Facoltà di Scienze Matematiche, Fisiche e Naturali

Dipartimento di Chimica

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(XIII Ciclo)

Proteomic study with mass spectrometry techniques

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CONTENTS

Abstract

Over the recent years, proteomics has moved from an effort to identify the proteins of biological systems to targeted strategy aiming to identify key proteins, such as biomarkers that can provide reliable diagnostic and prognostic indicators of disease progression, stress condition or treatment effects. By quantitative proteomics approach, in fact, it is possible to investigate the protein expression level changing due to particular external environmental. This is due to the dynamism of proteome that is influenced to stress conditions, instead of genome that result a static biological element. For these reasons in the present thesis were development three different method for differential proteomic study in biological matrix with mass spectrometry techniques.

First was a method for carbonic anhydrase II (CA II) absolute quantification in human serum. This method is based on high-performance liquid chromatography (HPLC)-Chip microfluidic device incorporating a nanoelectrospray source interfaced to a triple quadrupole mass spectrometer. The fraction containing CA II was isolated by preparative reversed-phase HPLC, and peptides obtained from the tryptic digest of the protein mixture were separated by the HPLC-Chip system. The multiple-reaction monitoring acquisition mode of a selected suitable CA II peptide and peptide internal standard allowed the selective and sensitive determination of a CA II. Recovery of the method was 81±10%. A comparison among three regression lines type which were obtained by external calibration, matrix-matched calibration, and standard addition method, respectively, demonstrated that the first one is adequate in obtaining good accuracy and

precision. Method quantification limit for CA II in serum was estimated to be 3,7 pmol/mL.

Second method was based on a magnetic bead-based platform amenable to high-throughput protein carbonic anhydrase II (CA II) capture. The key steps in this approach involved immunoaffinity purification of the target protein from serum followed by on-bead digestion with trypsin to release a surrogate peptide. This tryptic peptide was quantified by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) operating in multiple reaction monitoring acquisition mode. Using a synthetic peptide standard and a structural analogue free-labeled internal standard, the resulting concentration was stoichiometrically converted to CA II serum concentration. The analytical steps, such as preparation of immunobeads, protein capture, proteolysis, and calibration, were optimized. The method was validated in terms of recovery (77%), reproducibility (relative standard deviation [RSD] < 12%), and method detection limit (0.5 pmol ml/1). The developed method was applied to determining the CA II in eight healthy subjects, and the average concentration measured was 27.3 pmol/ml.

Third method was based on multidimensional chromatography. These techniques have emerged as a powerful tool for the large-scale analysis of such complex samples as biological samples. In order to evaluate these separation techniques, microgram quantities of protein extracted from mouse heart tissue were fractionated by four different chromatographic methods. Regarding peptide-level fractionation the first dimension of separation was performed with High-pH Reversed-Phase Chromatography (pH-RP) and Strong Cation Exchange

Chromatography (SCX). Regarding protein-level fractionation instead C8 Protein Reverse Phase (C8-RP Prot) and High Recovery Protein Reverse Phase (hr-RP Prot) were used. The second dimension consisted of a reversed-phase nano-HPLC on Chip coupled to an electrospray ionization quadrupole time-of-flight mass spectrometer for tandem mass spectrometric analysis. The performance and relative fractionation efficiencies of each technique were assessed by comparing the total number of proteins identified by each method. The peptide-level pH-RP and the hr-RP Prot peptide-level separations were the best method, identifying 1338 and 1303 proteins, respectively. Worst method instead was the peptide-level SCX with 509 proteins identified.

CHAPTER 1: General introduction

1.1 Proteomics

Proteins are vital parts of living organisms, as they are the main components of the physiological metabolic pathways of cells. The term proteomics was coined in the middle of ’90 in analogy with genomics, and trascrittomics and is defined as “The analysis of the entire protein complement expressed by a genome.”[1].

Unlike the genome that is constant, the trascrittome and proteome differ from cell to cell and may varies with time or cellular conditions. So proteomics may be considered the next step in the study of biological systems, after genomics and trascrittomics since proteins are the main effectors of most cellular functions.

Genome and trascrittome studies may be incomplete for several reasons. First, often the gene transcription level gives only a rough estimate of its level of expression into a protein. In fact in several cases, there is poor or no correlation between trascriptome and protein abundance. The mRNA produced in abundance may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. Second, there are other biological changes that may affect the correlation between gene expression and protein synthesis. For instance many transcripts give rise to more than one protein, through alternative splicing or alternative post-translational modifications [2].

By proteomics is it possible to obtain many important information about structures and functions of proteins. In particular functional proteomics is addressed to the study of cellular compartments, multi-protein complexes and signalling pathways. The knowledge of structure or conformational changing of

proteins may be of aid in understanding the role of proteins. The most common functional proteomics study point out to the analysis of the phosphoproteome by means of localization of the phosphorylated residues, and the determination of the phosphorylation degree [3,4].

The post-translational modifications may profoundly affect protein activities. For example some proteins are not active until they become phosphorylated. Then functional proteomics allow the comprehension of different protein activities depending to their phosphorylation state.

At present time, protemics is in high development also because it allows the knowledge of the molecular effects due to particular cellular conditions.

This application is employed by expression proteomics, which aims to measure the changing of protein levels. Indeed, by applying comparative characterizations, proteomics is a powerful tool for finding out which particular proteins are involved in an organism reaction mechanism when environmental conditions change. Then expression proteomics is science that show the exactly cellular phisiologic state. For these reasons it may recover many application in several scientific fields.

One of the most promising application of proteomics is the identification of potential new drugs for the treatment of disease. This relies proteome information to identify proteins associated with a disease, also called biomarker, which can be used as targets for new drugs. In addition protein may be used to diagnose disease. Under pathological conditions some proteins may be up or over-expressed. So by studying the proteins expression level changes it is possible to

find a particular protein related to pathologic state. There are more example of applications of disease biomarker for medical diagnosys.

1.2 Analytical techniques in proteomics

Over the past two decades, mass spectrometry (MS) has become an important tool for the analysis of proteins [5,6]. One current method for the analysis of protein mixtures is proteolytic digestion followed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). This approach overcomes many difficulties associated with protein mixture MS analysis [7]. MS/MS analysis has been particularly effective because the data can be directly used to identify peptides and subsequently infer which proteins are in the mixture [8]. This type of approach for the analysis of protein mixtures is often referred to as "shotgun" proteomics. Because increasingly complicated biological structures are studied by MS/MS, the need for more powerful and highly resolving separation methods has grown. Infact, proteins are identified by mass-to-charge ratios of peptides and their fragments and sufficient separation is required for unambiguous identifications. Therefore proteins MS is closely linked to and depends largely on the separation technologies to simplify incredibly complex biological samples prior to analysis of the mass. Front-end separation is also required to detect low-abundance species that would otherwise be overshadowed by a higher abundance signal. Therefore, both accuracy and sensitivity of a mass spectrometric experiment rely on efficient separation. There is a very strong conceptual link between chemical separation and MS in which the latter is viewed as the mass resolution dimension of molecules separation [5]. Selection of appropriate separation methods is often the

first step in designing the proteomic application. Two major approaches to separation widely used in proteomics are gel based and gel free. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is the historic centerpiece of the gel-based separation methods [9-12]. There are many excellent reviews that cover 2D PAGE and gel-based approaches to proteomics [13-16]. Gel-based methods have been traditionally used with pulsed ionization MALDI instruments in which the protein band can be excised, digested, and off-line sampled with MALDI source [17]. The limitations of 2-DE in detecting low abundance proteins, very small or large proteins, as well as basic and membrane/hydrophobic proteins [18-20], as well as difficulties with automation of the process, have forced researchers to look for other methods of protein separation, such as multidimensional liquid chromatography (MDLC). MDLC is by no means a new concept and has a long history, it has been enjoying a renaissance in proteomics [9]. MDLC combines two or more forms of LC to increase the peak capacity, and thus the resolving power, of separations to better fractionate peptides prior to entering the mass spectrometer. Furthermore for adequate representation of the proteome, only multidimensional separation techniques can provide resolving capability of thousands of protein species and have proven to be superior to one-dimensional approaches. These techniques have emerged as a powerful tool for the large-scale analysis of such complex samples [21-24]. It better resolves peptides differing in charge and hydrophobicity to minimize ion suppression and improve ionization efficiency, and it simplifies the complexity of peptide ions entering into the mass spectrometer to minimize undersampling. This last aspect is important because the tandem MS process is driven by data-dependent-acquisition (DDA) and has a

finite cycle time. A higher peak capacity and better resolving power improve the acquisition of data and can lead to a better representation of the proteins in the mixture and permit the identification of low-abundance proteins [21,22,25,26]. For example various techniques prefractionation orthogonal on the level of protein and peptide level have been utilized for the characterization of part of yeast proteome leading to the identification of thousands of proteins [27,28].

1.3 Proteins or Peptides Level Separation?

The protein-level and peptide-level separations have relative advantages and disadvantages. Proteins are sensitive to precipitation upon exposure to high salt concentrations, to basic pH values, and organic solvents. Peptides, on the other hand, are relatively stable in solution and generally do not exhibit solubility issues. However, peptide-level separations also have limitations, including the scattering of tryptic peptides from a single parent protein into multiple fractions, which can potentially reduce protein identification scores. Furthermore for adequate representation of the proteome, only multidimensional separation techniques can provide resolving capability of thousands of protein species and have proven to be superior to one-dimensional approaches.

1.4 Multidimensional Chromatographic Techniques

Currently in the literature are given a variety of multidimensional combinations that lead to an increase in the resolving power of the technique [29,30]. These methods can use different chromatographic techniques and a different number of dimensions. There are important factors to be considered as the amount of time

required for analysis, compatibility with MS buffer used for the chromatographic separation, and the effective integration of two dimensions. Usually the last stage of separation, which usually is the step directly interfaced to a mass spectrometer, is the RPLC, which can provide high resolution, desalting of samples, and the compatibility of the phases with the ESI source and MS detection. The basis of RP method is the hydrophobic interaction between peptides and stationary phase. The stationary phase is the most common C18 covalently linked with a basic material of silica, these phases are called RP, C18, silica or octadecyl (better known as ODS). Peptides are loaded onto an RP column in a solution with a low content of organic phase, which allows on-line desalting and concentration at the same time. During the chromatographic run is gradually increased the amount of organic modifier in the mobile phase so that the peptides may elute according to the strength of hydrophobic interactions with the stationary phase. The peptides separation by RP chromatography has been widely studied in recent decades and significant progress has been made in this technique [31]. Because of its separation efficiency, superior to other LC techniques, and its excellent compatibility with ESI, RP remains an important method of peptides or protein separation. An important consideration for the development of multidimensional separations is the orthogonality of coupled techniques. The resolution can be maximized by combining chromatographic methods based on different principles of separation. While the RP chromatography is mainly used as second dimension in proteomics applications, a variety of chromatographic techniques were used for the first dimension. The most commonly used techniques are exclusion chromatography, strong cation exchange (SCX) and strong anion exchange

[30,32-36]. Some factors are important in the first dimension, should have a large carrying capacity, to be configurable with the second dimension, and should use a solvent compatible with the second mode. Many of the methods listed above reflect these criteria, while others are more suited for off-line. One of the most widely used combinations for MDLC is SCX and RP. SCX keeps peptides based on electrostatic interactions. Sulfonyl end groups of the resin coat the surface and create a strong negative charge that is largely resistant to pH changes. Peptides are loaded onto an SCX column with a low pH buffer (3-4), which permits, block the dissociation of peptide's carboxylic groups and promote interactions between the protonated basic amino acid residues and the sulfonate groups of SCX resins. Peptides are eluted by increasing the strength of the salt buffer, which disrupts the interaction between peptides and sulfonate groups. To break the stronger interaction between peptide and SCX resin, the greater salt concentration is needed. Also experimentally showed that the phase SCX has additional features, such as hydrophobicity [37]. To minimize the hydrophobicity role and to facilitate the peptides denaturation, 10-15% of organic modifier is often added to the elution buffer. Fractionation of electrostatic interaction provides a degree of orthogonality to RP separation and therefore is an excellent complement. Multidimensional techniques discussed above can be coupled in off-line mode or in on-line mode. The first is the simplest and provides the fractions collection after the first dimension and a further separation of these with the second dimension interfaced to mass spectrometer. On-line mode, instead, is refers to a system in which the transfer of the analyte between the first and second dimension is automated and does not involve any disruption of flow [38]. To do this,

generally a switching valve is placed between the two dimensions. The main advantages of these mode are the ease of automation and a reduced risk of sample loss and contamination than off-line mode. Use of switching valves often involves use of a intermediate column such as a trap for on-line sample desalting and this makes the configuration relatively flexible compared to the integrated column. However, the passage of the sample in the switching valves and then exposure to surfaces and to additional connections can lead to loss of analyte. MDLC in on-line mode can also be performed using biphasic column. This integrated system is a simple system where the first 10-15 cm of the column is packed with RP material, followed by ~ 3-5 cm of SCX material. A final portion of RP can be added to act as desalting phase or as a further separation phase [39,40]. Peptides are loaded manually onto the column [41]. The manual loading of samples directly onto the column minimizes any loss of analyte that could occur through the valve system. The end of the capillary column RP usually forms a conical tip end so that the column serves as the ESI emitter, so as to have a minimum postseparation dead volume [42]. Multidimensional separation is achieved by passing a series of buffers in the column [32]. The peptides related to the SCX phase are first eluted with a 2-5 minutes pulse of saline solution and second are separated by a RP gradient. A second pulse of saline solution is then used to move another population of peptides on the RP column, this process is repeated a number of times. Because the sample transfer between phases occurs within a single section of the column, the dead volume becomes negligible. Generally, the on-line approaches are ideal when the sample available is limited and the losses must be minimized.

1.5 Aim of the thesis

The aim of this research was to develop three different method for differential proteomic study in biological matrix with mass spectrometry techniques.

First was a method for carbonic anhydrase II (CA II) absolute quantification in human serum. This method is based on high-performance liquid chromatography (HPLC)-Chip microfluidic device incorporating a nanoelectrospray source interfaced to a triple quadrupole mass spectrometer and we applied a label-free absolute quantification method based on quantitative equivalence between the protein and a selected peptide coming from protein tryptic digestion. Second method was based on a magnetic bead-based platform amenable to high-throughput protein carbonic anhydrase II (CA II) capture. In the current study, in fact, a Protein G magnetic bead-based antibody platform is proposed for the selective enrichment of CA II in human serum, followed by its accurate and reliable quantification by a label-free procedure performed on a less technologically sophisticated LC–ESI–tandem mass spectrometry (MS/MS) system. Third method was based on multidimensional chromatography. These techniques have emerged as a powerful tool for the large-scale analysis of such complex samples as biological samples. In order to evaluate these separation techniques, have been systematically compared the following chromatographic techniques: offline SCX combined with RP, mixed-mode pH RP-RP for peptide-level separation, and RP with two different columns for protein-level separation. We have also compared the following identification parameters: total proteins identified, total number of peptides identified (including redundant identifications), total number of unique peptides (only nonredundant

identifications), average protein sequence coverage, and protein *E*-value (probability score).

CHAPTER 2: HPLC-CHIP coupled to a triple quadrupole mass spectrometer for carbonic anhydrase II quantification in human serum

2.1 Introduction

Over the recent years, proteomics has moved from an effort to identify the proteins of biological systems to targeted strategy aiming to identify key proteins, such as biomarkers that can provide reliable diagnostic and prognostic indicators of disease progression or treatment effects. However, till now, this tool has been only partly exploited. Rapid immunotest development and clinical validation are time consuming and costly steps which restrict the new biomarker adoption in clinical praxis. Due to its flexibility, mass spectrometry (MS) may constitute a more accessible analytical tool for preclinical protein biomarker studies. However, it remains difficult to identify biomarkers using conventional proteomic approaches because of their low abundance [43]. In addition to the initial identification of phenotypic expression and protein characterization, a key parameter in proteomic analysis is the ability to quantify proteins of interest. To date, a majority of the quantitative proteomic analyses have been performed using stable isotope labeling strategies such as isotope coded affinity tag [44], isobaric tags for relative and absolute quantitation (iTRAQ™) [45], stable isotope labeling by amino acids in cell culture [46], ^{18}O labeling [47,48], and stable isotope standards with capture by anti-peptide antibodies [49,50]. These methodologies require complex, time-consuming sample preparation and can be relatively expensive [51]. One of the most powerful and used proteomics approaches for

low-abundance protein identification involves the ultrasmall-bore liquid chromatography coupled to nanoelectrospray MS (nano-LC-MS). Recently, it has been presented a microfabricated approach to nano-LC that integrates all the components on a single LC Chip, eliminating the need for conventional LC connections [52]. LC-Chip-MS technology allows by itself multi-dimensional LC because it has an integrated sample enrichment column, a reversed phase (RP) LC separation column, an electrical contact for electrospray (ESI) and a nano-ESI tip. Miniaturization has radically reduced sample size and cost, has improved sensitivity, and has increased speed [53]. Carbonic anhydrases (CAs) represent a group of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate [54]. In mammals, CAs occur in 14 different isoforms [53] that differ in their subcellular localization; for example, CA I is cytosolic, CA V is mitochondrial, while CA VI is membrane associated [55–58]. CAs are involved in important physiological processes such as, for example, transport of CO2 [54], intracellular pH control [59], renal and male reproductive tract acidification, electrolyte secretion, and formation of gastric acids [60–62]. The carbonic anhydrase II (CA II—molecular weight ca. 29 kDa, and theoretical pI 7.5) [63] is expressed in a wide variety of normal cells including erythrocytes (where it is the main isozyme of the CA family), pancreatic, as well as kidney and gastrointestinal tract epithelial cells. Finally, CA II has multiple, complex functions in the human brain where its deficiency has been associated with pathological consequences such as mental retardation and brain calcification [64,65], as well as Down Syndrome (DS) [54,63]

and Alzheimer' disease [63]. Increased CA II levels in some brain tissues have been found in infant with DS, and a marked CA II up-regulation in oligodendrocytes subject to demyelinating condition in humans has been also reported [66,67]. In previous researches, CA II purification has been performed by affinity chromatography followed by mono-dimensional gel electrophoresis [57,68]. Sensitive competitive enzyme-linked immunosorbent assay [56], two-dimensional gel electrophoresis followed by matrixassisted laser desorption/ionization time-of-flight (MALDITOF) MS [21], and Western blot [54, 55] have been adopted for its detection and to assess relative concentration in human [55] and equine [56] blood erythrocytes, as well as in human [63] and mouse brain [54]. To our knowledge, there are no papers dealing with the CA II determination by means of LC/ESI-MS in real samples. In addition, with the exception of the erythrocytes, CA II levels have been investigated only in postmortem [54] and tumor specimens [69]. As a consequence of the cellular expression, CA II might be also present in serum, although low concentrations would be expected in normal, healthy subjects. The aim of this research was to develop a method for the CA II absolute quantification in human serum. For this purpose, we applied a label-free absolute quantification method based on quantitative equivalence between the protein and a selected peptide coming from protein tryptic digestion. In a previous work [70] dealing with troponin T quantification in mouse heart tissue, small-bore highperformance liquid chromatography (HPLC) coupled with pneumatically assisted ESI-triple quadrupole (QqQ) MS/MS, operating in multiple reaction monitoring (MRM) mode, and the matrix-matched calibration curve were used. In this work, the

capability and the high sensitivity of the automated HPLC-Chip technology coupled with a QqQ mass spectrometer was exploited to identify and quantify CA II in serum. Although present at very low levels, due to the low matrix effect allowed by this kind of instrument, it was demonstrated that the external calibration can be used for CA II quantification in serum.

2.2 Experimental part

2.2.1 Reagents and chemicals

Bradford reagent and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tris (hydroxymethyl)aminomethane, 1,4 dithiothreitol (DTT), iodoacetamide (IAA), and urea were purchased from GE Healthcare (Uppsala, Sweden). All organic solvents were the highest grade available from Carlo Erba Reagents (Milan, Italy) and were used without any further purification. Ultrapure water was produced from distilled water by a Milli-Q system (Millipore Corporation, Billerica, MA, USA). C18 silica Bond Elut EWP (Extra Wide Pore) was obtained from Varian (Palo Alto, CA, USA). Cellulose Acetate Spin Filters (0.22 μm pore size) were purchased from Agilent Technologies (Wilmington, DE, USA). Protein LoBind tube were obtained from Eppendorf (Hamburg, Germany). Modified porcine trypsin, sequencing grade, was commercialized by Promega (Madison, WI, USA). A Sequazyme peptide mass standards kit, with the following composition: bradykinin fragment 1-5 (573.3150), angiotensin II human (1046.5424), neurotensin (1672.9176), ACTH fragment (2465.1989), insulin B chain oxidized (3494.6514), was obtained from LaserBio Labs (Sophia Antipolis Cedex, France). A α-cyano-4-hydroxycinnamic

acid (CHCA) for MALDI-TOF analysis was obtained from Applied Biosystems (Concord, ON, Canada). Synthetic peptide standards (5 mg each, certified title ≥95%), corresponding to GGPLDGTYR (CA II-specific peptide) and GGPLEGTYR (internal standard, IS), respectively, were purchased from the CRIBI Center (Padova University, Italy). Their masses were verified by MALDITOF MS instrument (Applied Biosystems), and no contaminants were detected. Peptide stock solutions were prepared in 0.1% TFA at 1 g/L and stored at −20°C. The human CA II standard (≥80%) was purchased from Sigma-Aldrich, reconstituted with 0.1% TFA at 1 g/L, and storedat −80°C. Its actual title was tested by the Bradford assay and was found to be 85±5%.

2.2.2 Samples

Eight human serum samples were obtained from San Raffaele Hospital (Roma) by venipuncture of healthy male and female volunteers (age between 20 and 40), with collection in a Becton Dickinson Vacutainer tube (VWR, West Chester, PA) with serum separator tube gel and BD clot activator. After clot formation, the sample was

centrifuged at 1,000×g for 15 min. The serum was removed and aliquots stored at −80°C. All serum samples were checked by Hemoglobin Ittero Lipemia test on Architect C 8000 system (Abbott, Irving, TX, USA) to verify absence of hemolysis. Total time for serum processing was less than 60 min. A protease inhibitor cocktail, Complete (Roche Biochemicals, Indianapolis, IN, USA) was added to serum to reduce proteolytic degradation.

2.2.3 Sample preparation

Carbonic anhydrase II isolation by RP-HPLC

The serum sample was transferred to a 0.22 μm pore size spin filter for removing particulates by centrifugation (centrifuge from ALC-Milan, Italy) at 16,000×g for 1 min at room temperature. Thereafter, 20 μL of filtered serum were diluted to 1 mL with a solution of water, urea, and acetic acid (final concentration, 6 mol/L urea, 1% v/v acetic acid). The solution was permitted to equilibrate for at least 30 min before RP-HPLC separation. Liquid chromatography was performed using Perkin-Elmer series 200 pumps (Norwalk, CT, USA), including a vacuum solvent degassing unit, and coupled to a UV detector (Varian-Walnut Creek, CA, USA). CA II chromatographic isolation was carried out on a Zorbax 300SBC8 column (150×4.6 mm i.d., 300 Å pore size, 5 μm particle size) from Agilent Technology (Santa Clara, CA, USA) with a securityguard octadecylsilica, 4×3 mm i.d. precolumn, supplied by Phenomenex (Torrance, CA, USA). The column was maintained at 50°C in a thermostatted oven (Timberline Instruments, Boulder, CO, USA). The injection volume was 100 μL of diluted sample (ca. 120 μg of proteins estimated using Bradford assay). Compounds were separated using a gradient of acetonitrile 0.08% (v/v) TFA (B) and water 0.1% (v/v) TFA (A). The gradient, at a flow rate of 0.5 mL/min, started with 30% eluent B and was linearly increased to 80% in 100 min. The eluted proteins were monitored at 215 nm by UV detection. A fraction was collected from 29.0 to 30.5 min based on the CA II retention time obtained by chromatographic analysis of 35 pmol of CA II standard solution in urea and acetic acid. Then the fraction, collected in a protein LoBind tube, was lyophilized (Thermo Fisher Scientific, Waltham, MA, USA) and stored

at −80°C. The chromatographic profile relative to a serum sample fortified with 20 nmol/mL CA II standard is reported in Fig. 1.

Enzymatic digestion and peptide desalting

The lyophilized protein fractions were redissolved with 100 μL of a solution of 100 mmol/L Tris/HCl, 6 mol/L urea, pH=7.8. The sample was reduced with 200 mmol/L DTT, alkylated with 200 mmol/L IAA, diluted to 1 mL, and then digested with 200 ng trypsin at 37°C for 20 h. The digestion was quenched by adding 1% (v/v) TFA. Then, 50 μL of a 3-pg/μL IS solution was added to the tryptic digest to be carried through all further processing. Peptides were desalted using a C18 silica cartridge (2 mL polypropylene tube with 50 mg of the adsorbent phase). Before use, the cartridge was attached to a vacuum manifold apparatus (Millipore), washed with 2 mL of water/acetonitrile (50:50, v/v), and equilibrated with 2 mL of solution consisting of 100 mmol/L Tris/HCl, 0.6 mol/L urea, pH 7.8, and 0.1% (v/v) TFA. The tryptic digest was passed through the C18 silica cartridge, and salts were removed with 1 mL of water 0.1% (v/v) TFA. Finally, the peptides were slowly eluted with 500 μL of water/acetonitrile (20:80, v/v), 0.1% (v/v) TFA, and collected in a 2 mL tube (Protein LoBind). Thereafter, solvents were removed in a water bath at 37°C under a nitrogen stream, and the sample was successively reconstituted with 200 μL of water/acetonitrile (95:5v/v), 0.1% (v/v) formic acid.

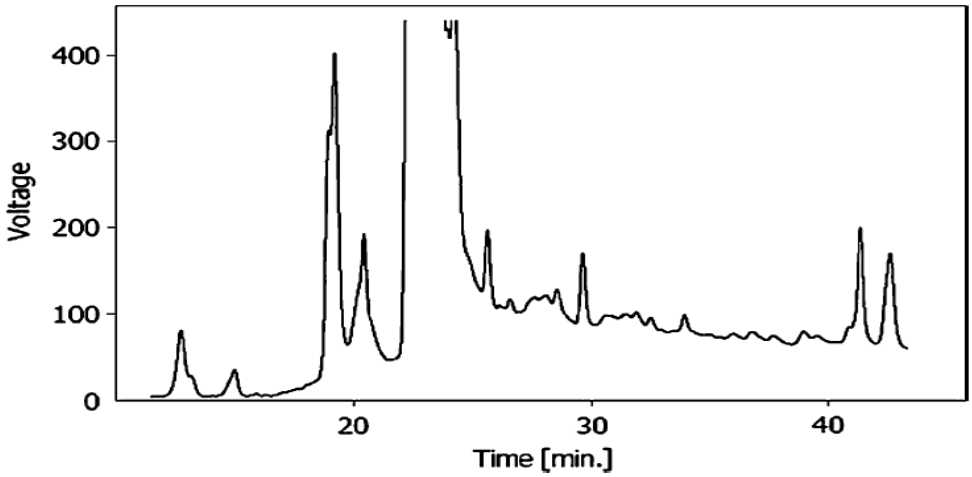

Fig. 1: Reversed-phase chromatographic profile relative to a diluted serum sample fortified with 20 nmol/mL carbonic anhydrase II standard. Zorbax 300SB-C8 column (150×4.6 mm i.d., 300 Å pore size, 5 μm particle size), T=50°C. UV detector at 215 nm

2.2.4 LC/ESI-MS/MS analysis

HPLC-Chip-MS system

Liquid chromatography was performed using an Agilent series 1200 instrument (Agilent Technologies, Waldbronn, Germany) consisting in 1200 Series nanopump with degasser; 1200 Series capillary pump; 1200 Series thermostatted microwell-plate autosampler, and HPLC-Chip-MS interface. The following components are integrated onto the HPLCpolymeric Chip (G4240-65001-Agilent Technologies, Santa Clara, CA, USA): a 40-nL enrichment column packed with ZORBAX 300 SB-C18, 5 μm particle size; a 50 μm (d)× 75 μm (w)×43 mm (l) RP-LC separation column packed with ZORBAX 300 SB-C18, 5 μm particle size; the nanospray emitter (50 μm i.d.). The HPLC-Chip is inserted into the HPLC-Chip-MS interface. It mounts directly on the MS source and includes a miniature camera for spray visualization, the HPLC-Chip loading and ejection mechanism, a

microvalve for flow switching, and fluid connection ports for the nano-LC and microwell-plate autosampler. The nanospray emitter was connected to the Agilent 6410 QqQ equipped with an ESI source. The Agilent Mass Hunter ChemStation software (version B01.03) was used for data acquisition and processing.

Chromatographic conditions

Solvent A was water, and solvent B was acetonitrile, both containing 0.1% (v/v) formic acid. The mobile phase gradient, started with 5% B, reached 31% B after 6 min, then was brought to 95% within 3 min and held constant for 1 min to rinse the column. Finally, the starting condition was restored over 1 min and the column re-equilibrated with a post run time of 2 min. Flow rate was set to 300 nL/ min. Enrichment of the analytes prior to gradient start was performed by capillary pump running at 4 µL/min 0% B connected to a µ-well plate autosampler for sample loading on the Chip. A 2.5-µL aliquot of digested sample was loaded onto the Chip devise by the autosampler. The intelligent sample loading feature of the HPLC-Chip ChemStation menu was used. This feature allows sample loading onto the enrichment column during the pre-run time. After sample enrichment was completed, the rotary valve interface was switched to the load position and then elution gradient started.

Mass spectrometric conditions

ESI stability was tested by delivering a test sample at a constant pressure and by monitoring the MS signal. The MS response of a 0.5 pg/µL reserpine solution was visualized both as the total ion chromatogram and the extracted ion mass m/z 609.2. The signal exhibited stability of 2% at a flow rate of approximately 114

nL/min. Ionization and mass spectrometric conditions were optimized for both CA II-specific peptide and IS by infusing at 0.6 μL/min flow-rate a 100 pg/μL solution in water/acetonitrile 95:5 (v/v) containing 0.1% (v/v) formic acid, using a MS calibration and diagnostic Chip (G4240- 61001—Agilent Technologies). The analyses were performed in positive ionization mode with a capillary voltage set at 1,750 V and a delta electro multiplier voltage of 500 V. The drying gas flow was 4 L/min of nitrogen, and the drying gas temperature was 350°C. Because the HPLC-Chip interface uses an enclosed source design to eliminate background contamination from the laboratory air, to achieve low background conditions, an additional 1.5 L/min of filtered air was added to the drying gas. The position of the nano-ESI Chip tip was optimized for low spray flow rate to produce a good direct ESI spray under the correct voltage and so to achieve high MS sensitivity and a stable spray. Peptide diprotonated molecules $[M + 2H]^{2+}$ (m/z 468.4 and 475.4) were mass-selected by the first quadrupole and fragmented. Two suitable transition pairs were chosen for acquisition in MRM mode for both CA II-specific peptide and IS peptide. The fragmentor and collision energy (CE) were optimized for each ion. Identification was performed on the basis of retention times and spectra matching respect to standards. Parameters are summarized in Tab. 1. The Chip can be used for many hours, even after several hours of no-use, in contrast to standard commercial nano-ESI tips.

Peptide	Retention time (min)	Precursor ion	MRM transition	Relative abundance (%)
GGPLDGTYR (CA II marker)	5.04	$[M + 2H]^{2+}$	468.4→611.4	53
			468.4→411.4	100
GGPLEGTYR (Internal standard)	4.97	$[M + 2H]^{2+}$	475.4→625.4	100
			475.4→418.2	92

Tab. 1: Instrumental parameter settings under Multi Reaction Monitoring conditions for the two peptides (CA II specific peptide and internal standard peptide

Calibration

Standard solutions for calibration were prepared by drawing the appropriate volume of the CA II-specific peptide GGPLDGTYR working standard solution, 50 μL of a 3- pg/μL IS peptide GGPLEGTYR working standard solution, evaporating at 37°C under a gentle N2 stream. Then the solution was reconstituted with 200 μL of the starting chromatographic mobile phase in order to obtain nine concentration levels in the range of 0.01–50 pmol/mL. Volumes of 2.5 μL of standard solutions were injected. The ion current profiles of the selected transition pairs for the two peptides were extracted from the LC-MRM dataset, the resulting peak areas were measured, and the ratios of CA II specific peptide to IS peptide areas were plotted. The matrix-matched calibration line was constructed by using a serum pool obtained by mixing 25 μL serum aliquots from the eight healthy subjects. As each 2 μL of sample give rise to a 200 μL of final extract, for matrix-matched calibration line, three extracts were mixed and subdivided in 50 μL aliquots, processed as reported above, and spiked with the suitable standard

amounts (GGPLDGTYR and IS standard solutions) in order to obtain the same concentrations as for the external calibration. After evaluating the analyte mean concentration by using the matrix-matched calibration line, the standard addition method was also performed. Two hundred microliters of the processed sample were subdivided in four aliquots of 50 μL each. One of them was analyzed unspiked, while 20, 30, and 40 pg, respectively, of the CA II-specific peptide (corresponding to 43, 64.5, and 86 pmol/mL in serum) were added to the remaining three aliquots before injection. All samples were run in triplicate, results were averaged, and the unweighed regression lines were constructed. In a separate experiment, the linear range was tested by an eight-point calibration graph. The response of ESI-MS/ MS was linearly related to the injected amounts up to 40 ng (3 nmol/mL serum), ($R2>0.9985$).

Recovery experiments and statistical evaluation

Absolute recoveries (for both the whole procedure and the selected representative peptide), and analytical recoveries were evaluated separately. Eight human serum aliquots were pooled and then split again into 12 aliquots (divided in three sets of four aliquots each). Then, three aliquots of the first set were fortified with suitable volumes of the CA II standard solution to obtain the spiked concentration levels of 103, 206, and 345 nmol/mL, respectively, while the fourth aliquot was kept unspiked. The criterion adopted for fortification was spiking at three different levels: about twice, four, and ten times the amount of endogenous protein (roughly evaluated by using external calibration). Afterwards, all the first set aliquots were handled as described above, but the IS was added just before injection. The second set samples were submitted to HPLC fractionation,

liophylization and tryptic digestion, then molar amounts of the representative peptide corresponding to those used for intact CA II fortifications were added to three aliquots of second set, while the fourth was left unspiked. The IS was added to the four aliquots just before injection. The third sample set was handled as the second one with the difference that the IS was added after tryptic digestion. Analyses of spiked and unspiked samples were replicated six times. Recoveries were assessed for each concentration by measuring the resulting peak area of the CA II-specific peptide (GGPLDGTYR), calculating the peak area ratio relative to that of the IS, subtracting the mean ratio value for unspiked samples and comparing this result with that obtained by adding both CA II-specific and IS peptides just before injection. Statistical evaluation was performed by the analysis of variance ($p=0.05$).

Peptide adsorption to surfaces

Peptide adsorption is an important attribute to take into consideration also for reproducibility. Because the steel passivation did not allow further peptide adsorption, reproducibility of LC-MS data was improved by periodically treating the autosampler needle (the only metallic components of the LC system) with a water/nitric acid solution 50:50 (v/v). To reduce peptide adsorptive processes, suitable storage vessels were used (protein low-retention tubes) [70].

2.3 Results and discussions

2.3.1 CA II-specific peptide and IS selection

After the RP-HPLC separation step, the sample complexity was still very high. When in proteomic research identification of proteins depends on identification of peptides resulting from enzymatic digests and the concentration of a selected peptide is stoichiometrically related to the concentration of intact protein [71–73], the choice of the target peptide appears crucial. Figure 2a, b report the MALDI-TOF spectra obtained by analyzing 0.2 nmol/mL CA II standard solution, and a serum sample extract spiked with the same concentration, respectively. The most abundant peptides coming from CA II tryptic digestion are indicated by their m/z values. Among tryptic cleavage peptides of CA II, GGPLDGTYR (corresponding to 81–89 sequence) was selected as the protein marker. In fact, this peptide amino acid sequence is associated exclusively to CA II; its signal intensity was reproducible and does not contain any reported posttranslational modification sites, thus its monoisotopical mass of 934.5 Da is not subjected to variations. Moreover, a preliminar LC/ESI-MS analysis of peptide mixture deriving from CA II enzymatic digestion showed an intense ion current corresponding to the diprotonated GGPLDGTYR molecule. The synthetic peptide GGPLEGTYR, having chemical and physical properties very similar to those of GGPLDGTYR, was chosen as IS. In fact, GGPLEGTYR structure (monoisotopic molecular mass of 948.5 Da) differs only for one amino acid (a glutamic acid residue instead of an aspartic acid residue) from GGPLDGTYR structure and is absent in unspiked samples. In this way, the use of a labeled peptide for protein quantification could be avoided. The advantages and limits of non-isotopically labeled IS has been

discussed previously [70]

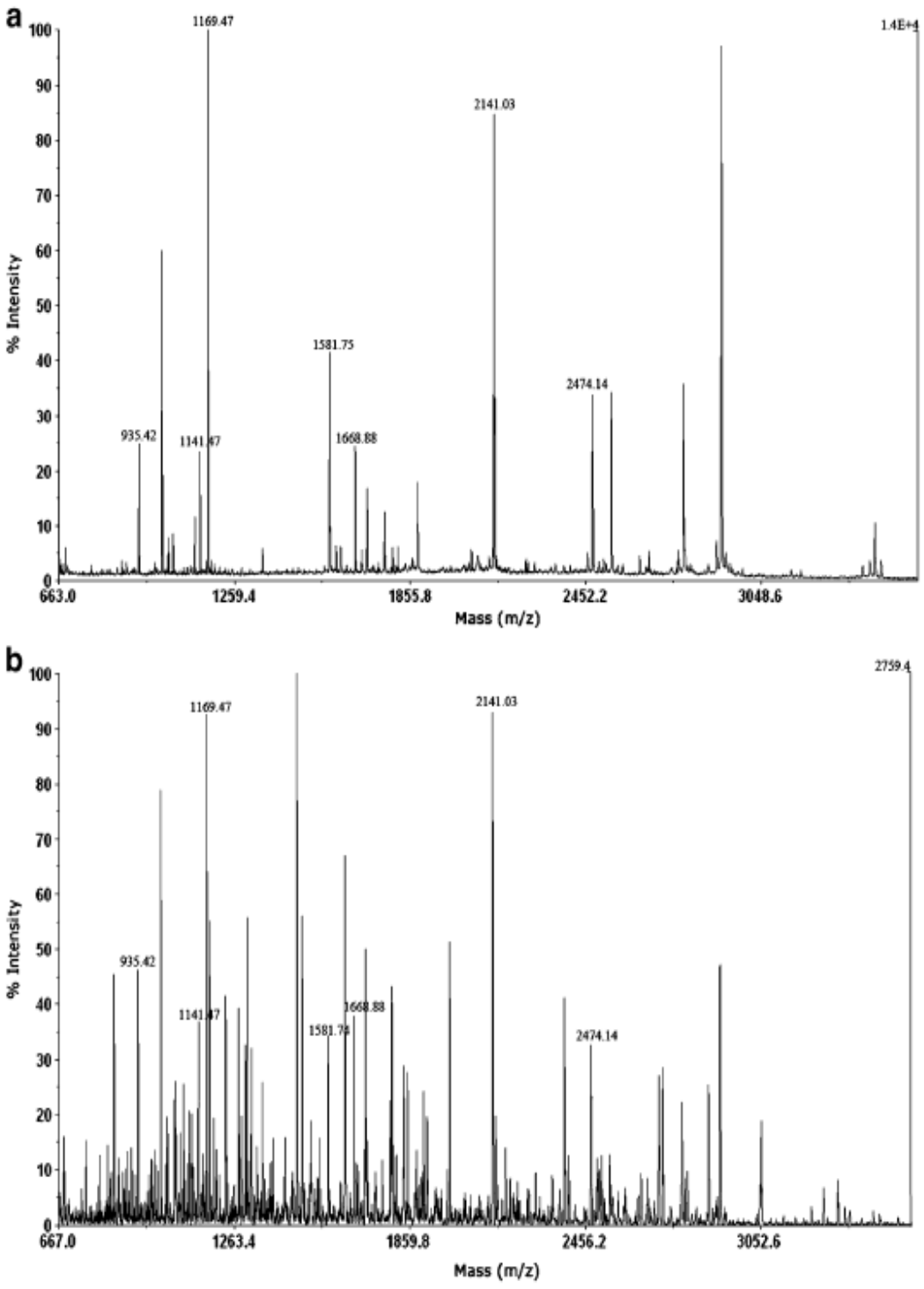

Fig. 2: MALDI-TOF spectra of trypsin digest. a 0.2 nmol/mL carbonic anhydrase II standard solution; b 0.2 nmol/mL CA II added to a serum sample

2.3.2 Sample preparation optimization

Complex serum protein samples were fractionated by RPHPLC. Solvent composition, flow rate, and gradient profile were optimized for CA II isolation from the serum most abundant proteins in a relatively short run time. By this separation, it was possible to select a narrow fraction according to the retention time (tR) of the standard protein. By injecting 1 μg of CA II, both standard and added to the serum samples, the chromatographic peak base width was ca. 40 s (see Fig. 1). Retention time reproducibility was attained by thermostatting the column; however, to assure a total protein recovery, its corresponding fraction was collected over 1.5 min. Identification of CA II was achieved only in the collected fraction by peptide mass fingerprinting of its tryptic digest, performed by a MALDI-TOF instrument. Considering that the IS can operate only after the peptide generation, it is possible to directly correlate target peptide amount to the parent protein amount only when the enzymatic cleavage gives high and reproducible yields. The amount of trypsin to be added to the serum fraction containing CA II was optimized by maximizing the target peptide peak area in a series of experiments accomplished in quadruplicate on aliquots of a purified serum pool. Two hundred nanograms trypsin assured the largest peptide generation which did not increase further on by increasing the trypsin amount. Absolute recovery of CA II for the whole procedure was performed by spiking serum samples with the standard, at three concentration levels as described in the "Experimental" section (first set of samples). The overall absolute mean recovery for the method was 52% with relative standard deviation not larger than 12% and was not dependent on the fortification levels tested. Peptide desalting and solvent

exchange steps were also very important for mass spectrometric performances (in particular signal suppression and reproducibility). Because the commercial zip tips for peptide mixture desalting have a low sample size throughput, 50 mg C18 (large pore) solid-phase extraction cartridges were preferred. The elution optimization has been described in a previous work [74]. The absolute mean recovery for the steps involving the selected peptide was evaluated by adding the peptide standard at three concentration levels after protein digestion and the IS just before injection (second set of samples). The peptide recovery was 65% with relative standard deviation not larger than 8% and was independent from the fortification levels tested. As expected, the third set of samples gave a mean recovery of 82% with relative standard deviations less than 15%. Overall recovery experiments demonstrated that the procedure critical step is the peptide desalting and evaporation/reconstitution, while the digestion step yield was about 80%, and recovery from chromatographic purification was about 100%.

2.3.3 Chromatographic performance of the HPLC-Chip

The advantages of HPLC-Chip-MS with on-Chip chromatography and spray tip in proteomic research have been discussed previously [75,76]. In Chip, all connections are ports rather than tubes, volumes are only a few nanoliters, and this minimizes the dead volumes, resulting in reduced band dispersion. Moreover, surface areas and contact surfaces are inert polyimide in place of steel or glass, thus reducing the chemical activity with peptides. As the band broadening is not longer due mainly to dead volumes, the mobile phase flow rate may contribute significantly to resolution. The HPLC-Chip device can be operated in the flow rate range from 100 to 400 nL/min [52]. The LC flow rates delivered by the

nanopump during gradient elution were set to 100, 150, 200, 300, and 400 nL/min and 2.5 μL of 34 fmol/mL CA II standard digest was injected three times in each run. Although the highest MS detection sensitivity could be achieved at 100 nL/min, reduced separation efficiencies were observed at this flow rate. Moreover, in agreement with previously reported results [52], by increasing the flow rates up to 300 nL/min, the sample throughput increased without significant increase in limit of detection. Another important consideration about Chip technology is the ruggedness and ease of operation of the system. In our hands, the nano-LC-Chip-MS system was operated in an uninterrupted manner for more than 600 injections of complex proteins digests on a single Chip without any noticeable performance degradation.

2.3.4 Chip-nano-ESI-MS/MS condition optimization

Nano-ESI-MS/MS detection was essential to ensure the necessary limit of quantification (LOQ) and sensitivity for serum CA II determination which cannot be obtained by a conventional ESI source. For robust, sensitive, and reliable quantitative determination of target peptides, the Chip device was coupled with a QqQ mass spectrometer working in MRM mode. As expected [77] in infusion experiments, for both peptides the double-charged molecules (m/z 468.4 and 475.4) gave a more intense signal in the mass spectrum than the monoprotonated molecules (m/z 935.4 and 949.5, respectively). Moreover, when selecting the mono-charged molecule as precursor in MRM acquisition mode, fragmentation occurred at high CE, and the relative abundances of transition pair signals in the spectrum showed a great variability. Thus, diprotonated molecules were selected as parent ions. Operating at the optimal instrumental conditions for $[M + 2H]^{2+}$

detection, the $[M + H]^+$ signal was not detectable in the spectrum. In product ion experiments, the two most intense

fragment ions of GGPLDGTYR peptide (m/z 611.4— y-series ion, and m/z 411.4—bicharged y-series ion) and of IS peptide (GGPLEGTYR; m/z 625.4—y-series ion, and m/z 418.2—bicharged y-series ion) were chosen for MRM experiments. CA II-specific peptide and IS peptide retention times differed of less than 0.1 min. In Fig. 3, the masschromatogram relative to the sum of the selected transitions for each peptide, obtained by analyzing CA II in a human serum sample, is reported.

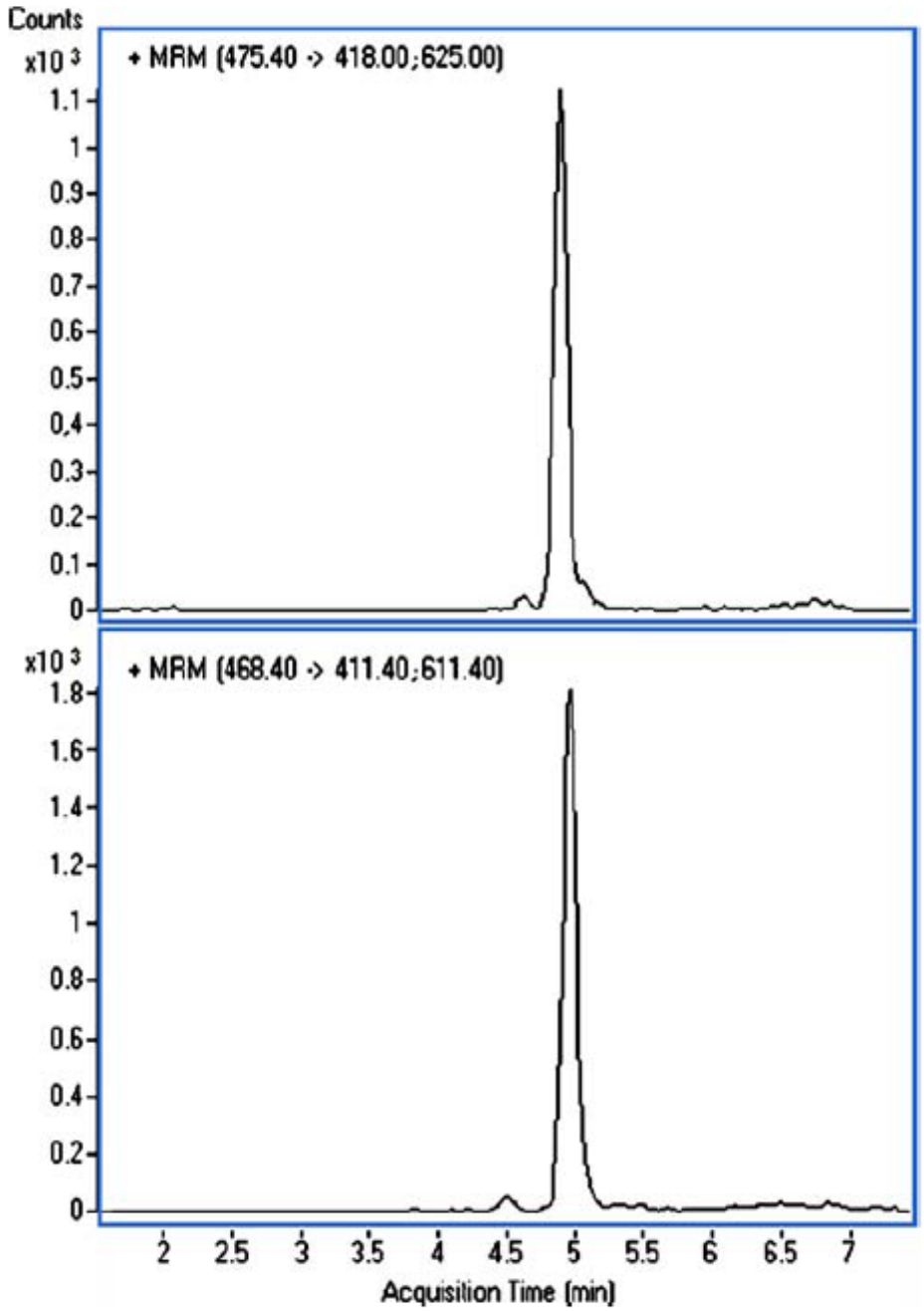

Fig. 3: Positive electrospray mass-chromatogram in multiple reaction monitoring mode relative to the sum of the selected transitions for carbonic anhydrase II-specific peptide (GGPLDGTYR, m/z 468.4 → 411.4; 611.4) and IS (GGPLEGTYR, m/z 475.4 → 418.2; 625.4)

2.3.5 Method performances

Calibration regression line and matrix effect evaluation

Calibration graphs were constructed as described in "Experimental" section. Three different regression line types were considered: (1) external calibration; (2) standard additions; (3) matrix-matched calibration. The standard addition regression lines were constructed taking into account both the absolute signal areas and the areas normalized for the IS response. Every dataset was obtained by triplicate measurements, interleaving three blank samples before the next series of injections. The results was evidenced the standard addition method was preferred to the matrix-matched calibration performed in pooled sample aliquots in order to obtain the matrix effect variability originated by non-homogeneity of serum sample. Most remarkably, the standard addition, the matrix matched, and the external calibration regression lines, after normalization by IS response, did not evidence significant differences in slope: mean $b_{matrix}/b_{standard}$ was 0.95, and mean $b_{standard\ addition}/b_{standard}$ was 0.93.

Accuracy and precision

Accuracy and precision were estimated from the analytical recovery calculated by adding known amounts of authentic CA II to the serum samples and the IS after tryptic digestion, as reported in the "Experimental" section (third set of experiments). Precision was also measured as the between-day relative standard deviation (RSD, %) from spiked and unspiked samples analyzed six times. A mean recovery of 81±10% was obtained, and differences among the three spiked levels were not significant at $p<0.05$. By comparing these values with absolute recoveries reported above, it appears that the selected IS compensates for

incomplete peptide recoveries, whereas a correction for intact protein loss, likely from the digestion step, can be accomplished. Between-day precision for unspiked samples seems appropriate for the purpose of biomarker determination [52]. Reproducible tR, in association with m/z ratio, are important factors for characterizing a peptide sequence. For this reason, tR reproducibility for the HPLC-Chip-QqQMS/MS system was also tested. Target peptide tR was measured over 1 month obtaining an average value of 5.01±0.42 min. As can be seen, there is an excellent reproducibility usually not achieved by conventional nanoHPLC/nano-ESI configurations.

Detection and quantification limits

The instrumental limit of detection (LOD) was estimated by the MRM LC-MS/MS chromatogram resulting from the analysis of 0.1 fmol of the synthetic CA II-specific peptide from a standard solution injected. The selected transitions from data set were extracted obtaining the resulting traces. Thereafter, the peak height-to-averaged background noise ratio (S/N) was measured. The background noise estimate was based on the peak-to-peak baseline close to the analyte peak. For GGPLDGTYR peptide, a definition of LOD as the amount giving S/N=3 for the second most intense

transition was adopted. Data are shown in Table 2, together with instrumental LOQ, estimated considering the sum of the two transition ion currents selected for the peptide and defining it as the amount giving S/N=10. Method identification limit (MIL) [78] and method quantification limit (MQL) were estimated in the same way of LOD and LOQ, respectively, by analyzing a physiological sample.

	LOD[a]	MIL[b]	MQL[c]
GGPLDGTYR	6 amol	2 pmol/mL	3 pmol/mL
CA II	–	2.5 pmol/mL	3.7 pmol/mL

[a] Instrumental limit of detection, expressed as attomols injected

[b] Method identification limit (*S*/*N*=3 for the second most intense transition in MRM), expressed as pmol/mL of serum

[c] Method quantification limit (*S*/*N*=10), expressed as pmol/mL of serum

Tab.2 Method performances

CA II quantitation in human serum samples

External calibration and standard addition method were employed for CA II-specific peptide quantification. The molar concentration of the peptide was assumed equal to the molar concentration of CA II. Recovery correction was applied. Eight human serum samples from apparently healthy subjects (five men and three women, aging 20–40) were analyzed, and for each sample, three RP-HPLC runs were done. The CA II concentration in serum was estimated to be 56 pmol/mL (RSD=21%) and 61 pmol/mL (RSD=24%) by using external calibration and standard addition, respectively.

2.4 Conclusion

The explosive growth in proteomic research for biomarker discovery, with its huge number of samples, demands high throughput, high sensitive, and high reproducible nano-LCMS technologies. In this work, we developed a method able in quantifying in serum CA II, a protein present at concentration level about five orders of magnitude lower than that of the most abundant proteins, by using nano-

LCnano- ESI-MS/MS. The same scheme, based on the molar equivalence of a specific proteolytic peptide with the peptide-producing protein and a nonlabeled peptide as IS experimented for a protein present in cardiac tissue samples at concentration of about hundreds of picomoles per gram [70], can be used for a protein in serum at few picomoles per milliliter levels, providing that a suitable nanospray MS device is adopted. In addition, using a chromatographic purification step instead of a most abundant serum protein depletion kit, protein loss due to aspecific interactions with the proteins bonded by antibodies can be avoided. The hardware miniaturization introduced by Chip technology has radically reduced analysis time and cost and has increased efficiency and sensitivity. Moreover, using this device allows to reduce matrix effect; then it is possible to use the external calibration for quantification.

CHAPTER 3: Immunoprecipitation on magnetic beads and liquid chromatography–tandem mass spectrometry for carbonic anhydrase II quantification in human serum

3.1 Introduction

Most of the studies dealing with biomarker discovery on blood are focused on the cell leakage products potentially present in that matrix; indeed, increased serum levels of various proteins are routinely used for diagnostic purposes [79–82]. The analysis of serum is challenging because of the large dynamic range of protein concentrations, spanning more than 10 orders of magnitude [79,83]. In particular, the presence of high-abundance proteins such as albumin and immunoglobulins, whose concentrations represent 60–90% of the total serum protein content, enhances the difficulty of detecting the low-abundance proteins of interest [83]. Recently, a multisite assessment of the precision and reproducibility of multiple reaction monitoring (MRM)1-based measurements of target proteins in plasma demonstrated that this platform may be very reproducible for proteins present at moderate to high abundance (>2 mg/ml) in nondepleted, nonfractionated plasma [84]. On the contrary, the reduction of sample complexity is an essential first step in the analysis of the serum low-abundance proteins, and it is often achieved by high-abundance protein depletion [85]. A common approach is the affinity removal method using antibody- based resins (both monoclonal and polyclonal) or affinity dye-based resins [85–87]. There are several affinity removal columns or kits commercially available for depleting up to 20 major abundant serum proteins [87,88]. Although this strategy is highly specific, even after depletion the

remaining proteins are still sufficiently abundant to hamper the low-abundance protein determination. Moreover, some authors have recently pointed out the risk of losing low-abundance proteins and biomarkers when using affinity-based depletion due to the association of the targeted proteins with the abundant ones [83,87,89,90], but different results have also been published [90–93]. Another method of reducing serum sample complexity is the filtration through molecular weight cutoff membranes [87,89] for removing the high-molecular-weight proteins, including albumin. Solvent precipitation has also been employed, in particular for albumin depletion [87]; the method is nonspecific but presents the advantage of being rapid and cheap compared with the immunoaffinity- based methods. For isolation of the fraction containing the target proteins, liquid chromatographic and electrophoretic fractionation techniques have been used [87]. A promising approach is the employment of affinity reagents for specific enrichment or isolation of target proteins [83,94]. Recently, increasing attention has been given to the development and application of separation techniques employing small magnetic particles. Magnetic carriers bearing an immobilized affinity or hydrophobic ligand or ion exchange groups, or magnetic biopolymer particles having affinity to the isolated structure, are mixed with the sample containing the target compound [95]. Following an incubation period in which the target compound binds to the magnetic particles, the whole magnetic complex is easily and rapidly removed from the sample using an appropriate magnetic separator. The isolated target compoundcan then be eluted and used for downstream applications and detection methods. Several types of mass spectrometry (MS) techniques in conjunction with various liquid chromatography

(LC) separation methods are adopted for proteomic measurements. In particular, the MRM acquisition mode is the most employed one in targeted proteomics [79,83,96–98]. For absolute quantitative analysis, labeling and labelfree strategies [99] are currently carried out, both aiming to correlate the mass spectrometric signal of proteotypic peptide with the relative or absolute protein quantity directly [100]. Carbonic anhydrase II (CA II), a single polypeptide chain of 29 kDa molecular weight, is present in the cytosol of most tissues even if the highest concentration is found in erythrocytes. It catalyzes the hydration of CO2 and the hydrolysis of esters, and its deficiency has been associated with pathological consequences such as mental retardation and cerebral calcification, osteoporosis, and renal tubular acidosis [101,102] as well as Down syndrome [103,104] and Alzheimer's disease [104]. Although serum is not the natural site of CA II, in consequence of cell release, this protein could be present at low concentration in serum. Furthermore, because CA II is involved in serious diseases, it represents an interesting model to investigate the separation, identification, and quantification of proteins present in low concentration in complex matrices. In a recent study by our laboratory [105], CA II quantification in serum was performed by automated LC chip technology coupled with an electrospray ionization (ESI) source and a triple quadrupole mass spectrometer, operating in MRM acquisition mode, after purification by reversed phase LC, enzymatic digestion, and surrogate peptide selection. The highly sensitive nanoelectrospray chip technology was necessary because only 1 μL of serum could be submitted to the LC isolation step without column overloading. In the current study, a Protein G magnetic bead-based antibody platform amenable to high throughput is proposed for the selective

enrichment of CA II in human serum, followed by its accurate and reliable quantification by a label-free procedure performed on a less technologically sophisticated LC–ESI–tandem mass spectrometry (MS/MS) system. Magnetic beads bound to polyclonal anti-CA II antibodies as affinity probes to isolate the specific target protein, and a conventional LC–ESI–MS/MS apparatus amenable to small molecule quantitation, were used for the determination of the CA II proteotypic peptide produced through proteolysis.

3.2 Experimental part

3.2.1 Reagents and chemicals

Anti-CA II polyclonal antibody was purchased from U.S. Biological (Swampscott, MA, USA), and Dynabeads Protein G were obtained from Invitrogen (Carlsbad, CA, USA). Synthetic peptide standards (5 mg each, certified title P95%), corresponding to GGPLDGTYR (CA II proteotypic peptide) and GGPLEGTYR (internal standard [IS]), were purchased from CRIBI Center (Padova University, Italy). The human CA II standard (<80%), dimethyl pimelimidate dihydrochloride (DMP), triethanolamine, Tween 20, Bradford reagent, ammonium bicarbonate, phosphate-buffered saline (PBS, pH 7.2), and trifluoroacetic acid (TFA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane, 1,4-dithiothreitol (DTT), iodoacetamide (IAA), urea, and protease inhibitor mix were purchased from GE Healthcare (Uppsala, Sweden). All organic solvents were of the highest grade available from Carlo Erba Reagents (Milan, Italy) and were used without any further purification. Ultrapure water was produced from distilled water by a Milli-

Q system (Millipore, Billerica, MA, USA). Modified porcine trypsin, sequencing grade, was commercialized by Promega (Madison, WI, USA). Peptide stock solutions were prepared in 0.1% TFA at 1 g L^{-1} and stored at -20 °C. The human CA II standard was reconstituted with 0.1% TFA at 1 g L^{-1} and stored at -80 °C. Its actual title was tested by the Bradford assay and was found to be 85 ± 5%.

3.2.2 Samples

Serum samples were obtained from the Department of Experimental Medicine at Sapienza University of Rome (Italy) by venipuncture of healthy volunteers (20–40 years of age), with collection done in a BD P100 Blood Collection System (Becton Dickinson, Franklin Lakes, NJ, USA) with K2EDTA anticoagulant and protease inhibitors mix (GE Healthcare). After clot formation, the sample was centrifuged at 1000g for 15 min. The serum was removed, and aliquots were stored at -80 °C. All serum samples were checked to verify the absence of hemolysis.

3.2.3 Immobilization of antibody on magnetic beads

The antibody solution (400 μL of 1μg μL^{-1} anti-CA II) was added into an Eppendorf microcentrifuge tube containing 200 μL (6 mg) of prewashed Dynabeads Protein G and incubated by gentle mixing at room temperature (RT) for 2 h to allow time to attach onto the surface of the beads. Following incubation, residual unbound antibody was removed by washing two times with 0.5 ml of citrate phosphate buffer (pH 5.0) with 0.05% Tween 20. Approximately 10 μg of antibody was bound per milligram of beads. For crosslinking of antibody to beads, the Dynabeads Protein G with immobilized polyclonal antibody were washed with

1 ml of 0.2 mol L^{-1} triethanolamine (pH 8.2) and resuspended in 1 ml of freshly prepared cross-linking buffer (20 mmol L^{-1} DMP in 0.2 mol L^{-1} triethanolamine, pH 8.2). The mixture was incubated under gentle rotation for 30 min at RT and placed on a magnet, and then the supernatant was discarded. To stop the reaction, the beads were resuspended in 1 ml of 50 mmol L^{-1} Tris (pH 7.5) and incubated for 15 min, and then the beads were rinsed three times with 1 ml of 100 mmol L^{-1} PBS with 0.05% Tween 20 before use.

3.2.4 CA II capture and digestion

To capture CA II, the Dynabeads Protein G with immobilized antibody were incubated at 4 °C for 1 h, under gentle mixing, with 20 μL of serum diluted with 100 mM PBS (1:49, v/v), and then the beads were recovered from the sample and washed three times with 1 ml of 100 mmol L^{-1} PBS. Immunocomplex was resuspended in 40 μL of 6 mol L^{-1} urea solution in 25 mmol L^{-1} ammonium bicarbonate and 2 μL of 10 mmol L^{-1} DTT and incubated at 37 °C for 1 h, under slight agitation, to denature captured antigen. Then 8 μL of 10 mmol L^{-1} IAA was added, and the immunocomplex was incubated at RT for 1 h in the dark. Subsequently, 8 μL of 10 mmol L^{-1} DTT was added and incubated at 37 °C for 1 h, under slight agitation, to consume any leftover alkylating agent and to avoid trypsin alkylation. The mixture was then diluted with 25 mmol L^{-1} ammonium bicarbonate to obtain a final urea concentration of 1 mol L^{-1}. Reconstituted trypsin solution (20 μg ml^{-1} in 25 mmol L^{-1} ammonium bicarbonate) was added to a final concentration of 5.8 μg ml^{-1}. The samples were allowed to digest under gentle mixing overnight at 37 °C, the digestion was quenched by adding 4 μL of formic acid, and then 50 μL of 3 pg ml^{-1} IS solution

was added. The supernatant (~400 μL) containing the peptides coming from both antigen and antibody digestion was recoveredù using a magnet, and an aliquot was subjected to LC–MS/MS analysis.

3.2.5 Chromatographic and mass spectrometric conditions

The LC apparatus consisted of a series 200 binary LC micropump, a series 200 binary LC pump, two vacuum degassers, an autosampler (PerkinElmer, Norwalk, CT, USA), and an eight-port Valco valve equipped with a 100 μL peek loop (VICI AG International, Schenkon, Switzerland). The LC separation was carried out on a Jupiter Proteo 4 μm C12 column (150 x 1 mm i.d., 4 μm average particle size, 90 Å pore size) equipped with a 2.1-mm i.d. guard column from Phenomenex (Torrance, CA, USA). A C18 trap column (4.0 x 2.0 mm i.d.) supplied by Phenomenex was used for on-line solid phase extraction (SPE) with 100 μL of samples being injected. ESI–MS was carried out on an API 3000 triple quadrupole instrument equipped with a TurboIonSpray (TISP) interface and with a built-in software-controlled eight-port valve (Applied Biosystems/MDS Sciex, Concord, ON, Canada). The LC–MS system, data acquisition, and processing were managed by Analyst software (version 1.4.2, Applied Biosystems/MDS Sciex). The scheme of the on-line system is shown in Fig. 4. The two alternate positions of the software-controlled eight-port valve allowed flows witching in the trap column. The LC binary pump (pump 1) was used to deliver a 300 μL/min flow rate through the trap column for loading and washing the injected sample, whereas the LC binary micropump (pump 2) was used to deliver a 70 μL/min flow rate for eluting the analyte from the trap column in back flushing mode, for carrying out the chromatographic run, and subsequently for flushing and

equilibrating the column. The mobile phases used for sample loading and washing (pump 1) were water (A) and acetonitrile (B), with both containing 1% (v/v) formic acid. Mobile phases for analyte elution (pump 2) consisted of water (C) and acetonitrile/ methanol (60:40, v/v) (D), with both containing 0.1% (v/v) formic acid. The sample was loaded onto the trap column and desalted by washing with 2% B for 2 min. Elution was performed by switching the eight-port valve; after an isocratic step at 10% D for 1 min, D was linearly increased to 40% within 7 min and then the eight-port valve was resettled to the starting position and B and D were brought to 95% within 1 min and held constant for 4 min to rinse the column and trap column. Finally, the D content was lowered to 10% and the B content was lowered to 2% over 1 min, and both the column and trap column were reequilibrated for 15 min. The mass spectrometer was calibrated using polypropylene glycol as standard (Applied Biosystems). Ionization and mass spectrometric conditions were optimized for both of the peptides (CA II-specific peptide and IS peptide) by infusing at a 5 μL min^{-1} flow rate a 0.1 ng μL $^{-1}$ solution prepared in water/acetonitrile/formic acid (70:30:0.1, v/v/v). TISP interface was operated in the positive ionization mode by applying to the capillary a voltage of 5500 V. Nitrogen was used as curtain, nebulizing, and turbo spray gases (heated at 300 °C), and the gas pressures were set at 20, 30, and 40 psi, respectively. Nitrogen, kept at medium pressure (arbitrary units), also served as collision gas. Diprotonated molecules ($[M + 2H]^{2+}$) were mass selected by the first quadrupole and fragmented. In the product ion scan mode, the range of m/z 200–1000 was monitored for both CA II proteotypic peptide and IS.

A flow chart of the analytical procedure is shown in Fig. 5.

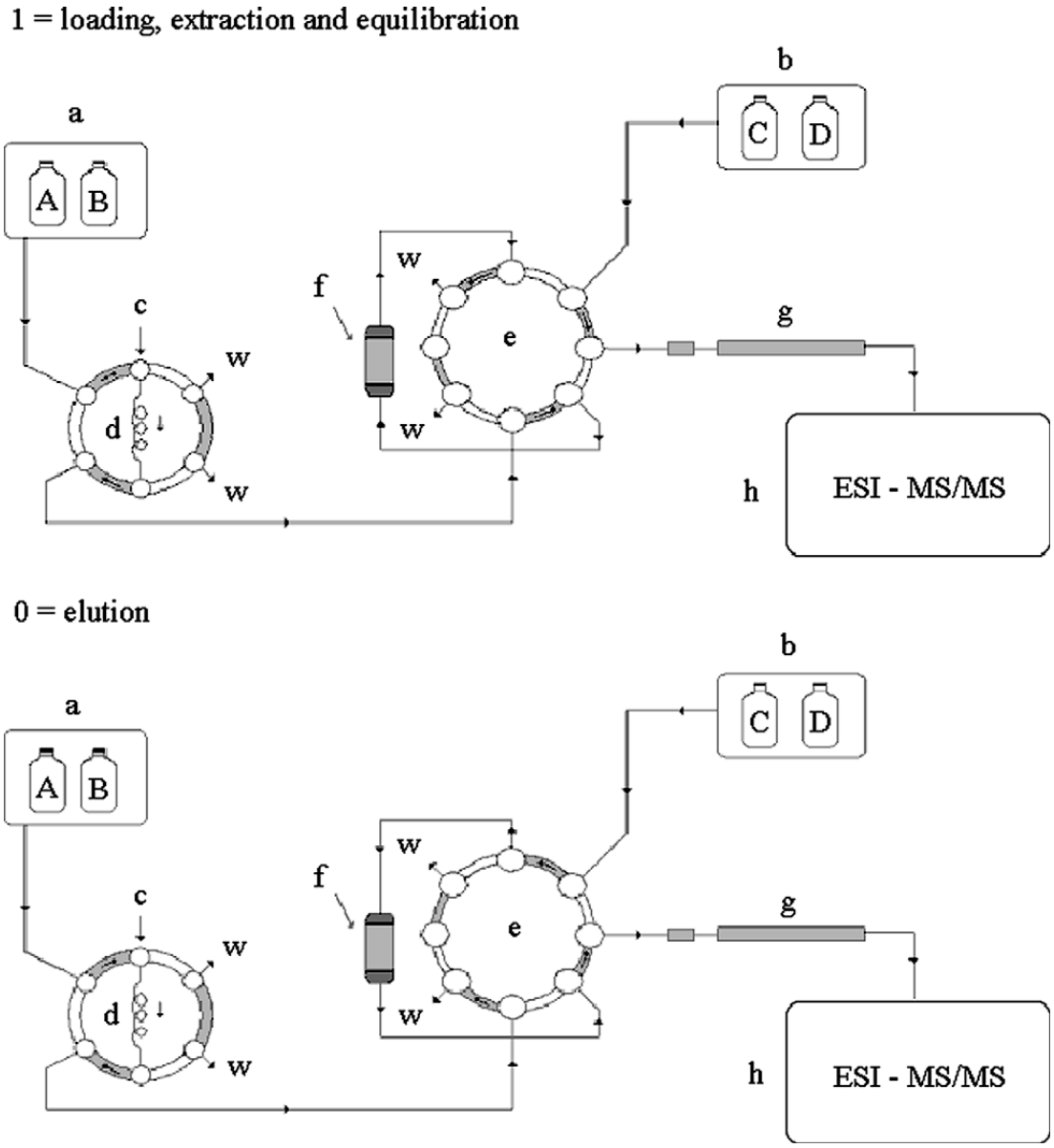

Fig. 4: On-line LC–MS/MS CA II proteotypic peptide analysis after off-line immunoextraction and tryptic digestion. Analytical system scheme: 1, position used for sample loading, extraction, analytical column equilibration, and cartridge regeneration; 0, position used for peptide elution from the extraction/enrichment trap column and peptide separation by the analytical column. Mobile phases: A, water; B, acetonitrile (containing 1% [v/v] HCOOH); C, water; D, acetonitrile/methanol (60:40, v/v, both containing 0.1% [v/v] HCOOH). a, pump 1; b, pump 2; c, autosampler; d, 100 µL loop; e, eight-port valve; f, extraction/concentration trap column; g, analytical column; h, mass spectrometer; w, waste.

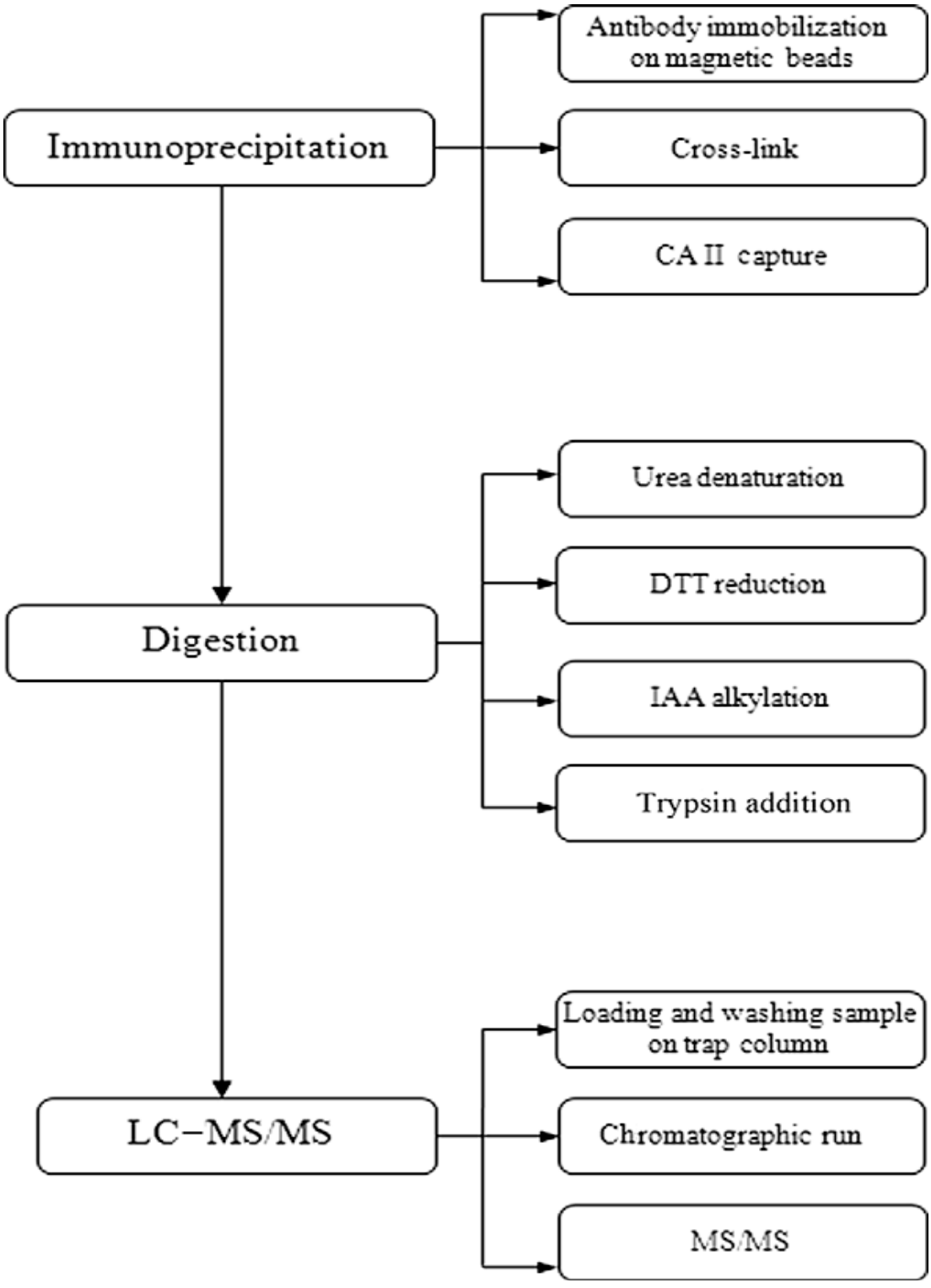

Fig. 5: Flow chart of the analytical procedure.

3.2.6 Calibration and recovery studies

Standard solutions for calibration were prepared by drawing the appropriate volume of the CA II-specific peptide GGPLDGTYR working standard solution so as to obtain nine concentration levels in the range of 0.05–100 pg μL^{-1} and 50 μL of a 3 pg μL^{-1} IS peptide GGPLEGTYR working standard solution. The solvent was evaporated at 37 °C under a gentle N_2 stream, and then the solution was reconstituted with 400 μL of 25 mmol L^{-1} ammonium bicarbonate acidified with formic acid (25 mmol L^{-1}). Volumes of 100 μL were injected. The ion current profiles of the selected transition pairs for the two peptides were extracted from the LC MRM dataset, the resulting peak areas were measured, and the plot of the ratio between the peak areas of the CA II synthetic peptide and the IS peptide versus concentration was obtained. The matrix- matched calibration line was also constructed by using a serum pool obtained by mixing serum aliquots from the eight healthy subjects. Nine 20 μL aliquots were processed as reported above. Next, to obtain the same concentrations as for the external calibration, the samples were transferred into vials spiked with the

suitable volume of standard and IS solutions after solvent evaporation. All samples were run in triplicate interleaving three blank samples before the next series of injections, and results were averaged. From the initial set of candidates, two suitable transition pairs were chosen for acquisition in MRM mode for both peptides. The declustering, entrance, and focusing potentials were maintained at the optimal values of 28, 7, and 370 V, respectively, whereas collision energy was optimized for each ion. The LC–ESI–MS/MS parameters are summarized in Table 3. Recovery studies were conducted by spiking human serum samples with

different amounts of CA II standard at different procedure steps while maintaining the IS amount constant.

Peptide	Retention time (min)	Precursor ion	MRM transition	Relative abundance (%)
GGPLDGTYR (CA II marker)	4.2	$[M + 2H]^{2+}$	468.3→496.3	100
			468.3→411.2	30
GGPLEGTYR (Internal standard)	4.0	$[M + 2H]^{2+}$	475.3→625.3	100
			475.3→418.2	90

Tab.3: Instrumental parameter settings under MRM conditions for the two peptides: CA II proteotypic peptide and IS peptide.

3.3 Results and discussion

3.3.1 Abundant protein depletion

To avoid significant interference due to nonspecific interaction of the most abundant serum proteins with the antibody capture of CA II, in a first attempt, immunoaffinity depletion of serum samples was carried out as the first analytical step before immunoprecipitation. Depletion was performed on the Agilent Human 14 Multiple Affinity Removal System (MARS) immunoaffinity spin cartridge according to the recommendations of the manufacturer. After depletion, the sample was handled as described in Materials and methods. Although recoveries from spiked samples were 80–90%, quantitative determinations on unspiked individual serum samples gave both lower concentrations and lower between-sample standard deviations (16.3 pmol ml^{-1} and relative standard deviation [RSD]

= 18%, respectively, n = 8) compared with the previous results obtained in our laboratory [27]. This result suggested that serum depletion may lead to loss of CA II to an unpredictable extent. Probably, the CA II is concomitantly removed during depletion due to the nonspecific binding to depleted proteins [106]. Thus, immunoprecipitation of CA II was optimized on whole serum.

3.3.2 Development of magnetic separation technique

Among the different commercially available magnetic bead systems tested, characterized by a variety of surface chemistries, the Dynabeads Protein G were chosen for the CA II antigen capture procedure. Indeed, this magnetic bead system showed high antibody coupling efficiency, good functional orientation of antibody for antigen capture, low background binding of serum peptides and proteins, and appropriate specificity for the succeeding MS analysis. During method development, each step was optimized to maximize the final recovery of CA II. An increasing amount of anti-CA II polyclonal antibody (25, 50, 100, and 150 μg) was incubated with a fixed amount of Dynabeads Protein G (50 μL, corresponding to 1.5 mg of beads) in a fixed sample volume of 100 μL, keeping the incubation time constant (5 h). An estimate of the antibody amount bound to Protein G magnetic beads was performed by comparing protein concentrations measured by Bradford assay by using an immunoglobulin G (IgG) standard. The supernatants containing the unbound antibody were discarded, and the complex was washed twice with the washing buffer to remove unbound antibody. Then the antibody was recovered after complex dissociation with citrate buffer (pH 2.5), and its concentration was determined spectrophotometrically. The bonded amount of anti-CA II polyclonal antibody increased up to 14.9 ± 0.5 μg for 50 μL of bead

solution using 100 μg of antibody. This ratio (6.7 μg antibody/mg beads) is very similar to that reported by Berna and coworkers [107] for a different antibody (8 μg antibody/mg beads). Next, keeping constant the amount of anti-CA II polyclonal antibody (100 μg) and the amount of Dynabeads Protein G (50 μL), the efficiency of the Dynabeads Protein G/anti-CA II polyclonal antibody binding reaction was tested by increasing the incubation time (1 h, 2 h, 4 h, 8 h, and overnight). No significant increase in the binding reaction was observed from 2 h to overnight incubation times. The effect of cross-linking between Dynabeads Protein G and anti-CA II polyclonal antibody was also investigated. In all cases in which the anti-CA II polyclonal antibody was covalently bound to the magnetic beads by a cross-linking reaction, the LC–MS/MS analysis of the peptide at m/z 468.2 ($[M + 2H]^{2+}$) exhibited a significant peak area increase (>30%). Probably, when cross-linking was not performed, a fraction of the immunocomplex was lost during washing. After the best conditions for obtaining the complex antibody beads had been determined, we optimized the incubation volume and the incubation time for serum samples. The same amount of magnetic beads with immobilized antibody (200 μL) was used for extracting 20 μL of serum spiked with 0.5 pmol of CA II standard and diluted in different volumes (200, 500, 750, and 1000 μL) of PBS. The samples were incubated overnight at 4 °C. The best LC–MS/MS result in terms of recovery was obtained using 20 μL of serum diluted to 500 μL with PBS buffer. Moreover, the effect of sample incubation time was investigated (30 min, 1 h, 2 h, 4 h, 8 h, and overnight) using 20 μL of diluted serum. Recovery did not further increase after 1 h of incubation, whereas for higher incubation times an increase of background signal in the MRM mass

chromatogram was noted, probably due to the extraction of other proteins by means of nonspecific interactions.

3.3.3 Digestion efficiency

The enzymatic digestion protocol was optimized to obtain the best peptide formation yield. For this purpose, the digestion efficiency was checked by spiking immunocomplex sample after immunoprecipitation with a known amount of CA II standard (2 nmol) while maintaining a constant volume (400 µL), and the recovery of surrogate peptide by increasing the trypsin concentration was evaluated by LC–MS/MS. Experiments were done in quadruplicate, and the results are reported in Tab. 4. A recovery of 97 ± 3% was obtained by adding to approximately 250 µL of sample 100 µL of a trypsin solution (20 ng μL^{-1}), suggesting that in these conditions the proteotypic peptide released from CA II digestion could stoichiometrically represent the absolute amount of its parent protein in serum. This concentration of enzyme is higher than that usually adopted in other protocols, including those adopted by our laboratory in a previous study [105,107].

Trypsin (mg)	Peptide (%)	RSD (%)
0.5	48	18
1.0	83	8
2.0	97	3
3.0	95	5

Note. CA II standard added: 2 nmol, total volume = 400 µl.

Tab. 4: Hydrolysis yield.

3.3.4 Method validation

To determine the performance characteristics of the magnetic beads-based capture system coupled to LC–ESI–MS/MS analysis, we assessed linear range, method detection limit (MDL), method quantification limit (MQL), recovery, accuracy, and precision. In addition to ionization efficiency and reproducibility, the selected proteotypic peptides also need to meet the proteotypic selection criteria described in literature [108,109]. In our previous work based on protein isolation by reverse phase high-performance liquid chromatography (RP–HPLC) before protein digestion, the peptide GGPLDGTYR was selected as an appropriate peptide of CA II and the peptide GGPLEGTYR, having very similar chemical and physical properties, was selected as the relative IS. The benefits and limits of a nonlabeled peptide as IS have been discussed previously [110]. For this reason, they were also the first candidates for this method. From the product ion MS/MS spectrum of diprotonated GGPLDGTYR peptide at m/z 468.3 (shown in Fig. 6) and diprotonated GGPLEGTYR at m/z 475.3, the two most intense transitions were chosen for MRM acquisition. The supernatant containing the tryptic cleaved peptide mixture was separated from magnetic beads, and an aliquot was injected into the LC–ESI–MS/MS system. The mass chromatograms relevant to the two selected transitions for CA II-specific peptide are shown in Fig. 7 A and B, and we see that background compounds present in the sample give, for the transition 468.3→212.3 (Fig. 7A), a signal poorly resolved from that relevant to the proteotypic peptide. This problem was solved by changing the selected transition 468.3→212.3 to 468.3→496.3 (Fig. 7C) that did not show background peaks. The IS was eluted approximately 0.2 min earlier and did not show any background

interference. Peptide adsorption is an important factor that may affect accuracy and reproducibility. To reduce peptide adsorptive processes, protein low-retention tubes were used. Surface adsorption phenomena are ruled by the adsorption constant, so the adsorbed compound amount decreases as the solvent volume increases. In addition, salt removal by means of C18 mini-columns is a timeconsuming critical step in the peptide analysis protocols. Bearing in mind these considerations, we devised an on-line injection system that permitted us to inject by an autosampler a relatively large volume (100 μL) into a small bore (1 mm i.d.) column. Our system is more complicated than that previously used for a similar study [107], but the current system has some advantages: (i) the injection and trap washing with water can be made during analytical column equilibration, (ii) highly retained compounds did not enter the analytical column, and (iii) the trap washing with strong solvent can be made during sample separation. Calibration graphs were constructed as described in Materials and methods. Two different regression lines were considered: external calibration and matrix-matched calibration. When the areas of the standard peaks were not normalized for the IS response, the ratio between the slopes (b_{mm}/b_{ext}) was 0.73 ± 0.6, whereas after normalization it was 1.04 ± 0.05. These data showed that ion suppression due to matrix compounds is moderate but not negligible and that the unlabeled IS was adequate. The memory effect was nearly 100% when the most diluted standard solution (0.05 pg μL^{-1}) was injected after the most concentrated one (100 pg μL^{-1}) and became negligible only after three blank injections. The response was found to be linear for the calibration range used, with R2 = 0.9990 and 0.9863 for the external and matrixmatched calibrations, respectively. Recovery, accuracy,

and reproducibility of the method were calculated by analyzing six aliquots of a serum pool unspiked and spiked with CA II at three concentration levels during 3 weeks, with three LC–MS/MS analyses being performed for each immunoprecipitated sample. Results are shown in Table 5. If we consider that the tryptic digestion regarding the representative peptide recovery is quantitative, the roughly 23% analyte loss should be due to an incomplete capture by the antibody. The RSD (<12%) was comparable to the RSDs of similar studies [107,111,112]. Instrumental limit of detection (LOD), calculated as three times the intercept of the external calibration regression line, was 2 pg of the injected synthetic peptide (~2 fmol). The method identification limit (MIL) and method quantification limit (MQL) were estimated from the serum samples, with samples being run in triplicate and results being averaged. MIL (signal/noise [S/N] = 3 for the second most intense transition in MRM) and MQL (S/N = 10 for the sum of the two selected transitions), expressed as pmol ml^{-1} CA II in serum, were 0.3 and 0.5, respectively. Although the performances of the method based on immunoprecipitation by magnetic beads were not superior to those based on reversed phase LC fractionation, it present two advantages: (i) many samples can be treated contemporaneously and (ii) due to the higher selectivity and loadability, a less sophisticated MS/MS platform can be used.

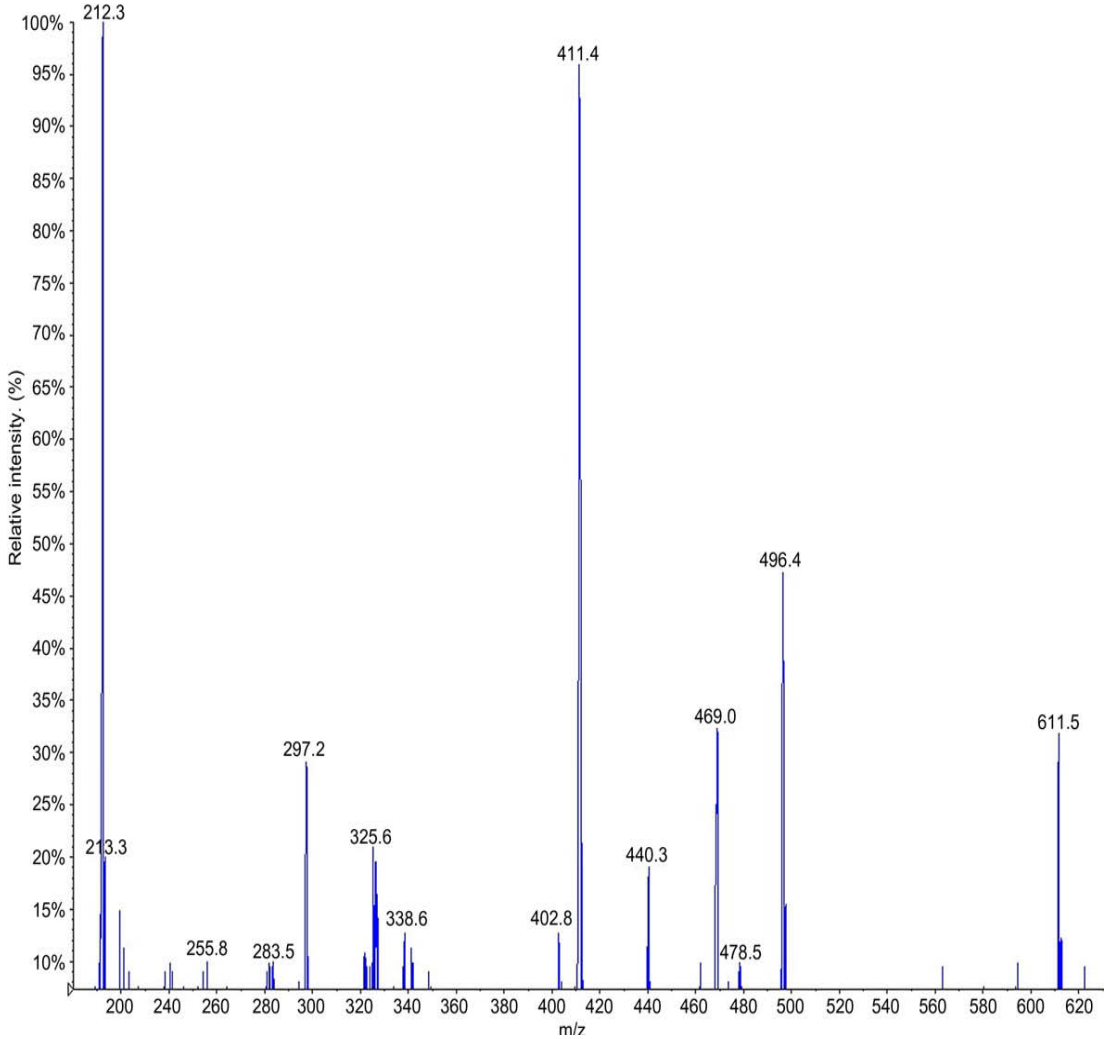

Fig.6: Product ion spectrum of the diprotonated molecule of CA II specific peptide GGPLDGTYR (m/z 468.3) acquired at 24% relative collision energy, obtained by analyzing a 10 pg μL^{-1} standard solution in infusion mode, at a 5 µL min^{-1} flow rate.

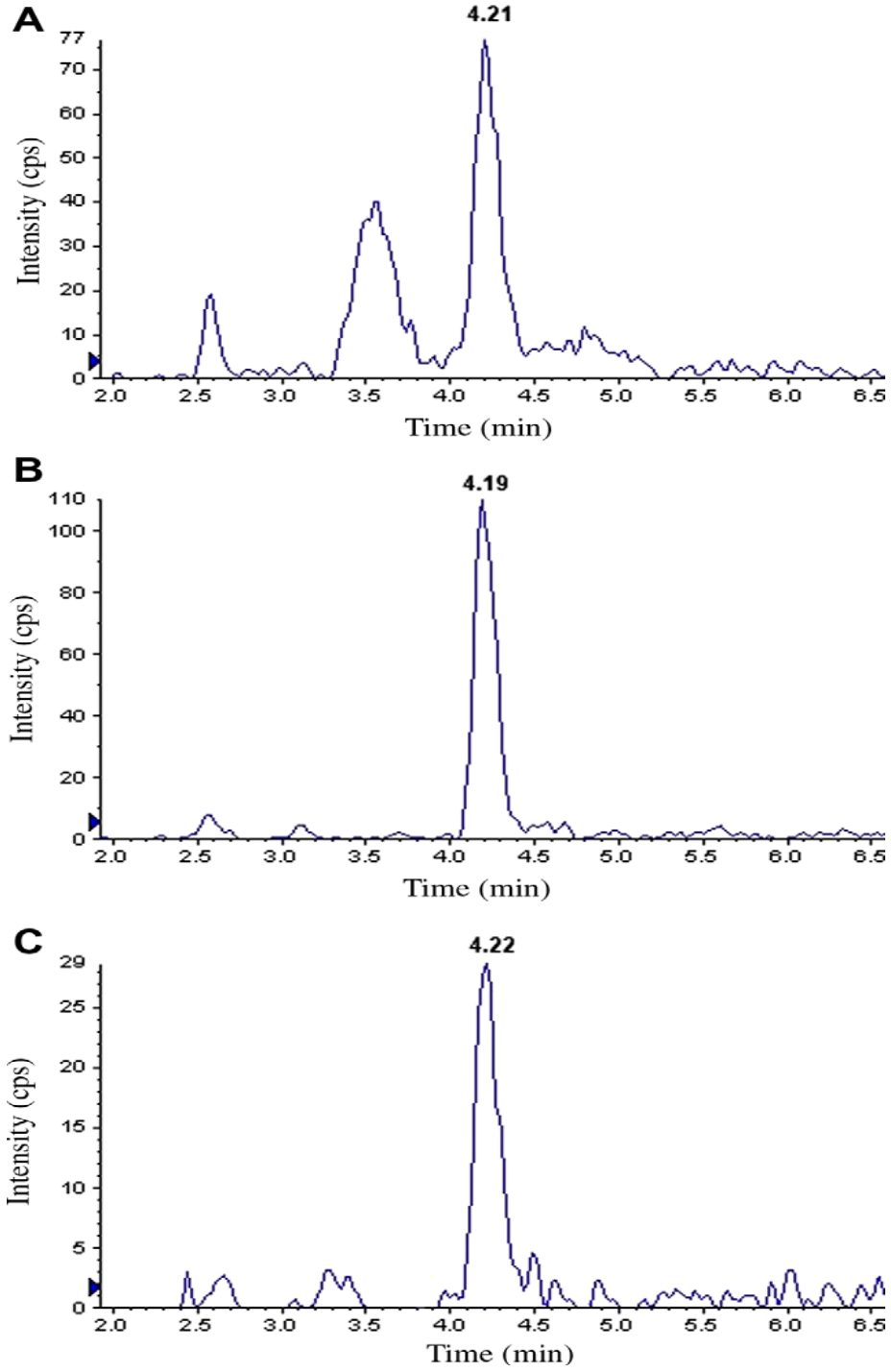

Fig. 7: LC–ESI–MS/MS extracted ion current in MRM mode deriving from the analysis of an unspiked sample relative to 468.3→212.3 transition (A), 468.3→411.2 transition (B), and 468.3→496.3 transition (C).

CAII added (pmol ml^{-1})	–	30	100	150
CAII found (pmol ml^{-1})	15.5	37.7	95,5	132.5
Recovery	–	74	80	78
RSD% ($n = 6$)	12	7	9	5

Tab. 5: Method recoveries and precision.

3.3.5 CA II quantitation in human serum samples

Eight human serum samples from apparently healthy subjects (seven males and one female, 20–40 years of age) were analyzed, and for each sample three RP–HPLC runs were done. For CA II-specific peptide quantification, external calibration was employed and recovery correction was done. The concentration of the endogenous CA II in serum was found to be 27.3 pmol ml^{-1} (RSD = 65%). Both the mean concentration and RSD were very different from those found in a previous study by our laboratory (56 pmol ml^{-1} and 21%, respectively). Because both methods were validated in a similar way and the sampled population was different but similar, a possible explanation may be that the external addition of standard CA II was not similar to the endogenous CA II situation at the isolation step in both cases. The chromatographic isolation used in the previous work fractionated denaturated samples, whereas the immunologic capture is performed in native samples. If the protein were present in different forms (modified or associated in complex structures), the affinity for the antibody would be different, so the recovery of the standard may give erroneous indications.

3.4 Conclusions

As demonstrated by the first attempts to conduct a multilaboratory assessment of the precision and reproducibility of MRM-based measurements of proteins in plasma published recently [84,113], the preclinical validation of candidate biomarker by LC–MS/MS is becoming a hot topic. In the method described in this work, the protein of interest, CA II, was isolated from serum by immunoprecipitation. A proteotypic peptide, produced stoichiometrically through proteolysis with trypsin, was ultimately quantified, using a synthetic peptide and a structural analogue free-labeled synthetic peptide as IS, by LC– ESI–MS/MS in MRM acquisition mode. An analytical column having 1 mm internal diameter and a triple quadrupole instrument were employed. A column switching system was used for on-line SPE sample cleanup. The assay was validated by recovery studies of both intact proteins and proteotypic peptide. Good precision and MDL as low as 0.5 pmol ml^{-1} were obtained. This strategy, based on isolation of target protein by immunoaffinity and its absolute quantification by quantifying one of its proteotypic products, could represent a very specific and sensitive analytical approach to reach the analytical goal of low-abundance protein determination in clinical samples such as serum. Nevertheless, a comparison of physiological CA II concentrations in sera of eight apparently healthy subjects obtained by using this approach with those found in a previous study by using chromatographic isolation of the fraction containing the target protein followed by a more technologically advanced platform, such as chip LC–MS/MS [109], showed very different values. If our data are not affected by an unrecognized error, these results pose a question. A protein may be present in a certain biological specimen in

different forms (e.g., free and involved in a complex with other molecules). Is the analysis of spiked samples ever a correct way for making validation? When trying to isolate the target from the most abundant proteins, what form is enriched?

CHAPTER 4: Evaluation of different two-dimensional chromatographic techniques for proteomic analysis of mouse cardiac tissue

4.1 Introduction

The general strategy in proteomic research includes sample preparation, protein or peptide separation, their identification, and data interpretation. Sample preparation is the first critical step that affects the outcome of the entire proteomic analysis. The next step is protein or peptide separation. The protein-level and peptide-level separations have relative advantages and disadvantages. Proteins are sensitive to precipitation upon exposure to high salt concentrations, to basic pH values, and organic solvents. Peptides, on the other hand, are relatively stable in solution and generally do not exhibit solubility issues. However, peptide-level separations also have limitations, including the scattering of tryptic peptides from a single parent protein into multiple fractions, which can potentially reduce protein identification scores. Furthermore for adequate representation of the proteome, only multidimensional separation techniques can provide resolving capability of thousands of protein species and have proven to be superior to one-dimensional approaches. Until recently, two dimensional gel electrophoresis (2-DE) was the technique most often used for protein separation. The limitations of 2-DE in detecting low abundance proteins, very small or large proteins, as well as basic and membrane/hydrophobic proteins [114-116], as well as difficulties with automation of the process, have forced researchers to look for other methods of

protein separation, such as multidimensional liquid chromatography coupled to mass spectrometry (MDLC-MS) or tandem mass spectrometry (MDLC-MS/MS). These techniques have emerged as a powerful tool for the large-scale analysis of such complex samples. [117-120]. MDLC combines two or more forms of LC to increase the peak capacity, and thus the resolving power of separation, to better fractionate peptides prior to entering into the mass spectrometer. It better resolves peptides differing in charge and hydrophobicity to minimize ion suppression and improve ionization efficiency, and it simplifies the complexity of peptide ions entering into the mass spectrometer to minimize undersampling. This last aspect is important because the tandem MS process is driven by data-dependent data acquisition and has a finite cycle time. A higher peak capacity and better resolving power improve the acquisition of data and can lead to a better representation of the proteins in the mixture and permit the identification of low-abundance proteins [117,120-122]. Various orthogonal prefractionation techniques on the protein and peptide level have been utilized for the characterization of a part of the yeast proteome leading to the identification of thousands of proteins [123-125]. A variety of separation modes have been employed to achieve protein-level separation, including size exclusion chromatography, [126,127] ion exchange chromatography, [128,129] isoelectric focusing (IEF), [130-134] gel separation (SDS-PAGE), [135,136] and reversed-phase chromatography (RP) [137-144]. In contrast to a protein-level separation followed by a peptide separation, a two-dimensional peptide-level separation can be performed by employing two or more methods with different separation selectivities. A number of separation modes have been implemented to this end, including strong cation exchange (SCX),

[124,145-149] IEF, [150,151] capillary electrophoresis, [152,153] capillary isoelectric focusing (CIEF), [154,155] and mixed-mode pH reversed phase (RP-RP) [156-159].

Development of better method that leads to the greatest number of identified proteins has great scientific importance, in fact a full understanding of the molecular mechanisms involved in health and disease progression will require the identification of all forms of each protein involved in cellular processes.

In health study the proteomic approach has proved to be particularly interesting for cardiovascular diseases and thereby improving our understanding of the mechanisms involved and identifying new biochemical factors and biomarkers associated in these diseases.

Heart disease infact is the leading cause of mortality in industrialized countries and the development of novel therapeutic strategies is largely dependent on our understanding of the molecular basis of cardiac function and dysfunction [160]. With the availability of animal models mimicking human disease and expression profiling to detect changes in gene expression has been made considerable progress toward understanding the molecular basis of contraction, sarcomere assembly, regulation of muscle gene expression, and metabolism. The current focus on RNA in expression profiling has generated extensive datasets relating to cardiac and skeletal muscle disease, but protein expression and post-translational modifications (PTM) determine cardiac function too. Thus, the molecular analysis of heart disease would greatly benefit from a proteomics approach that combines the advantages of high throughput analysis (as in expression profiling) and the focus on protein levels and modifications. Accordingly, proteomics has now been

applied to investigate the molecular basis of cardiovascular and muscle disease, such as cardiac hypertrophy, atrophy, and ischemia [161-164].

The goal of this work is to develop an appropriate method to study the proteome of cardiac system hence differential study of heart disease. To achieve this goal have been systematically compared the following chromatographic techniques: offline SCX combined with RP, mixed-mode pH RP-RP for peptide-level separation, and RP with two different columns for protein-level separation. We have also compared the following identification parameters: total proteins identified, total number of peptides identified (including redundant identifications), total number of unique peptides (only nonredundant identifications), average protein sequence coverage, and protein *E*-value (probability score).

4.2 Experimental

4.2.1 Reagents and chemicals

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), protease inhibitor cocktail for mammalian tissue, ethylenediaminetetraacetic acid (EDTA), sodium vanadate, sodium fluoride, Bradford reagent (Bio-Rad Protein Assay Reagent, Bio-Rad, Hercules, CA, USA), trifluoroacetic acid (TFA), formic acid (FA) and trypsin from porcine pancreas were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris), 1,4-dithiothreitol (DTT), iodoacetamide (IAA), and urea were purchased from GE Healthcare Amersham Biosciences (Uppsala, Sweden).

Methanol and acetonitrile 'Plus' (ACN) of HPLC grade were obtained from Carlo Erba (Milano, Italy). All other solvents and chemicals were of analytical grade (Carlo Erba) and were used as supplied. Deionized water was further purified using a Milli-Q apparatus (Millipore, Bedford, MA, USA).

4.2.2 Samples

Mice (Mus musculus) employed in the experiment were 24-week-old C57blk healthy males from the Department of Histology and Medical Embryology of "Sapienza" Università of Rome (Italy). Mice were sacrificed and hearts were dissected and snap-frozen in liquid nitrogen. The tissue was stored in a freezer at –80°C until use.

All procedures were carried out in compliance with the Italian Legislative Decree 116/1992 in application of the EU Directive 86/609/EEC26 concerning the use of experimental animals.

4.2.3 Protein extraction

Protein extraction was carried out according to Nerenova et al. [165] with some modification.

Heart tissues ground under liquid nitrogen were homogenized for 1 min using a little pestle. Then the sample was mixed with a buffer solution consisting of 20 mmol L^{-1} Tris (pH 6.8), 0.2 mmol L^{-1} sodium vanadate, 50 mmol L^{-1} sodium fluoride, 2 mmol L^{-1} EDTA, 1% (v/v) protease inhibitor mixture. The better ratio of wet tissue weight : buffer solution volume (whole tissue homogenate) was 1: 4.

The suspension was centrifuged at 4 °C at 18000 x g for 20 min. After centrifugation, the supernatant was collected and the pellet was re-extracted with the same buffer. The procedure was repeated and the supernatant was combined to the first (neutral extract).

The remaining pellet was extracted by mixing with a acid buffer of 15 mmol L^{-1} aqueous TFA and 1 mmol L^{-1} TCEP (initial tissue weight : buffer volume = 1:4). The suspension was centrifuged at 4 °C at 18000 x g for 20 min. After centrifugation, the supernatant was collected and the pellet was re-extracted with the same buffer. The procedure was repeated and the supernatant was combined to the first (acid extract). Extracts were stored at –80 °C. Total protein concentration in each sample was determined by Bradford assay.

4.2.4 Protein digestion

After extraction, solvent was removed by vacuum centrifugation. The protein were redissolved with 100 µL of 100 mmol L^{-1} Tris (pH=7.8), and 6 mol L^{-1} urea solution.

Cysteinyl disulfides were reduced with 200 mmol L^{-1} DTT for 30 min at 37 °C. Reduced disulfides were then alkylated with 200 mmol L^{-1} IAA for 30 min in the dark. Trypsin was added at a 1:8 enzyme-substrate (w/w) ratio and incubated for 12 h at 37 °C. The digestion was quenched by adding 1% (v/v) TFA.

4.2.5 Offline Strong Cation Exchange Chromatography (Off-SCX)

100 μg sample of protein digest was vacuum-centrifuged to dryness and reconstituted in 100 μL of 75% (v/v) H_2O, 25% (v/v) ACN, 5 mmol L^{-1} K_2HPO_4

at pH 3 (HPLC buffer A). A Rainin Dynamax HPLC equipped with a 100 μL sample loop, binary pump, UV detector, and fraction collector was used to deliver the reconstituted digest to a PolySulfoethyl A SCX column (2.1 × 250 mm, 5 μm, 300 Å) from PolyLC (Columbia, MD).

Peptides were eluted with a linear gradient of 75% (v/v) H_2O, 25% (v/v) ACN, 500 mmol L^{-1} K_2HPO_4 at pH 3 (HPLC buffer B) from 5 to 100% over 60 min. Fractions were collected every 5 min for a total of 12 fractions.

Each fraction was vacuum-centrifuged to dryness and reconstituted in 20 μL of 0.1% (v/v) FA; 2 μL of each reconstituted fraction was analyzed by LC-MS/MS (see below for details).

4.2.6 Offline High-pH Reversed-Phase Chromatography (pH-RP)

100 μg sample of protein digest was vacuum-centrifuged to dryness and reconstituted in 100 μL of 200 mmol L^{-1} ammonium formate at pH 10 (HPLC buffer A). A Rainin HPLC (see Off-SCX for description) was used to deliver the reconstituted digest to a Gemini C18 RP column (2 × 150 mm, 3 μm, 110 Å) from Phenomenex (Torrance, CA). Peptides were eluted with a linear gradient of 100% ACN (HPLC buffer B) from 5 to 35% over 60 min. Fractions were collected every 5 min for a total of 12 fractions. Each fraction was vacuum centrifuged to dryness and reconstituted in 20 μL of 0.1% FA (v/v); 2 μL of each reconstituted fraction was analyzed by LC-MS/MS (see below for details).

4.2.7 Offline C8 Protein Reverse Phase (C8-RP Prot)

A 40 μg sample of undigested protein extract in 100 μL of water was delivered to a Zorbax 300SB-C8 column (150 mm × 4.6 mm i.d., 300 Å pore size, 5 µm particle size) from Agilent Technology (Santa Clara, CA, USA) with a securityguard ODS, 4 mm × 3 mm i.d. precolumn, supplied by Phenomenex (Torrance, CA, USA). To increase recovery and decrease protein adsorption, the column was heated to 60 °C. Compounds were separated using a gradient of ACN 0.08% (v/v) TFA (B) and water 0.1% (v/v) TFA (A). The gradient, at a flow rate of 0.5 mL min^{-1}, started with 15% eluent B and was linearly increased to 55% in 55 min. The eluted proteins were monitored at 215 nm by UV detection. Fractions were collected every 5 min for a total of 12 fractions. Each fraction was vacuum-centrifuged to ~50 μL, diluted to 100 µL of a solution 200 mmol L^{-1} Tris (pH=7.8), and 12 mol L^{-1} urea and digested according to the solvent-assisted protein digestion procedure. Each digested fraction was vacuum centrifuged to dryness. The digested fractions were reconstituted in 20 μL of 0.1% FA; 2 μL of each reconstituted fraction was analyzed by LC-MS/MS.

4.2.8 Offline High Recovery Protein Reverse Phase (hr-RP Prot)

The procedure for hr-RP Prot was followed exactly as well as C8-RP Prot except for different chromatographic column was used. Agilent (Santa Clara, CA) macroporous mRP-C18 column (4.6 × 50 mm, 5 μm) heated to 80 °C was employed instead of the C8 column from Agilent Technology.

4.2.9 LC/Chip-MS/MS

Liquid chromatography was performed using an Agilent series 1200 instrument (Agilent Technologies, Waldbronn, Germany) consisting in 1200 Series nanopump with degasser; 1200 Series capillary pump; 1200 Series thermostatted microwell-plate autosampler, and HPLC-Chip/MS interface.

Reverse phase nanoLC separation was performed on a μ-fluidic HPLC chip cube system (C18 reversed phase column, 300 Å pore size, 5 μm particle size, 75 μm i.d., 43 mm length) interfaced to a Q-ToF MS instrument (Agilent Technologies, Santa Clara, CA).

Separation was performed at 300 nL min^{-1} flow rate using a 50 min gradient from 5 to 45% B (eluent A, 95% (v/v) H_2O, 5% (v/v) ACN, 0.1% (v/v) FA and eluent B, 95% (v/v) ACN, 5% (v/v) H_2O, 0.1% (v/v) FA). Enrichment of the analytes prior to gradient start was performed by capillary pump running at 4 µL min^{-1} 0% B (v/v).

The analyses were performed in positive ionization mode with a capillary voltage set at 1,750 V and a delta electro multiplier voltage of 500 V. The drying gas flow was 4 L min^{-1} of nitrogen, and the drying gas temperature was 350°C. Because the HPLC-Chip interface uses an enclosed source, to achieve low background conditions, an additional 1.5 L min^{-1} of filtered air was added to the drying gas. The position of the nano-ESI Chip tip was optimized to produce a good direct ESI spray under the correct voltage and so to achieve high MS sensitivity and a stable spray.

To maintain the mass accuracy, a short regular full mass range scan is performed in MS mode without filtering. Acquisition cycles were set as follows: first

acquisition was run in regular MS mode, second acquisition was run in special mode with a cutoff value at *m*/*z* 300 and no collision energy followed by a third (or more) acquisition in special mode with a *m*/*z* cutoff value of 300 and a low collision energy. Acquisition time for MS and special mode scans was set to 65 ms and to 1 s, respectively.

4.2.10 Database Search

Agilent Mass Hunter was used to convert the files into .xml text files for database searching. Peptide masses were used to search the Swiss-Prot database using Mascot. The following parameters were used for both search engines: taxonomy was limited to *Mus musculus (House Mouse)*, parent mass tolerance was 5 ppm, fragment mass tolerance was 0.4 Da, a maximum of one missed cleavages was allowed, carbamidomethylation was set as a fixed modification and oxidation was set as a variable modification. For the Mascot search results, the significance threshold was set at $p < 0.05$ (indicates identity or extensive sequence similarities).

4.3 Results and Discussion

4.3.1 Phase extraction

Traditional methods of extraction are based on the use of detergents to break the cell membranes.

The high concentrations of detergent are needed to break down the structure of sarcomeres and completely dissolving sarcomeric proteins but they are incompatible with most of chromatographic separation techniques.

The extraction procedure used in this work is simple, fast and does not require use of detergents.

It has been optimized the ratio between buffer volume and weight tissue. Goodness of extraction has been so verified by the Bradford assay and the best ratio was found to be of 4mL of buffer extracting per gram of tissue (Tab. 6).

Weight tissue:buffer volume ratio	Extracted protein (µg)
1:1	1.50
1:2	2.05
1:4	3.83
1:8	2.90

Tab. 6: Amount of extracted protein at different weight tissue:buffer volume ratio. Values refer to neutral extraction.

4.3.2 Comparison of chromatographic techniques

In this work four different chromatografic techniques have been studied for separation in first dimention in order to choose the best procedure for the analysis of mouse tissue samples.

In order to compare the performance of the separation methods proposed, was compared the total number of identified proteins. It was tried to understand, moreover, the various reasons for the differences observed between the various

methods, including the resolution power, the orthogonality of the techniques, and the loss of sample. (Tab. 7) summarizes the total proteins identified by Mascot search engine, for each technique tested.

As is clear from this table, the largest number of identified proteins was obtained by the fractionation of tryptic digested by reversed phase chromatography at high pH (pH-RP).

In addition to the total proteins identified, was considered the total number of peptides identified (including redundancy), the total number of unique peptides (excluding redundancy), the average amino acid coverage (coverage) and the probability of error associated with identification (score).

While the technique hr-RP Prot is the one that provided the greatest number of peptides identified, in agreement with the large number of proteins identified, the separation pH-RP has a relatively low number of peptides in total, about half of that seen with the hr-RP Prot (2730 vs 5015). Method hr-RP Prot also presents coverage amino acids and a higher average score than the pH-RP method. Intuitively, these differences should lead to a greater number of proteins identified for the technical hr-RP Prot while, as already mentioned, the pH-RP has been the technique that has provided, even if slightly, the largest number of proteins identified.

The reason for this is that the pH-RP technique has provided a higher ratio of unique peptide compared to peptides total. The ratio of unique to total peptides is a measure of how often the instrument fragments the same peptide (MS/MS resampling). This resampling causes a loss in duty cycle (the instrument spends valuable MS/MS time refragmenting the same peptides) and results in a lower

number of protein identifications. The substantially higher percentage of unique/total peptides given by the pH-RP method is indicative of a low MS/MS resampling rate. So while the hr-RP Prot method yields more total peptides than the pH-RP method, the pH-RP method has a higher ratio of unique/total peptides. This is due to the hr-RP Prot method having a higher rate of resampling events, which results in a lower number of protein hits per peptide sequenced.

The reason why some methods have such a high rate of resampling the same transition is often dependent on mass-spectrometric instrumental parameters, but in this study, these parameters were kept constant for all the methodologies as well as variations in the resampling are the result separation in the first dimension. Regarding the technique SCX according to Dai et al. [145], the elution of peptides in the pH mode occurs according to the isoelectric point (pI) of the peptide. However, a peptide possessing multiple functional groups with different pKa's could interact with the SCX resin in amore complex manner, resulting in a peak elution profile that is not strictly dictated by isoelectric point. If this were the case, then peptides containing fewer ionizable functional groups would elute in a single peak while more complex peptides with multiple ionizable groups would elute in multiple peaks. However, the interaction between individual peptides and the SCX resin is complex and depends on both the pH and ionic strength of the elution buffer.

Based on the results obtained from this study the best technique is the pH-RP. This method has allowed the identification of 1338 proteins, of these 82 are present in both pools. An overlap of 6.53% provides an estimate of the selectivity

of the extraction procedure developed in this work resulting, therefore, be very high.

Separation techniques	Total proteins	Total peptides	Unique peptides	% unique/total	Av % sequence coverage	Av protein scores
C8-RP Prot	1267	3485	2341	67.2	13.0	74
hr-RP Prot	1303	5015	2948	58.8	18.4	97
SCX	509	1262	971	76.9	10.2	108
pH-RP	1338	2730	2282	83.6	16.6	88

Tab. 7: Comparison of different separation technique than number of Total proteins, Total peptides, Unique peptides identified and than protein identification values of Average sequence coverage and Average scores. (N) refer to neutral extract; (A) refer to acid extract.

4.4 Conclusions

All techniques examined in this study were suitable for a proteomics study. However, each has specific advantages and limitations depending on the available equipment, expertise and monetary resources. Peptide separations are easier than the protein-based methods owing to simple sample processing, i.e., en masse digestion versus digestion of individual fractionations. In addition, protein-level separations generally preclude the use of stable isotope labeling for quantitative shotgun proteomics. However, the protein-level separations are greater for the spectral counting due to the large number of total peptides identified. Furthermore, separation of the protein level appear to be larger for smaller proteins and are generally more effective at removing residual abundant proteins after immunodepletion of serum than peptide HPLC.

CHAPTER 6: Concluding remarks

The explosive growth in proteomic research for biomarker discovery, with its huge number of samples, demands high throughput, high sensitive, and high reproducible LC/MS technologies. In this work, we developed three different method able in differential proteomics study. First method was developed for serum CA II quantification, a protein present at concentration level about five orders of magnitude lower than that of the most abundant proteins, by using nano-LC-ESI-MS/MS. In addition, using a chromatographic purification step instead of a most abundant serum protein depletion kit, protein loss due to aspecific interactions with the proteins bonded by antibodies can be avoided. The hardware miniaturization introduced by Chip technology has radically reduced analysis time and has increased efficiency and sensitivity. Moreover, using this device allows to reduce matrix effect; then it is possible to use the external calibration for quantification. In second method the protein of interest, CA II, was isolated from serum by immunoprecipitation. A proteotypic peptide, produced stoichiometrically through proteolysis with trypsin, was ultimately quantified, using a synthetic peptide and a structural analogue free-labeled synthetic peptide as IS, by LC–ESI–MS/MS in MRM acquisition mode. This strategy, based on isolation of target protein by immunoaffinity and its absolute quantification by quantifying one of its proteotypic products, could represent a very specific and sensitive analytical approach to reach the analytical goal of low-abundance protein determination in clinical samples such as serum. Finally third method was based on multidimensional chromatography approach applied for a total protein

extract analysis. In particular a mouse cardiac tissue was analyzed by high pH LC at peptide level coupled with nanoLC-Chip Q TOF mass spectrometer. Moreover all techniques examined in this last reserch were suitable for a proteomics study. However, each has specific advantages and limitations depending on the available equipment, expertise and monetary resources.

References

[1] Wasinger VC et al , Electrophoresis 1995, 16: 1090-1094.

[2] Gygi S, Rochon Y, Fransz B, Aebersold R. Mol Cell Biol 1999, 19: 1720–1730.

[3] Angela Amoresano, Gianluca Monti, Claudia Cirulli and Gennaro Marino. Rapid Commun. Mass Spectrom. 2006, 20: 1400–1404.

[4] Amoresano A, Marino G, Cirulli C, Quemeneur E. Eur J Mass Spectrom. 2004, 10: 401-12.

[5] Yates III, J.R. Annual Review of Biophysics and Biomolecular Structure, 2004 33, 297–316.

[6] Aebersold, R. & Mann, M., Nature, 2003, 422; 198–207.

[7] Washburn, MP; Wolters, D; Yates III, JR. Natural Biotechnology, 2001, 19, 242–247.

[8] Eng, J; McCormack, A; Yates, J. Journal of the American Society for Mass Spectrometr, 1994, 5, 976–989.

[9] Iborra, F. & Buhler, JM., Analytical Biochemistry, 1976, 74; 503–11.

[10] Klose, J. Humangenetik, 1975, 26, 231–43.

[11] Scheele, GA. Journal of Biological Chemistry, 1975, 250, 5375–5385.

[12] Klose, J. & Kobalz, U., Electrophoresis, 1995, 16; 1034–59.

[13] Herbert, BR; Harry, JL; Packer, NH; Gooley, AA; Pedersen, SK; Williams, KL. Trends Biotechnology, 2001, 19, S3–S9.

[14] Monteoliva, L. & Albar, JP., Brief Function Genomic Proteomic, 2004, 3; 220–39.

[15] Braun, RJ; Kinkl, N; Beer, M; Ueffing, M. Analytical Bioanalytical Chemistry, 2007, 389, 1033–45.

[16] Cooper, JW; Wang, Y; Lee, CS. Electrophoresis, 2004, 25, 3913–3926.

[17] Eckerskorn, C; Strupat, K; Karas, M; Hillenkamp, F; Lottspeich, F. Electrophoresis, 1992, 13, 664–65.

[18] Gor, A; Obermaier, C; Boguth, G; Harder, A; Scheibe, B; Wildgruber, R; Weiss, W. Electrophoresis, 2000, 21, 1037-1053.

[19] Gorg, A. & Weiss, W., Methods Molecular Biology, 1999, 112; 189-195.

[20] Gygi, SP; Corthals, GL; Zhang, Y; Rochon, Y; Aebersold, R. Proceeding of the National Academy Science U.S.A., 2000, 17, 9390-9395.

[21] Krijgsveld, J; Gauci, S; Dormeyer, W; Heck, AJ. Journal of Proteome Research 2006, 5, 1721-1730.

[22] Reinders, J; Zahedi, R.P; Pfanner, N; Meisinger, C; Sickmann, A. Journal Proteome Research, 2006, 5, 1543-1554.

[23] Essader, AS; Cargile, BJ; Bundy, JL.; Stephenson, JL. Proteomics, 2005, 5, 24-34**.**

[24] Lemeer, S; Pinkse, MW; Mohammed, S; van Breukelen, B; den Hertog, J; Slijper, M; Heck, A. J. Journal Proteome Research, 2008, 7, 1555-1564.

[25] Yaneva, M. & Tempst, P., Methods Molecular Biology, 2006, 338; 291-303.

[26] Cargile, B.J; Sevinsky, J.R; Essader, AS; Stephenson, JL; Bundy, JL. Journal Biomolecular Technology, 2005, 16, 181-189.

[27] de Godoy, LM; Olsen, J.V; Cox, J; Nielsen, ML; Hubner, NC; Frohlich, F; Walther, TC; Mann, M. Nature, 2008, 455, 1251-1254.

[28] Wilson-Grady, JT; Villen, J; Gygi, SP. Journal of Proteome Research, 2008, 7, 1088-1097.

[29] Link, AJ; Eng, J; Schieltz, DM; Carmack, E; Mize, GJ; Morris, DR; Garvik, BM; Yates, JR. Nat. Biotechnol, 1999, 17, 676–682.

[30] Mawuenyega, KG; Kaji, H; Yamauchi, Y; Shinkawa, T; Saito, H; Taoka, M; Takahashi, N; Isobe, T. J. Proteome Res, 2003, 2, 23–35.

[31] Claessens, H.A. & van Straten, M.A., J. Chromatogr. A, 2004, 1060; 23–41.

[32] Wolters, DA; Washburn, MP; Yates III, JR. Anal. Chem, 2001, 73, 5683–5690.

[33] Machtejevas, E; John, H, Wagner, K. J. Chromatogr., B: Anal. Technol. Biomed. Life Sci, 2004, 803, 121–130.

[34] Peng, J; Elias, JE; Thoreen, CC; Licklider, LJ; Gygi, SP. J. Proteome Res, 2003, 2, 43–50.

[35] de Godoy, LM; Olsen, JV; de Souza, GA; Li, G; Mortensen, P; Mann, M. Genome Biol, 2006 7, R50.

[36] Chen, J;. Lee, CS; Shen, Y; Smith, RD; Baehrecke, EH. Electrophoresis, 2002, 23, 3143–3148.

[37] Burke, TL; Mant, CT; Black, JA; Hodges, RS. J. Chromatogr, 1989, 476, 377–389.

[38] Dixon, SP; Pitfield, ID; Perrett, D. Biomed. Chromatogr, 2006, 20, 508– 529.

[39] McDonald, WH; Ohi, R; Miyamoto, DT; Mitchison, TJ; Yates III, JR.. J. Mass Spectrom, 2002, 219, 245–251.

[40] Wei, J; Sun, J; Yu, W; Jones, A; Oeller, P; Keller, M; Woodnutt, G; Short, JM. J. Proteome Res, 2005, 4, 801–808.

[41] Delahunty, C. & Yates III, JR., Methods, 2005, 35; 248–255.

[42] Gatlin, CL; Kleeman, GR; Hays, LG; Link, AJ; Yates III, JR. Anal. Biochem, 1998, 263, 93–101.

[43] Hardouin J, Joubert-Caron R, Caron M., J Sep Sci., 2007, 30:1482

[44] Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R., Nat .Biotechnol, 1999, 17:994

[45] Ross RL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ., Mol Cell Proteomics, 2004, 3:1154

[46] Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M., Mol Cell Proteomics,2002, 1:376

[47] Shevchenko A, Chernushevich I, Ens W, Standing KG, Thomas B, Wilm M, Mann M., Rapid Commun Mass Spectrom, 1997, 11:1015

[48] Yao X, Freas A, Ramirez J, Demirev PA, Fensalau C., Anal Chem., 2001, 73:2836

[49] Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW., J Proteome Res 2004, 3:235

[50] Whiteaker JR, Zhao L, Zhang HY, Feng L, Piening BD, Anderson L, Paulovich AG., Anal Biochem 2007, 362:44

[51] Silva JC, Gorenstein MV, Li G, Vissers JPC, Geromanos SJ., Mol Cell Proteomics, 2006, 5:144

[52] Yin H, Killeen K, Brennen R, Sobek D, Werlich M, van de Goor T., Anal Chem., 2005, 77:527

[53] Hardouin J, Duchateau M, Joubert-Caron R, Caron M., Rapid Commun Mass Spectrom., 2006, 20:3236

[54] Palminiello S, Kida E, Kaur K, Walus M, Wisniewski KE, Wierzba-Bobrowicz T, Rabe A, Albertini G, Golabek AA., Brain Res., 2008, 1190:193

[55] Wu-Hsien K, Shun-Fa Y, Yih-Shou H, Chiou-Sheng T, Wen-Li H, Shu-Chen C., Clinica Chimica Acta, 2005, 351:79

[56] Nishita T, Takahasi M, Kasuya T, Matsui K, Ichihara N, Murakami M, Asari M., J Vet Med Sci., 2005, 67(1):63

[57] Demür N, Demür Y, Coþkun F., Turk J Med Sci., 2001, 31:477

[58] Lund SG, Dyment P, Gervais MR, Moyes CD, Tufts BL., J Comp Physiol B., 2002, 172:467

[59] Li X, Alvarez B, Casey JR, Reithmeier RA, Fliegel L., J Biol Chem., 2002, 277:36085

[60] Alver A, Mentese A, Karahan SC, Erem C, Keha EE, Arikan MK, Eminagaoglu MS, Deger O., Exp Clin Endocrinol Diabetes, 2007, 115:287

[61] Sly WS, Hu PY., Annu Rev Biochem., 1995, 64:375

[62] Supirpan CT, Briganti F, Tilli S, Chegwidden WR, Scozzafava A () Bioorgan Med Chem., 2001, 9:703

[63] Vlkolinský R, Cairns N, Fountoulakis M, Lubec G., Neurobiol Aging, 2001, 22:547

[64] Sly WS, Hewett-Emmett D, Whyte MP, Yu YL, Tashian RE., Proc Natl Acad Sci USA, 1983, 80:2752

[65] Tashian RE, Hewett-Emmett D, Dodgson SJ, Forster RE, Sly WS., Ann N Y Acad Sci., 1984, 429:263

[66] Morris CS, Esiri MM, Sprinkle TJ, Gregson N., Neuropathol Appl Neurobiol., 1994, 20:272

[67] DeLuca GC, Nagy Z, Esiri MM, Davey P., Acta Neuropathol., 2002, 103:590

[68] Ozensoy O, Arslan O, Oznur Sinan S., Biochemistry, 2004, 269:216

[69] Yoshiura K, Nakaoka T, Nishishita T, Sato K, Yamamoto A, Shimada S, Saida T, Kawakami Y, Takahashi TA, Fukuda H, Imajoh-Ohmi S, Oyaizu N, Yamashita N., Clin Cancer Res., 2005, 22:8201

[70] Cavaliere C, Cucci F, Guarino C, Gubbiotti R, Samperi, Laganà A., Rapid Commun Mass Spectrom., 2008, 22:1159

[71] Barnidge DR, Dratz EA,Martin T, Bonilla LE,Moran LB, Lindall A., Anal Chem., 2003, 75:445

[72] Barnidge DR, Goodmanson MK, Klee GC, Muddiman DC., J Proteome Res., 2004, 3:644

[73] Kuhn E, Wu J, Karl J, Liao H, Werner Z, Guild B., Proteomics, 2004, 4:1175

[74] Bizzarri M, Cavaliere C, Foglia P, Guarino C, Samperi R, Laganà A., Anal Bioanal Chem., 2008, 391:1969

[75] Yin H, Killeen K., J Sep Sci., 2007, 30:1427

[76] Vollmer M, Miller C, Gauthier GL., Agilent Technologies Application 2005, Note 5989-3538EN

[77] Tamvakopoulos C., Mass Spectrom Rev., 2007, 26:389

[78] Bacaloni A, Cavaliere C, Faberi A, Pastorini E, Samperi R, Laganà A., J Agric Food Chem., 2005, 53:5518

[79] Schiess R., Wollscheid B., Aabersold R., Mol. Oncol., 2009, 3:33–44.

[80] Grossklaus D.J., Smith J.A., Shappell S.B., Coffey C.S., Chang S.S., Cookson M.S., Urol. Oncol., 2002, 7:195–198.

[81] Whitehouse C., Solomon E., Gynecol. Oncol., 2003, 88:S152–S157.

[82] Jaffe A.S., Clin. Chim. Acta, 2007, 381:9–13.

[83] Pernemalm M., Lewensohn R., Lehtiö J., Proteomics, 2009, 9:3414–3424.

[84] Addona T.A., Abbatiello S.E., Schilling B., Skates S.J., Mani D.R., Bunk D.M., Spiegelman C.H., Zimmerman L.J., Ham A.-J.L., Keshishian H., et al., Nat. Biotechnol., 2009, 27:633–641.

[85] Tanaka Y., Akiyama H., Kuroda T., Jung G., Tanahashi K., Sugaya H., Utsumi J., Kawasaki H., Hirano H., Proteomics, 2006, 6:4845–4855.

[86] Urbas L., Brne P., Gabor B., Barut M., Strliè M., Èerk Petriè T., Štrancar A., J. Chromatogr. A, 2009, 1216:2689–2694.

[87] Jmeian Y., El Rassi Z., Electrophoresis, 2009, 30:249–261.

[88] Melm C., Schuchard M., Chen D.E., Duewel H., Life Sci. Innov., 2008, 24:17–20.

[89] Zhou M., Lucas D.A., Chan K.C., Issaq H.J., PetricoinIII E.F., Liotta L.A., Veenstra T.D., Conrads T.P., Electrophoresis, 2004, 25:1289–1298.

[90] Gong Y., Li X., Yang B., Ying W., Li D., Zhang Y., Dai S., Cai Y., Wang J., He F., Qian X.J., J. Proteome Res., 2006, 5:1379–1387.

[91] N. Zolotarjova, J. Martosella, G. Nicol, J. Bailey, B.E. Boyes, W.C. Barrett, Proteomics, 2005, 5:3304–3313.

[92] Hannan N.J., Stoikos C.J., Stephens A.N., Salamonsen L.A., J. Proteome Res., 2009, 8:1099–1103.

[93] Stempfer R., Kubicek M., Lang I.M., Christa N., Gerner C., Electrophoresis, 2008, 29:4316–4323.

[94] Freije J.R., Bischoff R., Drug Discov. Today, 2006, 3:5–11.

[95] Safarik I., Safarikova M., Biomagn. Res. Technol., 2004, 2:7–24.

[96] Kitteringham N.R., Jenkins R.E., Lane C.S., Elliott V.L., Park B.K., J. Chromatogr. B, 2009, 877:1229–1239.

[97] Domon B., Aebersold R., Science, 2006, 312:212–217.

[98] Monaci L., Visconti A., Trends Anal. Chem., 2009, 28:581–591.

[99] Sechi S., Humana Press, Totowa, NJ, 2007. p. 359.

[100] Bantscheff M., Schirle M., Sweetman G., Rick J., Kuster B., Anal. Bioanal. Chem., 2007, 389:1017–1031.

[101] Sly W.S., Hu P.Y., Annu. Rev. Biochem., 1995, 64:375–401.

[102] Tashian R.E., Hewett-Emmett D., Dodgson S.J., Forster R.E., Sly W.S., Ann. NY Acad. Sci., 1984, 429:284–286.

[103] Palminiello S., Kida E., Kaur K., Walus M., Wisniewski K.E., Wierzba-Bobrowicz T., Rabe A., Albertini G., Golabek A.A., Brain Res., 2008, 1190:193–205.

[104] Vlkolinsky´ R., Cairns N., Fountoulakis M., Lubec G., Neurobiol. Aging, 2001, 22:547–553.

[105] Callipo L., Foglia P., Gubbiotti R., Samperi R., Laganà A., Anal. Bioanal. Chem., 2009, 394:811–820.

[106] Yocum A.K., Yu K., Oe T., Blair I.A., J. Proteome Res., 2005, 4:1722–1731.

[107] Berna M.J., Zhen Y., Watson D.E., Hale J.E., Bradley L.A., Anal. Chem., 2007, 79:4199–4205.

[108] Kamiie J., Ohtsuki S., Iwase R., Ohmine K., Katsukura Y., Yanai K., Sekine Y., Uchida Y., Ito S., Terasaki T., Pharm. Res., 2008, 25:1469–1483.

[109] Mallick P., Schirle M., Chen S.S., Flory M.R., Lee H., Martin D., Ranish J., Raught B., Schmitt R., Werner T., Kuster B., Aebersold R., Nat. Biotechnol., 2007, 25:125–131.

[110] Barnidge D.R., Dratz E.A., Martin T., Bonilla L.E., Moran L.B., Lindall A., Anal. Chem., 2003, 75:445–451.

[111] Careri M., Elviri L., Lagos J.B., Mangia A., Speroni F., Terenghi M., J. Chromatogr. A, 2008, 1206:89–94.

[112] Whiteaker J.R., Zhao L., Zhang H.Y., Feng L.C., Piening B.D., Anderson L., Paulovich A.G., Anal. Biochem., 2007, 362:44–54.

[113] McIntosh M., Fitzgibbon M., Nat. Biotechnol., 2009, 27:622–623.

[114] Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. Electrophoresis 2000; 21: 1037-1053.

[115] Gorg A and Weiss W. Methods Mol. Biol. 1999; 112: 189-195.

[116] Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R. Proc. Nat. Acad. Sci. U.S.A. 2000; 17: 9390-9395.

[117] Krijgsveld J, Gauci S, Dormeyer W, Heck AJ. J. Proteome Res. 2006; 5: 1721-1730.

[118] Essader AS, Cargile BJ, Bundy JL, Stephenson JL Jr. Proteomics 2005; 5: 24-34.

[119] Lemeer S, Pinkse MW, Mohammed S, van Breukelen B, den Hertog J, Slijper M, Heck AJ. J. Proteome Res. 2008; 7: 1555-1564.

[120] Reinders J, Zahedi RP, Pfanner N, Meisinger C, Sickmann A. J. Proteome Res. 2006; 5: 1543-1554.

[121] Yaneva M and Tempst P. Methods Mol. Biol. 2006; 338: 291-303.

[122] Cargile BJ, Sevinsky JR, Essader AS, Stephenson JL Jr, Bundy JL. J. Biomol. Tech. 2005; 16: 181-189.

[123] de Godoy LM, Olsen JV, Cox J, Nielsen ML, Hubner NC, Frohlich F, Walther TC, Mann M. Nature 2008; 455: 1251-1254.

[124] Washburn MP, Wolters D, Yates JR. Nat. Biotechnol. 2001; 19: 242-247.

[125] Wilson-Grady JT, Villen J, Gygi SP. J. Proteome Res. 2008; 7: 1088-1097.

[126] Opiteck GJ and Jorgenson JW. Anal. Chem. 1997; 69: 2283-2291.

[127] Gao J, Opiteck GJ, Friedrichs MS, Dongre AR, Hefta SA. J. Proteome Res. 2003; 2: 643-649.

[128] Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR. Nat. Biotechnol. 1999; 17: 676-682.

[129] Butt A, Davison MD, Smith GJ, Young JA, Gaskell SJ, Oliver SG, Beynon RJ. Proteomics 2001; 1: 42-53.

[130] Giorgianni F, Desiderio DM, Beranova-Giorgianni S. Electrophoresis 2003; 24: 253-259.

[131] Kachman MT, Wang H, Schwartz DR, Cho KR, Lubman DM. Anal. Chem. 2002; 74: 1779-1791.

[132] Walker AK, Rymar G, Andrews PC. Electrophoresis 2001; 22: 933-945.

[133] Wall DB, Kachman MT, Gong S, Hinderer R, Parus S, Misek DE, Hanash SM, Lubman DM. Anal. Chem. 2000; 72: 1099-1111.

[134] Yan F, Subramanian B, Nakeff A, Barder TJ, Parus SJ, Lubman DM. Anal. Chem. 2003; 75: 2299-2308.

[135] Lee CL, Hsiao HH, WLin C, Wu SP, Huang SY, Wu CY, Wang AH, Khoo KH. Proteomics 2003; 3: 2472-2486.

[136] Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. Proc. Natl. Acad. Sci. U. S. A. 2004; 101: 12130-12135.

[137] Evans CR and Jorgenson JW. Anal. Bioanal. Chem. 2004; 378: 1952-1961.

[138] Lee SW, Berger SJ, Martinovic S, PasaTolic L, Anderson GA, Shen Y, Zhao R, Smith RD. Proc. Natl. Acad. Sci. U. S. A. 2002; 99: 942-5947.

[139] Li W, Hendrickson CL, Emmett MR, Marshall AG. Anal. Chem. 1999; 71: 4397-4402.

[140] Marshall J, Jankowski A, Furesz S, Kireeva I, Barker L, Dombrovsky M, Zhu W, Jacks K, Ingratta L, Bruin J, Kristensen E, Zhang R, Stanton E, Takahashi M, Jackowski G. J. Proteome Res. 2004; 3: 364-382.

[141] Martosella J, Zolotarjova N, Liu H, Nicol G, Boyes BE. J. Proteome Res. 2005; 4: 1522-1537.

[142] Swanson RV and Glazer AN. Anal. Biochem. 1990; 188: 295-299.

[143] Wang Y, Balgley BM, Rudnick PA, Lee CS. J. Chromatogr. A 2005; 1073: 35-41.

[144] Zolla L and Bianchetti M. J. Chromatogr. A 2001; 912: 269-279.

[145] Dai J, Shieh CH, Sheng QH, Zhou H, Zeng R. Anal. Chem. 2005; 77: 5793-5799.

[146] Peng J, Elias JE, Thoreen CC, Licklider LJ, Gygi SP. J. Proteome Res. 2003; 2: 43-50.

[147] Shen Y, Jacobs JM, Camp DG, Fang R, Moore RJ, Smith RD, Xiao W, Davis RW, Tompkins RG. Anal. Chem. 2004; 76: 1134-1144.

[148] Vollmer M, Horth P, Nagele E. Anal. Chem. 2004; 76: 5180–5185.

[149] Wolters DA, Washburn MP, Yates JR. Anal. Chem. 2001; 73: 5683-5690.

[150] Cargile BJ, Talley DL, Stephenson JL Jr. Electrophoresis 2004; 25: 936-945.

[151] Xiao Z, Conrads TP, Lucas DA, Janini GM, Schaefer CF, Buetow KH, Issaq HJ, Veenstra TD. Electrophoresis 2004; 25: 128-133.

[152] Tong W, Link A, Eng JK, Yates JR. Anal.Chem. 1999; 71: 2270-2278.

[153] Figeys D, van Oostveen I, Ducret A, Aebersold R. Anal. Chem. 1996; 68: 1822-1828.

[154] Chen J, Balgley BM, DeVoe DL, Lee CS. Anal. Chem. 2003; 75: 3145-3152.

[155] Wang Y, Rudnick PA, Evans EL, Li J, Zhuang Z, Devoe DL, Lee CS, Balgley BM. Anal. Chem. 2005; 77: 6549-6556.

[156] Delmotte N, Lasaosa M, Tholey A, Heinzle E, Huber CG. J. Proteome Res. 2007; 6: 4363-4373.

[157] Gilar M, Olivova P, Daly AE, Gebler JC. Anal. Chem. 2005; 77: 6426-6434.

[158] Gilar M, Olivova P, Daly AE, Gebler JC. J. Sep. Sci. 2005; 28: 1694-1703.

[159] Nakamura T, Kuromitsu J, Oda Y. J. Proteome Res. 2008; 7: 1007-1011.

[160] Olson EN. A decade of discoveries in cardiac biology. Nat. Med. 2004; 10: 467-474.

[161] Zerkowski HR, Grussenmeyer T, Matt P, Grapow M, Engelhardt S, Lefkovits I. J. Proteome Res. 2004; 3: 200-208.

[162] Dunn MJ. Methods Mol. Biol. 2007; 357: 3-13.

[163] Gramolini AO, Kislinger T, Liu P, MacLennan DH, Emili A. Methods Mol. Biol. 2007; 357: 15-31.

[164] McGregor E and Dunn MJ. Circ. Res. 2006; 98: 309-321.

[165] Neverova I and Van Eyk JE. Proteomics 2002; 2: 22-31.

Acknowledgments

First of all, I would like to thank my supervisor prof. Aldo Laganà and prof. Roberto Samperi for their scientific support and teaching. I would like to thank Dr. Fiorella Guadagni (Scientific Institute for Research, Hospitalization and Health Care (SIRHHC or IRCCS in the Italian acronym) San Raffaele-Roma) for supplying the serum samples. A special acknowledgment is given to Waters and Agilent staff members, for their invaluable help and technical assistance. In particular, the authors wish to thank Ian Phipps (Waters), Francesco Migliaccio (Waters) and Alberto Stocco (Agilent Technologies) for his technical assistance and his infinite helpfulness. Finally I would like to thank my irreplaceable colleagues and friends, my girlfriend and my family for continued support given to me.

Publications and conference partecipations

The results obtained by this research allowed to three publications in international papers, one talk, and two posters presented in conferences.

Publications:

1. "HPLC-CHIP coupled to a triple quadrupole mass spectrometer for carbonic anhydrase II quantification in human serum" L. Callipo, P. Foglia, **R. Gubbiotti**, R. Samperi, A. Laganà. Analytical and Bioanalytical Chemistry 2009; 394: 811-820

2. "Immunoprecipitation on magnetic beads and liquid chromatography–tandem mass spectrometry for carbonic anhydrase II quantification in human serum" L. Callipo, G. Caruso, P. Foglia, **R. Gubbiotti**, R. Samperi and A. Laganà. Analytical Biochemistry 2010; 400: 195–202

3. "Evaluation of different two-dimensional chromatographic techniques for proteomic analysis of mouse cardiac tissue" L. Callipo, A. Capriotti, C. Cavaliere, **R. Gubbiotti**, R. Samperi and A. Laganà. Biomed Chromatogr 2010 jul 22, DOI: 10.1002/bmc.1487

Talk:

1. *3° Convegno Giovani "La Chimica Sostenibile",* Roma 18-19 Giugno 2008 "Sindrome di Down e patologie associate: studio quantitativo dell'anidrasi

carbonica II quale possibile biomarker" L. Callipo, G. Caruso, **R. Gubbiotti,** R. Samperi, A. Laganà. **(Premio SCI per la migliore relazione orale)**

Posters:

1. XXI Convegno Nazionale della Divisione di Chimica Analitica della società Chimica Italiana *"Il Ruolo della Chimica Analitica nella tutela della salute",* Rende (CS) 21-25 Settembre 2008 "Sindrome di Down e patologie associate: studio quantitativo dell'anidrasi carbonica II quale possibile biomarker" L. Callipo, G. Caruso, **R. Gubbiotti,** R. Samperi, A. Laganà. **(Premio come migilor Poster)**

2. 1st International Congress on Analytical Proteomics - ICAP, Caparica (Portogallo), 30 Settembre-3 Ottobre 2009 "*Differential proteomics for studying microgravity effects on cardiac tissue by nanoHPLC-CHIP QTOF"*
E. Corradini, L. Callipo, A.L. Capriotti, C. Cavaliere, P. Foglia, P. Giansanti, R. Gubbiotti, A. Laganà.