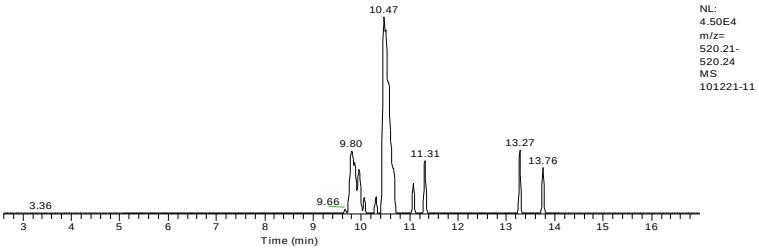


Fig 91: in file 101215-05 and 101215-05 the two 1x Rapdigest digestions, in file 101215-06 and 101215-07 the 2 10X Rapdigest digestions

Considering also the cost, we decided to use only 1x Rapdigest. With this new conditions set up, we performed the complete extraction procedure, from serum. 200 µL of spiked serum with the following concentrations of the protein: 1.2/2.4/4.8/12/24/48/120/240/480 ng/mL. The total volume of sample was diluted 1:1 in Rapdigest and after a 10 minutes manual pipetting digestion the samples passed to the SPE extraction procedure.



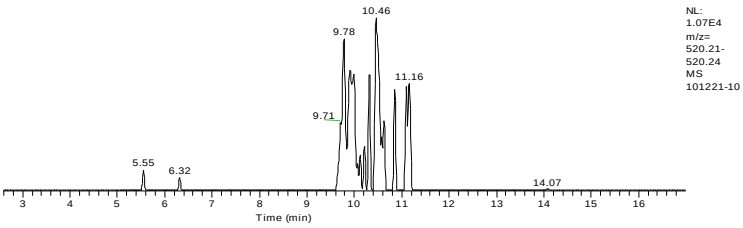


Fig 92: in file 101221-11 the 480 ng/mL concentration,
in file 101221-10 the 240 ng/mL concentration

For all the extracted concentrations, only the two highest were seen. This means that we still had some trouble passing from the buffer to the serum, maybe still due to the digestion procedure.

In order to develop the digestion procedure, we found many literature articles that use ultrasounds as digestion booster. With cavitation effect, ultrasounds give kinetic energy to the molecules without heating them. So we decided to test if ultrasounds could help us having a better digestion. We decided to perform this test in buffer, because we wanted to test the real effect of ultrasounds and so we wanted to avoid the purification steps. Ultrasounds were used at r.t. and at 40°C for 5 minutes, and checked with a control solution, normally digested, in order to see if we could

find some differences between these 3 different approaches.

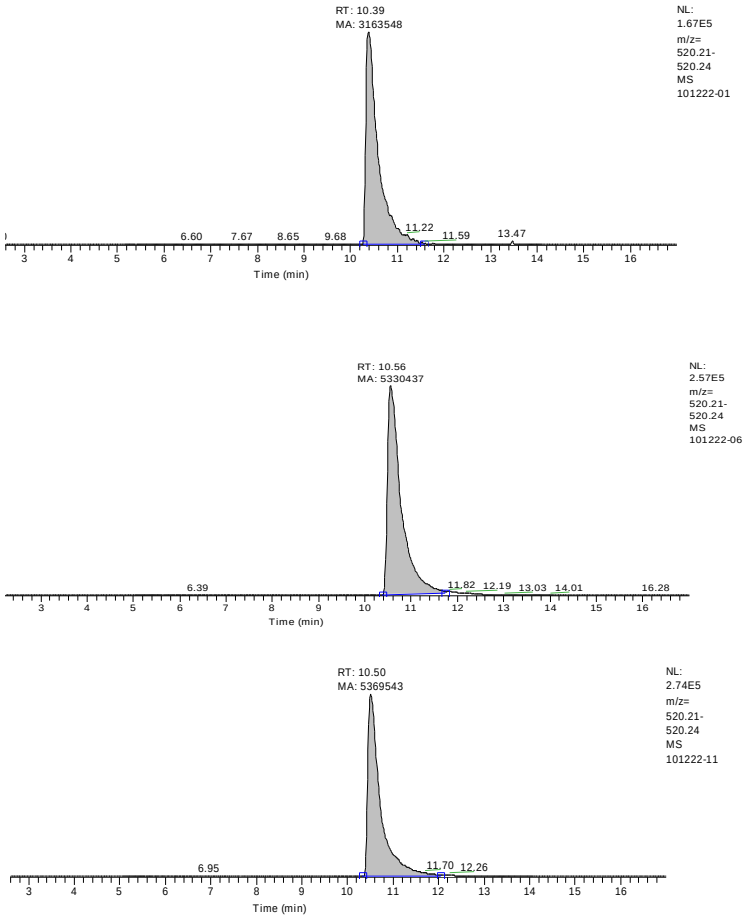


Fig 93: in file 101222-01 the r.t. ultrasounds digestion in file 101222-06 the 40°C ultrasounds digestion and in file 101222-11 the classic in solution digestion.

According to the area value found in the chromatograms Fig.93, we saw that ultrasounds had no effect, because the two area values of high temperature digestion were almost the same and the r.t. digestion was even less active than the normal one. At this point, internal standard was tested in order to have the complete method available for a theoretical validation. A Peptide was ordered, with a length of 21AA, so with 1 cleavage site, useful for us to check the digestion. To have a differentiation between the original peptide and the internal standard, 1 AA of primary sequence was substituted with an analogue, so we had a difference in m/z to distinguish our peptides. The internal standard peptide was digested, and then analyzed in order to optimize the tuning file also for the internal standard. Some analyses were conducted in order to find if the chromatographic condition were good even for the internal standard. A mixture of the two peptides was injected at different concentrations. The basic solution was at 31.6 μ/mL in protein and 26.6 μg/mL in buffer; both concentrations are referred to starting protein concentrations.

Fig 94: file 101226-07, the black line is the peptide signal and the red line is the internal standard signal

From the chromatogram Fig.94 we saw that the internal standard had almost the same response as the peptide and also the retention time is similar.

Protein Precipitation

As a second approach for peptide purification, we decided to use protein precipitation. Protein precipitation is the simples approach to purification, in fact an adequate amount of organic solvent is directly added to the biological matrix. In this way several biological proteins precipitate and after a step of centrifugation, the solution should be ready to be analyzed.

As a first approach to protein precipitation development, 200 µL of serum, at concentration of 600

ng/mL of protein and 500 ng/mL of internal standard were digested with Digestips with the previous procedure. We decided to add 700 µL of a MeOH/Water mixture 95/5. We decided to add a small amount of water, to be sure that, when we add the organic solvent, we still have a little amount of “local” water: in order to maintain in solution our peptide. As first condition, after this addition, the sample was centrifuged for 20 minutes at 14000g r.t. The liquid obtained after precipitation was brought to dryness. The result that we obtained for this procedure is in the chromatogram shown in Fig.95

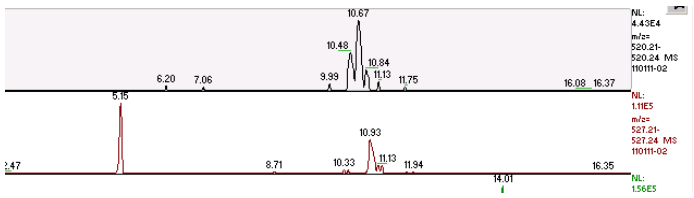


Fig 95: chromatogram of protein precipitation

As we can see, the peak obtained for our peptide had a very uncommon shape, maybe due to the large amount of undesired substances that enter in to the column with this purification method. To avoid strange phenomena in column, we decided to inject again the

same solution and put a blank after every injection in order to have a major column cleaning.

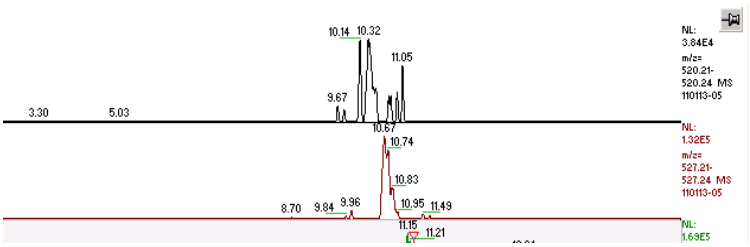


Fig 96: peak shape with blank injection before and after the sample

According to the peak area obtained, we saw that we had an improvement compared to SPE techniques, so we thought that protein precipitation is more efficient in peptide extraction. Unfortunately this technique seems to be dirtier than SPE and this fact impacts the analysis, because by injecting more undesired substances both the column and the instrument suffer of clogging phenomenon and lose sensitivity very rapidly. For this reason we tried to develop again other digestion procedures. Actually, with Digestips, the only part of digestion that could be modified is the time, so the step in the trypsin resin. To see what would be the time dependent improvement a method on the automatic liquid handling station Hamilton was developed in house. The fluid class necessary to

manage serum with these tips was provided by Proteogen Bio and with this automatic method we were able to improve 10 times the time of resin processing. We used the same concentrations as the day before, so we had a direct comparison between the two digestions, and then, after digestion, we purified the samples by protein precipitation. The results obtained showed that a time improvement is useless, because we obtained the same peak area with both techniques.

As last resource to modify the digestion protocol, we used a blank column in which we immobilized by ourselves trypsin, and then we performed an online digestion in the HPLC, by stopping the sample in the column volume. All the procedures done for this column conjugation were performed as suggested by the provider. As a first impression, the high concentration of NaCl, that is not a mass spectrometry friendly salt, could be a problem, because after some washes the amount of NaCl still present in the column seems to be higher than expected. Some buffer online digestion experiments were performed, but none of them showed any signal about our peptide and since it

is a very dirty technology for mass spectrometry standard, we decided to abandon this procedure.

The Magnetic beads digestion

When we developed the whole protein procedure, we did not know if the non specific binding could be due to beads or to the HPLC, or maybe both. Supposing that on magnetic beads we almost did not have any non specific binding, due to the procedure for beads preparation that includes washes with BSA, we tried to use this approach to purify the protein from the biological matrix and then we performed the digestion with the protein still on the beads. The beads were prepared in the same way as the whole protein methods, but we decided to modify some aspects, particularly the washing procedure after the antibody charge. We prepared 5 samples of beads:

- 3 samples in classic 0.1% BSA wash
- 1 sample in classic 0.1% BSA + 0.2% of Biotin
- 1 sample with Superblock used for ELISA test

The addition of biotin and the Superblock should help in coating beads and avoiding non specific binding. 200 µL of serum, at concentration of 600 ng/mL of protein

and 500 ng/mL of internal standard were placed on beads to form the immunocomplex. After two hours, the biological matrix was removed, and so the trypsin inhibitor was removed too. After this treatment the internal standard was added. The addition of internal standard after the matrix removal is due to the fact that the Ab for whole protein was not able to attach also this smaller peptide. After the internal standard addition, 100 µL of a 40 mg/mL trypsin solution was added to perform the digestion. The solution was placed O.N. at 37°C to have a complete digestion. The tree beads prepared in the classic mode were used in 3 different ways. One was used as explained just for a simple peptide catch. The second one was used for a sandwich approach, in fact we also use the secondary Ab used for the ELISA method in order to cover more protein surface and to reduce the non specific binding. The third approach with classic beads was slightly different. We made the immunocomplex before charging the Ab to the beads. So we had a free capture of Ab in solution, then we added the beads to this solution to form the binding between biotin-streptavidin. This long time for digestion was thought

because the protein captured on the beads would be less available to the cleavage enzyme. We thought that this approach could be useful for us, because we consider the N-term of the protein, an Ab free zone, because we imagined that the immunocomplex is formed in other parts of the protein.

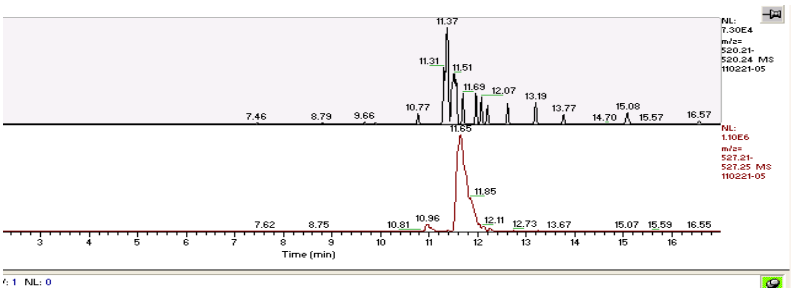


Fig 97: Magnetic beads sandwich approach

The analysis highlighted that the internal standard was always recovered, but the only chromatogram where we found a possible peak for our peptide was the one we digested with the sandwich approach. This could match with our theory that the monoclonal antibodies are able to cover our protein and so it is masked from non-specific binding.

In parallel with the magnetic beads capture before the digestion, we also tried one other approach to improve our signal: the double tip approach. We thought that if

the trypsin inhibitor is present in the solution, this should be captured by trypsin from the first tip, and so the second should operate much better. Unfortunately we could not consider this analysis because we had the peak from our peptide but the internal standard signal was missing.

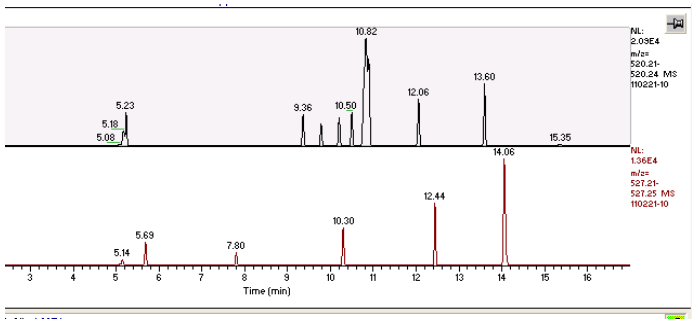


Fig 98: double tip digestion

Due to the problem found before, we changed column, because the performance was lowering and we had not anymore the required sensitivity. The column was changed with another one with the same characteristics. To find the best solution for digestion we set a number of parallel experiments:

- 2 Classic Digestips digestion + Protein precipitation

- 2 Double tip digestion + Protein Precipitation
- 3 Magnetic sandwich capture
- 2 High pH digestion
- 2 cytraconic anhydride

All these experiment were performed with 200 µL of serum, at concentration of 600 ng/mL of protein and 500 ng/mL of internal standard. The classic digestion was used as a control standard; we decided to use again the double tip to have a better comprehension of the behaviour of this technique. The magnetic beads capture was set up in triple to see if the results obtained before could be confirmed. We also decided to use an high pH, major than 10.5, in order to reduce the amount of positive charge on the protein and to reduce the non specific binding. This wasn't used before, because trypsin is active from 7 to 9, 10.5 is really higher than the normal working pH. The cytraconic anhydride is a substance used to tag the lysines present in the protein structure. We decided to set up an experiment with this tagging molecule, in order to mask the charge of lysine and to reduce the non specific binding of the protein. We performed the reaction with a large excess of the anhydride in order

to have a complete reaction. During the experiment we had some problem because one of the high pH experiments did not precipitate during the MeOH addition. This was due to the high pH. We corrected the pH on the second experiment by adding a little amount of HCl. The reaction with anhydride didn't work at all, because during the reaction, held at 37°C for 2h, the whole solution was transformed in a yellow precipitate that we were not able to digest. In order to be sure that all the experiment were analyzed with the same instrumental performance, we decided to load the sample with a blank and then a standard after the blank to check it. The results obtained showed that the only working method was the classic digestion method developed with Digestips and protein precipitation. The double tip strategy worked, because we had small improvement with digestion, around 10 %, but due to high cost of Digestips, we decided not to use 2 tips for 1 single digestion. In all the other experiment we didn't find any trace regarding our peptide, and so we cannot proceed anymore with that development. Another check with single and double tips was performed during the followings days and confirmed our previous

results: a small improvement that is not economically affordable. The focus on development was now targeted on protein precipitation. We did a small protein precipitation development with these experiments:

- Volume change from 0.7 to 1.0 mL
- Acidic modification with HCl or $HClO_4$
- Temperature of centrifugation
- Double Step of protein precipitation

According to other protein precipitation techniques, $HClO_4$ was known to be helpful for this technique because it has a caotropic effect that helps the protein precipitation since it's a large ion. We used different volumes to vary the final percentage of organic solvent in solution in order to find the major clearness of the sample. The temperature of centrifugation was changed because maybe the peptide could be suffering from higher temperature generated from centrifugal forge, so we decided to use a temperature controlled centrifuge to see if we had some improvement. We also tried to use a double step precipitation by using a second organic addition after the first, to see if this

could be an improvement for sample cleaning. Unfortunately none of these innovative approaches modified the situation. Indeed we did not have any signal relative to our peptide. For the double step procedure, we could imagine that it was the same situation as SPE purification: we passed above the organic percentage tolerated by peptide, which precipitated itself in the second step of purification. We still don't know why lower temperature affects the protein precipitation. The experiments performed at different volumes showed no significant differences, so we decided to stay in the starting condition. After this development we decided to test the final sensitivity of the whole method, to see if we could have some improvements from the SPE purification. We decided to use the double tip for this experiment and to compare with some experiments with single tip to see if there were significative differences at lower concentrations. We treated 200 µl of the following concentrations both in single or double tip digestion: 7.5/15/60/150/300 ng/mL.

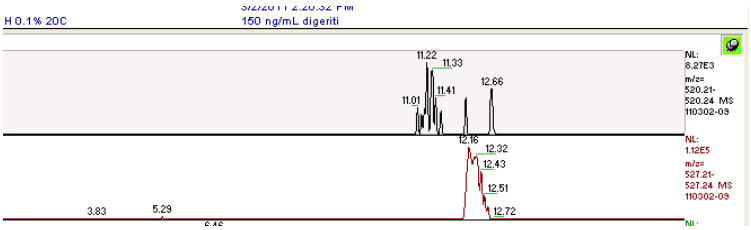


Fig 99: 150 ng/mL digested with single tip

We had as best results in term of sensitivity the 150 ng/mL digestion, that was the lower concentration digested and detected in mass spectrometry, all the other experiments didn't show any signal about our peptide. These results were an improvement respect to SPE, but this is also a dirtier procedure respect SPE, that affects the instrumental stability and creates several clogging phenomenon. For this fact we were looking for other more efficient purification techniques.

Polyclonal Antibodies

A specific polyclonal Antibody was produced to target specifically our peptide. We decided to maintain the magnetic beads purification, but changing the kind of Ab mounted on beads. At the beginning of this new

purification development, we chose two different approaches:

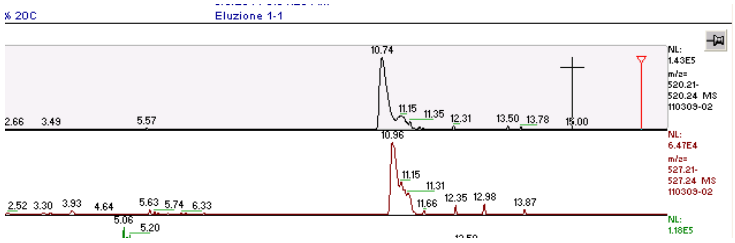
- Biotinilation of r-PoAb and conjugation on streptavidin beads
- Direct conjugation of r-PoAb with Protein-G beads.

The conjugation between biotin and PoAb was not successful, because we applied the procedure used for the whole protein, but unfortunately we did not have any results. This was due to a probable biotinilation of the antibody active site that is not able anymore to catch the peptide. The second approach required a new procedure development, so we had r.t. incubation of the polyclonal Ab with magnetic beads for 1 h then we performed a little wash step with PBS+Tween-20 0.02%. The incubation of the peptide was performed O.N. at 4°C and then the elution step involved the use of a 5% acetic acid solution. As starting point we decided to use an elevated concentration of peptide/Internal standard, because we prepared 4 samples in serum, in which we had a 6 μg/mL peptide concentration and a 5 μg/mL concentration of internal standard. This test was also important, because as said

before, the internal standard has a difference in primary sequence compared to the peptide in analysis and so we were not sure that the PoAb could capture also the peptide deriving from the internal standard. The first set of experiments involved also the knowledge that we developed before:

- Single tip digestion, Protein Precipitation and Immunocomplex formation (file 110309-02)
- Single tip digestion and immunocomplex formation (file 110309-04)
- Single tip digestion, protein precipitation, immunocomplex in solution formation, beads conjugation (file 110309-06)
- Single tip digestion, immunocomplex in solution formation, beads conjugation (file 110309-08)

After the incubation a washing step with PBS +0.02% of Tween-20 was set up in order to clean the sample before the elution.



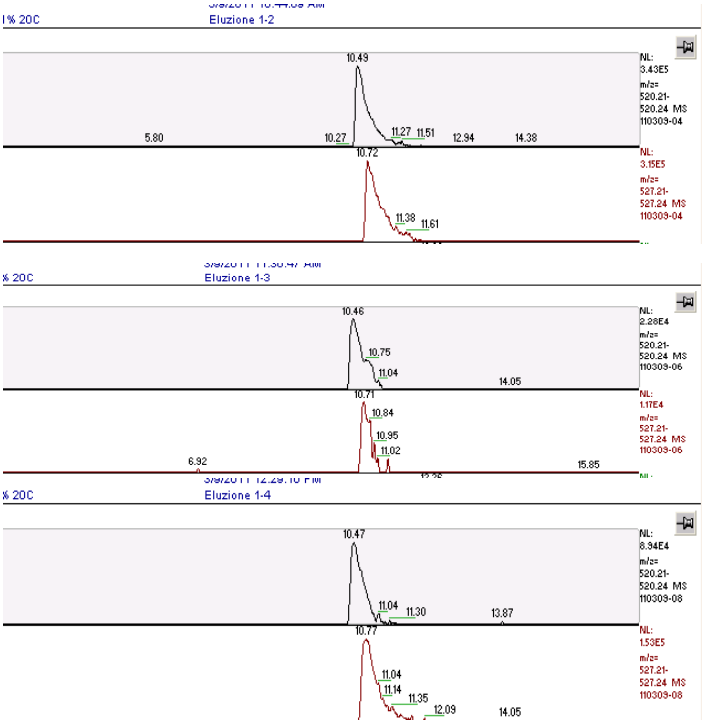


Fig 100: elution chromatogram from magnetic beads coated with protein-G

The chromatograms in Fig.100 showed results never obtained before: the performances of this kind of separation are better than the other purification procedures. By looking inside the data, we saw that when we performed the purification without the protein precipitation we had a better result. This could be due to the presence of MeOH, but a possible solvent change in order to aid the polyclonal antibodies could

be associated to some non-specific binding and so we decided to avoid this kind of purification step. The in solution capture, thought to avoid the non specific binding of the protein, seems to be less effective than the capture obtained when the PoAb is already coated on the beads. Since we had strong results from the beginning, we decided to test immediately the sensitivity of this actual method (single tip digestion + direct immunocomplex formation) by testing the following concentrations: 7.32/14.65/58.6/146.5/293 ng/mL and in every solution, composed by 200 µL of serum, a final concentration of 500 ng/mL of internal standard was reached.

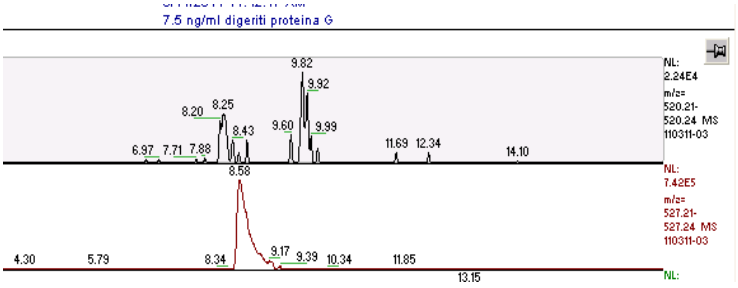


Fig 101: 7.5 ng/mL digested and extracted from serum

File	(ng/mL)	Peptide Area	Internal Standard Area	Corrected peptide area
110311-03	7.5	127016	12866240	148315.80
110311-04	15	108404	16394625	99340.06
110311-05	60	199302	14674996	204039.52
110311-06	146.5	425121	14374566	444322.65
110311-07	300	1163603	33617439	1040041.91

Tab 11: Resume of quantitative analysis

By the results, it's clear that something happened to the 15 ng/mL digestion, because we had a lower area than the 7.5 ng/mL injection. Unfortunately, by the analysis it was possible to observe that a double amount of internal standard was inserted in the 300 ng/mL solution. By excluding the 15 ng/mL concentration, we corrected the peak area for peptide, according to normalization by internal standard area.

With this new area value we built a standard calibration curve.

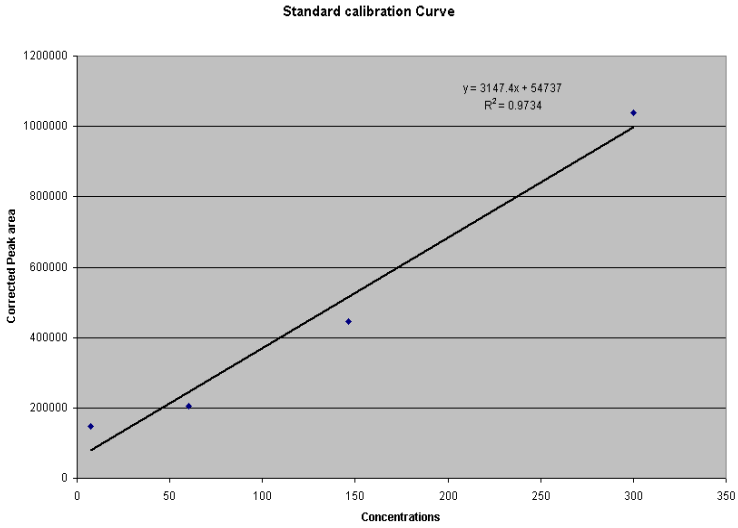


Fig 102: standard calibration curve extracted from serum

What we could see in Fig.102 is that we had a quite good linearity that, as first result, was promising for future development. Pushed by this result, we decided to start immediately with a new set of experiments, to see if we could extend the standard curve to the pg/mL range. A new set of concentrations was tested by the wholeprocedure:

150/300/600/1500/6000/15000/60000 ng/mL. In this set of experiments the amount of internal standard added was reduced to avoid carryover phenomena.

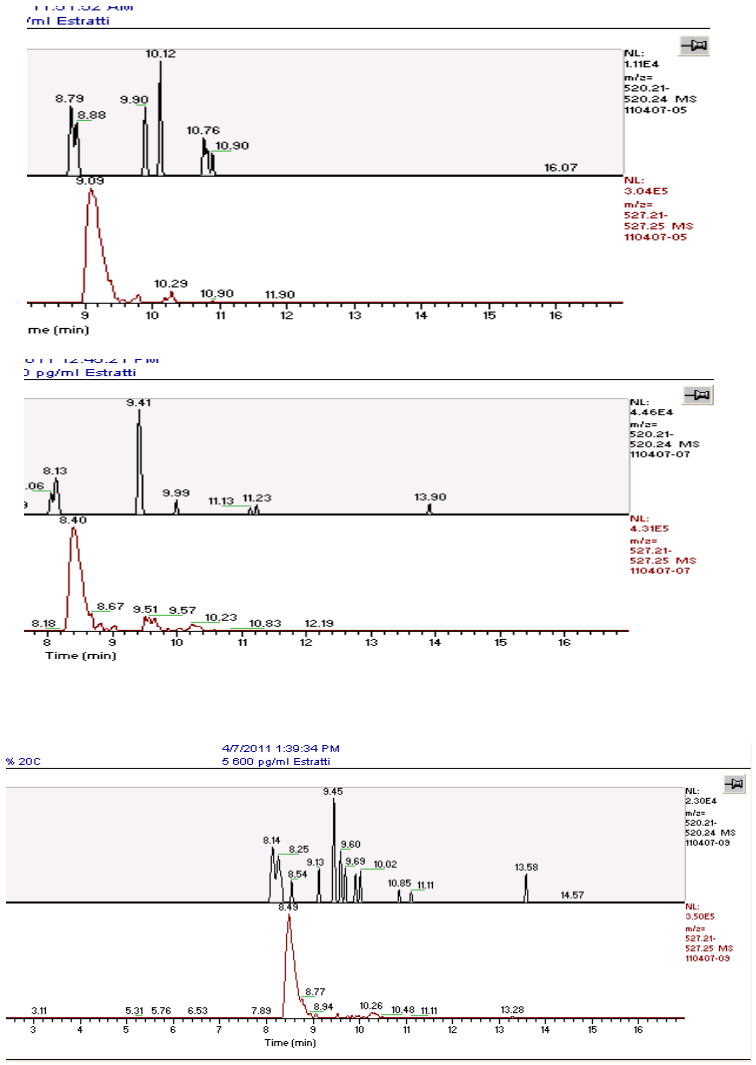


Fig 103: in file 110407-05 150 pg/mL extracted in file 110407-07 300 ng/mL extracted and in file 110407-09 600 ng/mL extracted

File	(pg/mL)	Peptide Area	Internal Standard Area	Corrected peptide area
110407-05	150	44140	4665429	148315.80
110407-07	300	90028	5846648	99340.06
110407-09	600	127327	4789519	204039.52

Tab 11: Resume of quantitative analysis

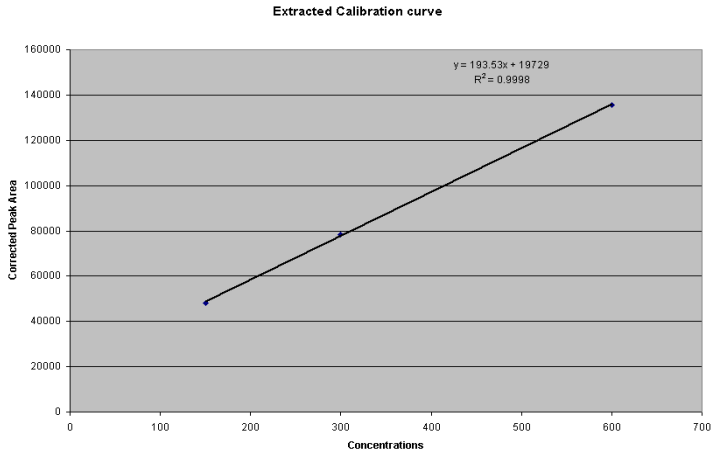


Fig 104: Extracted calibration curve

This time the results showed impressive sensitivity and linearity: we were able to reach 150 pg/mL as lower detectable concentration. Unfortunately, concentrations upper than 600 pg/mL completely disappeared. Both peptide and internal standard peaks

were not present at all in the chromatograms and so we thought that some instrumental problem occurred after these analyses. The same experiment with the same concentration was replicated in order to understand what happened to the previous standard curve. Unfortunately, due to some clogging phenomena we decided to change a post column pipe with one with a larger inner diameter, to avoid some blocking during the analysis. This new pipe should get us worse results because the molecules have more spreading inside it.

Name of File	(pg/mL)	Peptide Area	Internal Standard Area	Corrected peptide area
110408-06	150	18893	5368488	148315.80
110408-08	300	55372	4345547	99340.06
110408-10	600	76738	409234	204039.52

Tab 12: value obtained from chromatographic analysis

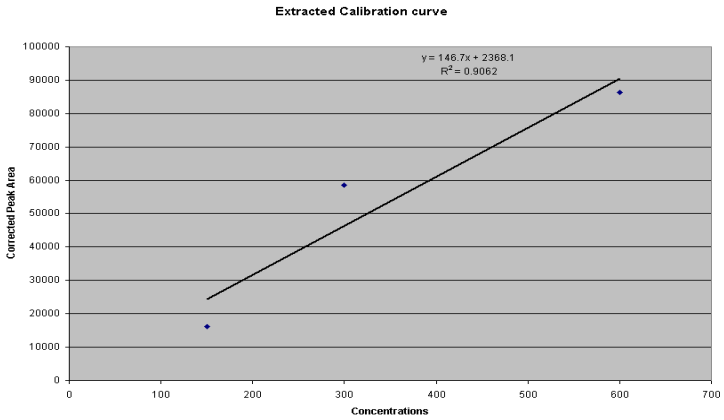


Fig 105: Corrected calibration curve from extracted samples

According to what we expected by pipe substitution, we had smaller peak area values, but unfortunately, now the linearity got really worse and we still had the same problem with higher concentration, that showed in this case, really bad results. One other difference from the previous analysis was that the sample were extracted and analyzed later than the previous one, so this could be an indication that samples are not completely stable at 4°C for more than 24h. According to the results of these analyses it was easy to understand that, maybe due to the very complex structure of the starting protein, the method is very susceptible to every variation that we bring in. This consideration should be kept always in mind for the

future analyses. Since we obtained very good results in serum, we decided to move our focus to synovial fluid. To keep the method as intact as possible, we decided to use the Digestips also for synovial fluid. Unfortunately synovial fluid, as it is, is too viscous to flow trough this tips. A sample pre-treatment is necessary in order to reduce the viscosity of this matrix. Synovial fluid is a viscous, non-Newtonian fluid found in the cavities of synovial joints. The principal role of synovial fluid is to reduce friction between the articular cartilages of synovial joints during movement. The inner membrane of synovial joints is called the synovial membrane and secretes synovial fluid into the joint cavity. The fluid contains hyaluronic acid secreted by fibroblast-like cells in the synovial membrane and interstitial fluid filtered from the blood. This fluid forms a thin layer (roughly 50 μm) at the surface of cartilage and also seeps into microcavities and irregularities in the articular cartilage surface, filling all the empty spaces. The fluid in articular cartilage effectively serves as a synovial fluid reserve. During movement, synovial fluid held in the cartilage is squeezed out mechanically

to maintain a layer of fluid on the cartilage surface. The functions of the synovial fluid include:

- reduction of friction - synovial fluid lubricates the articulating joints
- Shock absorption - as a dilatant fluid, synovial fluid is characterized by the rare quality of becoming more viscous under applied pressure; synovial fluid in diarthrotic joints becomes thick when moment shear is applied in order to protect the joint and subsequently thins to normal viscosity instantaneously to resume its lubricating function between shocks
- Nutrient and waste transportation - the fluid supplies oxygen and nutrients and removes carbon dioxide and metabolic wastes from the chondrocytes within the surrounding cartilage.

Synovial tissue is sterile and composed by vascularised connective tissue that lacks a basement membrane. Two cell types (type A and type B) are present: Type B produces synovial fluid. Synovial fluid is made of hyaluronic acid and lubricin, proteinases, and collagenases. Normal synovial fluid contains 3–4 mg/ml hyaluronan (hyaluronic acid), a polymer of

disaccharides composed of D-glucuronic acid and D-N-acetylglucosamine joined by alternating beta-1,4 and beta-1,3 glycosidic bonds. Hyaluronan is synthesized by the synovial membrane and secreted into the joint cavity to increase the viscosity and elasticity of articular cartilages and to lubricate the surfaces between synovium and cartilage. Synovial fluid contains lubricin secreted by synovial cells. Chiefly, it is responsible for so-called boundary-layer lubrication, which reduces friction between opposing surfaces of cartilage. There is also some evidence that it could help regulate synovial cell growth.

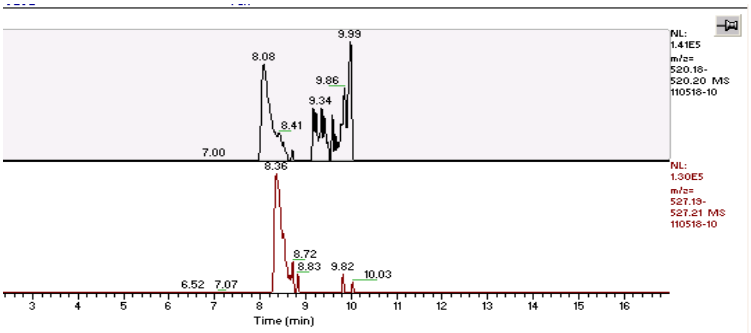
Several papers in literature deals with synovial fluid and the most common process for reducing the viscosity is made by centrifugation or enzyme that cut the polymers present in synovial fluid. We decided, after few developments, to combine these two treatments by a digestion step, followed by centrifugation. Our target was reached because we could successfully digest samples in synovial fluid, with Digestips, after this double treatment. To test the performance between these two matrices, different

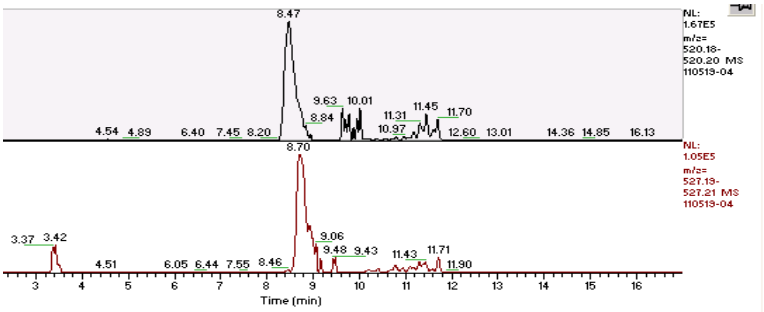
conditions were tested in order to find if this new treatment could harm the molecule.

Some experiments were performed in parallel:

- 3 Reduce viscosity treatment + digestips + Immunoaffinity purification in synovial fluid
- 2 Digestips + Immunoaffinity purification in serum
- 1 Reduce viscosity treatment + digestips + Immunoaffinity purification in serum

All these experiment were conducted with 200 µL of biological matrix, at concentration of 600 ng/mL of protein and 500 ng/mL of internal standard. The last experiment was performed on serum to see if there were any differences from the normal digested serum sample. In this way we had the same matrix and it was possible to test only the effect of the treatment.





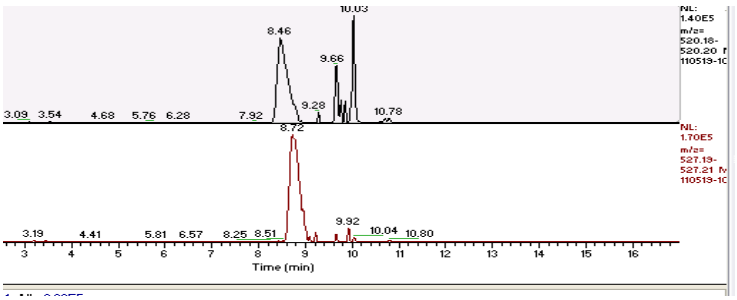


Fig 106: in file 100518-10 Synovial fluid extracted, in file 110519-04 the serum extracted sample as digestion control and
in file 110519-10 the serum extracted with viscosity treatment.

The results obtained were quite good because we were in the same range for every kind of condition, but there was still some difference that should be considered.

Conclusions

The advent of biopharmaceuticals brought clinical and pre-clinical analytics on a new era. Since FDA requests for new bio-drug approval are becoming stricter and stricter, Mass spectrometry could play a key role in this field. The power of mass spectrometry, coupled with Chromatography, to resolve complicate mixture of molecule is the key for the leading role in analytical chemistry.

Actually there are no quantification methods available on the whole protein, due to the non specific binding phenomenon, always present at low protein concentration, but since therapeutic proteins are becoming the main target for new drug research, analytical chemistry needs to make some steps forward in order to fill this gap. My research work takes place in this new emerging field, in which ELISA quantification is necessary but not sufficient to validate a clinical trial. Working with proteins, especially with this high IP one, is not a trivial problem, because there are variety of aspects that need to be taken into account. As I

demonstrated with my work, immunoaffinity procedure to clean up the sample (both for protein and peptide), seems to be the only way to obtain a valid mass spectrometry purification of this new bio therapeutics. In fact the complexity of the biological matrices coupled with the complexity of the protein really makes more difficult the research work. Different strategies are available for Ab binding on a stationary phase ad all of them have pros and cons. We worked very hard on magnetic beads technology, because they're versatile in term of binding stationary phase and the overcoming of matrix complexity, for example synovial fluid viscosity, because you can add the beads to the matrix and not vice versa.

The ability of the solid phase digestion method developed to overcome the presence of alpha-1-antitripsin, is in my opinion, the most valued technology developed during this Ph.D. experience. This is very important, because you can't do the depletion of most abundant protein in serum, when you have to treat with low concentration of a protein, and so, in this way you can digest the sample directly without any other step, in order to minimize the non

specific binding phenomenon. The several experimental designs performed during the study highlighted some important aspects on the instrument operation and let us build a good knowledge on this new instrumentation, especially the HPLC that will be helpful for other new studies. The knowledge of how acidic modification influences the nano-ESI and the ESI source should be applied to all the methods development procedures. The most important improvement of this new approach is that is universal. Both the digestion and the purification are not affected by the matrix, so they're applicable to a broad spectrum of molecules and matrices. Just producing the right Ab for the target molecule, the purification is usable for every kind of molecules or metabolite. There are still some concerns regarding the reproducibility, because some times the analysis doesn't show the expected result. The lack of the methods could be attributed to the Digestips or to the capture process, because they are custom preparation and sometimes the quality couldn't be enough for the strict FDA validation approval, but generally speaking, this kind of

methodologies could give a great support to bioanalytics for pharmaceutical companies.

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