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APPLICATIONS OF THE IMPACT II HIGH RESOLUTION QUADRUPOLE TIME-OF-FLIGHT (QTOF) INSTRUMENT FOR SHOTGUN PROTEOMICS

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ABSTRACT

Mass spectrometry is a central analytical method for protein research and other biomolecules which also demonstrated capability to detect peptides and proteins in a specific manner. Combined with appropriate sample preparation and/or enrichment, sensitivity is high enough to quantify peptides and proteins. In particular, the detection of iso-forms and different post-translational modifications is of highest interest for clinical applications, including discovery of novel biomarkers for early detection and targeted therapy of cancer and cardiovascular disease. The need to identify, characterize, and quantify proteins at ever increasing sensitivities and in even more complex samples has resulted in the evolution and development of a wide range of new mass spectrometry-based analytical platforms and experimental strategies. Among them, hybrid quadrupole time-of-flight (QToF) mass spectrometers are standard instruments in proteomic laboratories. Here, we discuss the basic concepts of mass spectrometry including performance characteristics, components of tandem mass spectrometer such as quadrupoles, ion traps, ToF mass analyzers, shotgun proteomics methods and bioinformatics analysis. The recent introduction of QToF Impact II Bruker mass spectrometer offers unrivaled mass accuracy (better than 1.45 ppm), high resolving power (40000 at m/z 1222) and a high dynamic range (1.7×10^5), without the need for a superconducting magnet and its associated maintenance requirements. A comparative analysis of basic performance characteristics of Impact II instrumentation such as resolving power, accuracy, mass range, optimal detection level and dynamic range is also presented in this review.

Keywords: Mass spectrometry (MS), Quadrupole Time-of-Flight (Q-ToF), proteomics, Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS), Sodium DodecylSulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE), MultiDimensional Protein Identification Technology (MudPIT), Strong Cation Exchange (SCX), Collision Induced Dissociation (CID), Electron Transfer Dissociation (ETD), Post-Translational Modifications (PTMs), High Performance Liquid Chromatography (HPLC)

INTRODUCTION

During the past decades, liquid chromatography – tandem mass spectrometry (LC-MS/MS) has progressively become a basic technique for the high-throughput analysis of large biomolecules, such as peptides and proteins [1–3]. This is most evident in the related fields of proteomics, metabolomics and metabonomics as well as in the emerging field of lipidomics. Proteomics can be regarded as the identification and quantification of all the expressed gene products of a cell type, tissue or organism [1].

In a typical LC-MS/MS experiment, protein sample is digested into peptides with proteolytic enzymes (e.g. trypsin, ArgC or other [4]), to break protein molecules into relatively short peptide sequences, and the resulting peptide mixtures are separated using Liquid Chromatography (LC) first, and subsequently ionized using or Electrospray Ionization (ESI) [5]. After ionization, the charged peptides are detected and separated in the first mass analyzer, and then fragmented and measured in the second mass analyzer. Usually two types of spectra are collected, MS spectra (or survey scans) in which the intensity and m/z are measured for intact peptides and MS/MS spectra where one of the ions detected in the MS spectra is isolated, fragmented and measured in a high-throughput manner [6].

One of the main practical applications of mass-spectrometry is related to clinical proteomics. The purpose of clinical proteomics is to characterize protein profiles of a plethora of diseases with the aim of finding specific biomarkers. These are particularly valuable for early diagnosis, and represent key molecules suitable for elucidation of pathogenic mechanisms. Samples derived from patients (i.e. blood, cerebrospinal fluid, saliva, biopsies) serve as source material for clinical proteomics. Due to the complexity of the extracted samples their direct analysis is usually impossible. Any analytical clinical proteomics study should start with the choice of the optimal combination of strategies with respect to both sample preparations and MS approaches. Peptide or protein fractionation (on-line or off-line) is essential to reduce complexity of biological samples and to achieve the most complete and reproducible analysis.

There is a critical need for the discovery of novel biomarkers for early detection and targeted therapy of cancer, a major cause of deaths worldwide [7]. In this respect, proteomic technologies, such as mass spectrometry (MS), enable the identification of pathologically significant proteins in various types of samples [8]. MS is capable of high-throughput profiling of complex biological samples including blood, tissues, urine, milk, and cells. MS-assisted proteomics has contributed to the development of cancer biomarkers that may form the foundation for new clinical tests [9]. It includes biomarkers of lung and thyroid [10], breast [11], ovarian [12], pancreatic [13], colorectal [14], gastric [15] and other types of cancers [16]. It can also aid in elucidating the molecular mechanisms underlying cancer [8]. Currently, most routine chemistry tests utilize spectrophotometric or immunologic detection. In contrast to enzymatic and antibody-based methods which are usually used in clinical practice, mass spectrometry (MS)-based proteomics measures the highly accurate mass and fragmentation spectra of peptides derived from sequence specific digestion of proteins. Because the masses and sequences of these peptides are unique, proteomics is inherently specific, circumventing a typical problem with colorimetric enzyme tests and immunoassays. Thus, mass spectrometry (MS) typically provides much greater analytical specificity relative to traditional methods.

Mass spectrometry-based proteomics methodology has become an important tool in elucidating some of the underlying mechanisms involved in cardiovascular disease [17]. The highly selective and specific mass spectrometry-based approaches have led to important new findings and provided new mechanistic information. For example the role of six proteins involved in the etiology of cardiovascular disease including acetylated platelet cyclooxygenase-1, serum apolipoprotein A1, apolipoprotein C-III, serum C-reactive protein, serum high mobility group box-1 protein, insulin-like growth factor I has been studied [17]. There are an increasing number of examples where highly selective mass spectrometry-based quantification has provided new important data that could not be obtained with less labor intensive and cheaper

immunoassay-based procedures. It is anticipated that these findings will lead to significant advances in a number of important issues related to the role of specific proteins in cardiovascular disease. The availability of a new generation of high-sensitivity and high-resolution mass spectrometers will greatly facilitate these studies so that in the future it will be possible to analyze serum proteins relevant to cardiovascular disease with levels of specificity and/or sensitivity that cannot be attained by immunoassay-based procedures.

Thus, proteomic technologies, such as mass spectrometry (MS), enable the identification of pathologically significant proteins in various types of samples. MS is capable of high-throughput profiling of complex biological samples including blood, tissues, urine, milk, and cells. The new impact II is one of the latest innovations in Bruker's unique UHR-QqTOF (Ultra-High Resolution Qq-Time-of-Flight) mass spectrometry product line with industry leading Resolving power among TOF instruments. This mass spectrometer with tremendous potential has been purchased and installed at NCB recently and is used for proteomic analysis of samples provided by other labs. Probably, it is first a fully functional instrument capable of solving tasks of modern proteomics which is available in Kazakhstan. It opens up enhanced analytical performance levels for all applications where trace analysis from complex, high-background matrices is a challenge - such as biomarker research, identification of impurities, or residue screening. The impact II sets a new technology standard where industry leading performance values are all simultaneously available in a single acquisition at full sensitivity (https://www.bruker.com/products/mass-spectrometry-and-separations/lc-ms/o-tof/impact-ii/overview.html).

Basic performance characteristics of mass spectrometers: mass resolution, mass accuracy, mass range, limit of detection, dynamic range

The mass spectrum of analyte species is represented by a bar graph that plots signal abundance or relative intensity of each of the ions against mass-to-charge ratio which is often abbreviated as m/z [18]. The term m/z is the parameter or property of the particle that is measured by the mass analyzer.

Mass resolution reflects the ability of the mass spectrometer to distinguish between two peaks with slightly different m/z values. It is conventionally defined as $R = m/\Delta m$ (also called the Resolving Power) in which the mass difference Δm can be defined as the width of the peak measured at a 50% of the peak height (figure 1A), which is also called the Full Width at Half Maximum (FWHM).

Larger resolution always indicates a better separation of peaks profiled in a mass spectrum. Resolving Power RP $\geq$ 5000 is considered to be high resolution with, that can greatly facilitate high precision measurements. The resolution obtained affects the ability to accurately determine the m/z of the analyte. The two plots of relative intensity versus m/z in Figure 1B correspond to a protonated ion of the peptide bradykinin (Mr = 1061.22). The plots are for bradykinin acquired when the maximum resolution observed is 1000 and 5000 $m/\Delta m$, respectively. The peak profile in the top part of Figure 1B is the sum of the various 13C isotopic contributions from the molecular formula whereas, in the bottom part of Figure 1B the isotopic contributions of ^{13}C are separated into discrete signals (https://msr.dom.wustl.edu/tof-ms-resolution-mass-measurement-accuracy/). The heavier isotopes of nitrogen and oxygen also contribute to the multiplet, but carbon is the most abundant element by percent in organic molecules like peptides and ^{13}C is the major contributor to the multiplet [19].

Mass accuracy defines how much accuracy of the mass value a mass analyzer can provide [20]. It is normally measured by millimass unit (mmu) or parts per million (ppm). A mmu is equivalent to 1/1000 of the unified atomic mass unit (u). The unified atomic mass unit (u) is displaced by the unit dalton, so 1 mmu equals to 1 millidalton (mDa). Mass accuracy expressed in ppm is calculated in the following way:

$$A_{ppm} = \frac{(|m_1 - m_2|)}{m_2} \times 10^6$$

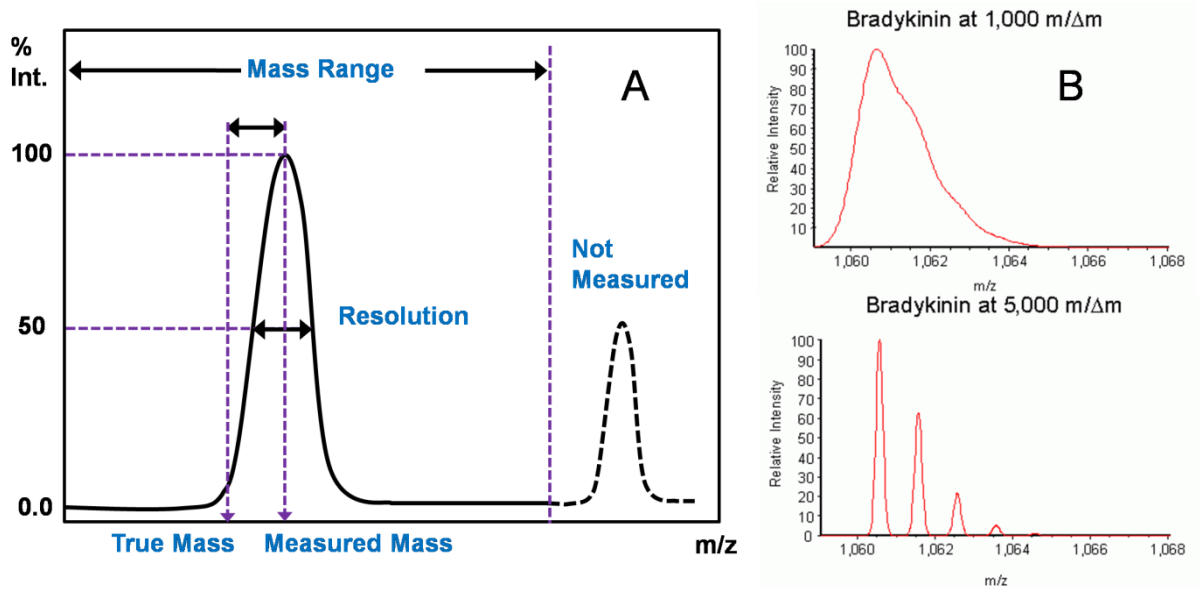
where m_1 is the real mass, and m_2 is the mass given by the mass spectrometer. So given the mass accuracy A_{ppm} and the mass value of an ion MW, we can covert the accuracy back to the unit of mDa by the formula

$$A_{mDa} = \frac{A_{ppm} \times MW}{10^3}$$

Mass range is the area of interest to be measured in an experiment or the capability of the analyzer (Figure 1).

Limit of detection (sensitivity) is an instrument ability to detect minimal amount of analyte. Usually it is measured in fg, pg (or amol, fmol).

The dynamic range can be defined as the ratio of the largest to smallest detectable signal with the instrument operating under the same conditions.



A: Mass resolution (Δm): is the ability of a mass analyser to separate one mass from an adjacent mass. Mass accuracy: is the measurement of the closeness of the given measurement to the true mass of the analyte. Mass Range: Mass analysers measure mass-to-charge ratio (m/z) and the difference between the highest and lowest measurable m/z denotes the mass analyser range. B: Plot of intensity versus m/z for bradykinin at a resolution of 1,000 m/Δm and 5000 m/Δm.

Fig. 1. Diagram showing the concepts of peak resolution, accuracy and mass range

Quadrupole mass analyzers

Quadrupoles are common components of many different mass spectrometers, where they are used as mass filters, ion guides or mass analyzers. They were invented in the 1950s by German physicist Wolfgang Paul [21]. A quadrupole is composed of a ring of four hyperbolic rods, each having an alternating radiofrequency potential applied to it (figure 2, A).

In quadrupole mass analyzing devices electric fields are used to separate ions according to their mass-to-charge ratio (m/z) as they pass along the central axis of four parallel equidistant rods (or poles) that have fixed (DC) and alternating (RF) voltages applied to them.

Magnitudes of these voltages can be adjusted so that only ions of certain masses are allowed to travel the whole length of the quadrupole, reaching the detector, the other ions being deflected onto trajectories that cause them to collide with the rods and remain undetected.

At any given time two of the rods will be positively charged and two will be negatively charged producing a potential difference between the two pairs [22]. An ion path is aimed through the centre of the device along the z axis and the radio frequency fields stabilize the trajectories of the ions along the x and y axes. The ions passing through the device will be momentarily attracted to the rods of the opposite potential, but will be repelled as soon as polarities switch.

The quadrupole ion trap is an extraordinary device that functions both as an ion store, in which gaseous ions can be confined for a period of time, and as a mass spectrometer of considerable mass range and variable mass resolution. As a storage device the ion trap acts as an "electric field test-tube" for the confinement of gaseous ions, either positively charged or negatively charged, in the absence of solvent. The confining capacity arises from the formation of a trapping potential well when appropriate potentials are applied to the electrodes of the ion trap. In its simplest form an ion trap permits the study of the spectroscopy and the chemistry of trapped ions. The quadrupole also can serve as a mass spectrometer, when combined with various ion selection and scanning techniques. The elucidation of ion structures by the use of repeated stages of mass analysis known as tandem mass spectrometry has added a new dimension to the armory of analytical techniques, especially in the biosciences. Quadrupole ion trap mass spectrometry has been a continuously growing technology since its invention in 1953 and, over the past two decades since its commercialization, has evolved into a work-horse instrumental technique in many analytical laboratories. With the advent of new methods by which ions can be formed in the gas phase, and introduced subsequently into an ion trap, the range of applications of the quadrupole ion trap mass spectrometer has increased enormously. In short, quadrupole devices have brought a revolution in the field of mass spectrometry during the past twenty years.

A quadrupole can be used as a standalone instrument but is often more powerful when used in an array of 3 quadrupoles in series known as a triple quadrupole (QqQ). The triple quadrupole mass spectrometer was first demonstrated in the late 1970s and opened up several new options for fragmentation [23]. In this set-up the second quadrupole acts as a collision cell and both the first and the third quadrupole can be used to select a specific m/z or scan the whole range – these setups are examples of tandem mass spectrometry experiments. The triple quadrupole was the first instrument available to put this idea into practice. Tandem mass spectrometry experiments available on triple quadrupole mass spectrometers include product ion scanning, precursor ion scanning, neutral loss scanning, and selected reaction monitoring experiments[22].

New instruments have appeared based on concatenations of quadrupole mass filters together with modified quadrupole rod sets that function as highly efficient ion pipes for the transmission of ions through various pressure regimes. Such concatenations, when combined with a quadrupole collision cell, constitute tandem mass spectrometers of high sensitivity. In the early stages of development of triple-stage quadrupole mass spectrometer, the ion beam moved continuously in the z-direction. However, with the development of quadrupole linear ion traps, the forward motion of the ion beam can be arrested and, when required, can be reversed for ion manipulation then reversed once again before being directed to the detector.

Ion trap mass spectrometry

The ion trap itself consists of a ring electrode and two end cap electrodes (Figure 2, B). Theoretical considerations suggest that to generate a quadrupole field in the ion trap the inner surfaces of all the electrodes are to have a hyperbolic profile and each electrode must be placed at a unique position. Using mass-selective ejection, an ion trap mass spectrometer takes a sample, ionizes it, and then traps ions over a large mass range of interest simultaneously. The trap can hold ions for surprisingly long periods of time, for example, up to 15 minutes or more for some stable ions [24].

Professor W. Paul and H. Steinwedel were the first to disclose a method for mass analysis by trapping a range of ion masses in the quadrupole ion trap and employing means to detect and measure the ions while stored. Their work is illustrated in Figure 2, C as the age of ion trap mass-selective detection.

The second age (mass-selective storage) took place during the late 1960s to the early 1980s. Scientists including P.H. Dawson, N.R. Whetten, John F. J. Todd and Raymond E. March were leaders in this age. This second scanning method involved producing a range of ion masses but operating the quadrupole storage field so as to store only a single mass in the ion trap at a time. This single ion mass is then ejected from the trap for detection by an external electron multiplier. The process is repeated rapidly until a complete mass spectrum is generated.

The third age is called mass-selective ejection, and its development started in 1979 by George Stafford [24] and included the work of other early researchers namely John E. P. Syka, Walter E. Reynolds, and Paul E. Kelley [25].

At the present period of time there are three major directions of trapping mass spectrometry (MS), specifically its radiofrequency (RF) ion trap, Fourier transform ion cyclotron resonance (FT ICR), and Orbitrap branches. The main unifying theme of trapping MS is the confinement of ions by electromagnetic fields for prolonged periods of time within limited volume with mass measurement taking place within the same volume [26].

Time of flight mass spectrometry

A time of flight (ToF) mass spectrometer contains a drift tube (Figure 2, D). An ion pulse is injected into the drift tube and the ions receive an acceleration pulse on injection in the tube. The tube has a detector such as a microchannel plate at the far end [27]. The applied field is constant so all ions no matter what m/z have the same kinetic energy. This acceleration results in an ion having the same kinetic energy as any other ion that has the same charge. The velocity of the ion depends on the mass-to-charge ratio (heavier ions of the same charge reach lower speeds, although ions with higher charge will also increase in velocity). Thus, the acceleration voltage separates out the ions in a spatial manner and as lower m/z as faster ions will reach the detector first. The time it takes each ion to reach the detector is measured and as the distance of the flight tube is known the velocity of the ions can be calculated[28]. The mass to charge ratio of the ion can then be calculated by the formula shown in following equation:

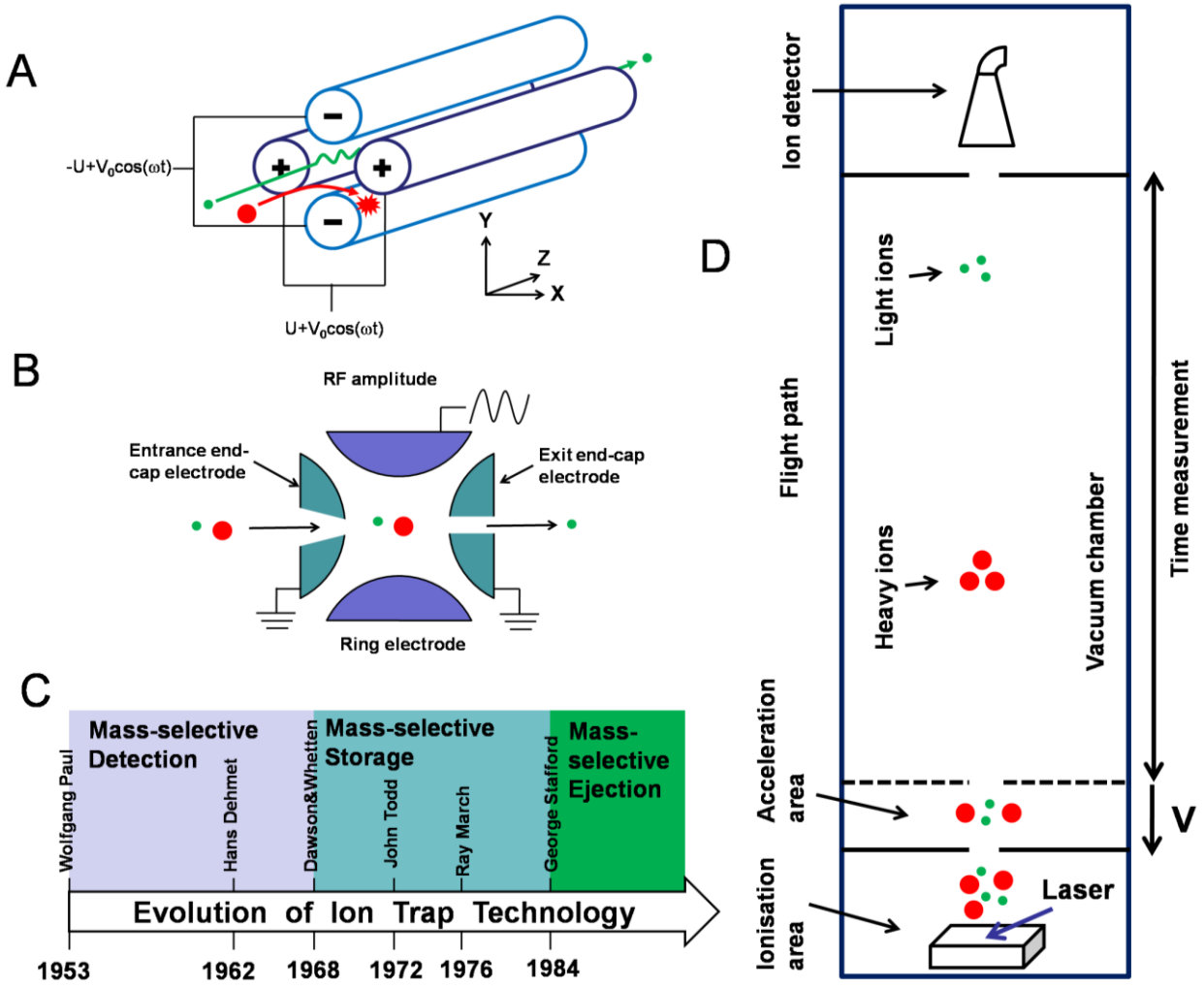
$$(m/z)^{1/2} = \left(\frac{\sqrt{2eV_S}}{L}\right) \times t$$

where V_S =accelerating potential, L = distance of ion path t = flight time [28].

Theoretically there is no upper m/z limit of a ToF mass analyzer. ToF mass spectrometers have undergone a number of refinements to improve their resolution. Early instruments had problems to apply completely uniform acceleration voltage across all ions. The ions would receive a range of different kinetic energies from the source so when the acceleration pulse was applied, the total kinetic energies of the ions would not be identical. Ions of the same m/z could have different kinetic energies and flight time so the peaks in a spectrum would be very broad [27]. A solution is to accelerate the ions after a short delay, which is known as delayed extraction. In the delay period ions with different kinetic energies separate slightly, so receive different energies from the acceleration pulse. The energy applied depends on the ions' proximity from the acceleration electrode and corrects for initial kinetic energies applied by the source. This ensures that the energy distribution of ions is more uniform and peaks became narrower. The resolution of a ToF can also be increased by adding a reflectron (or reflector) in the drift tube. A reflectron changes the ion path of the drift tube from a linear to a V shape. Both the accelerating field and the detector are at the same end of the drift tube and the reflectron is

placed at the other. Ions are injected into the drift tube are then reflected back to the detector by the reflectron which acts as an ion mirror. Reflectrons increase the path length without increasing the size of the drift tube. It helps increase mass resolution as ions separate more over a greater path and the reflectron further corrects the kinetic energy balance of ions with the same m/z. More energetic and faster moving ions spend more time in the reflectron so less energetic ions of the same m/z catch up. Instruments can contain multiple reflectrons for this purpose.

Combining a pulsed method of mass analysis with a continuous ion source such as ESI is a challenge which was solved by use of orthogonal acceleration [29]. In this case the flight tube is set orthogonally to the ESI source and the ion beam. A "pusher" is used which provides an accelerating voltage pulse off axis to the ion beam and injects ions into the drift tube. The velocity component is independent of that produced to the spray so it is not affected by initial kinetic energies formed upon ionization [29]. The ion pulse last only 10-100 nanoseconds, but a new population of ions is not injected until the ions with the highest m/z have reached the detector. In this time period the ion beam will begin to fill the orthogonal pusher before they are injected into the flight tube. Waiting for the previous scan to finish does mean the instrument has a slower scanning rate compared to conventional ToF mass spectrometer.



A: In quadrupole, electric fields are used to separate ions according to their m/z as they pass along the central axis of four parallel equidistant rods. Ion separation is performed by using controlled voltages applied to the mass analyzer rods which allow to pass one ion (colored green) and deflect another ion (red colored). B: 3D ion traps, apply their electric fields in three dimensions as opposed to the two dimensions of mass filters; this is achieved through the arrangement of electrodes in a sandwich geometry: two end-cap electrodes enclose a ring electrode. This arrangement allows ions to be trapped within the electric field. Mass selective instability is introduced by scanning the RF voltage applied to the device; as the voltage increases, the ions of sequentially higher m/z's are selected for detection by being ejected through an end-cap opening (green colored ion). C: Milestones in the mass spectrometric

development of the quadrupole ion trap. D: All ToF mass analyzers rely upon the acceleration of ions obtained from an ion source through a fixed potential into a drift region of a set length. This process of ion acceleration results in all ions of the same charge obtaining the same kinetic energy. Lower mass ions will obtain a greater velocity (green colored ions) than higher mass ions (red colored ions). Lower mass ions will therefore traverse the distance of the drift region in a shorter amount of time than heavier ions, resulting in the separation of ions according to their m/z. As the length of the drift region is known, ion velocities can be determined by measuring the time they take to reach the detector, allowing the m/z of the ions to be determined.

Fig. 2. Mass analyzers and ion traps. General principles and evolution

Orthogonal acceleration time-of-flight mass spectrometers have been used highly successfully as hybrid Q-ToF mass spectrometers. The ToF mass analyzer is used in tandem with two quadrupoles. The first quadrupole is used to select a specific m/z which is fragmented in the second quadrupole and then analyzed by the ToF [30]. The Bruker Impact II mass spectrometer is an example of such an instrument [31]. Quadrupole time of flight mass spectrometers have proved to be highly effective in analyzing high m/z ions of native protein complexes [32].

Tandem mass spectrometry

A tandem mass spectrometer has more than one analyzer (usually two), that are separated by a collision cell into which an inert gas (Ar, Xe, He) is admitted to provide fragmentation of selected sample ions through collision induced dissociation (CID). The collision cell is usually a hexapole or octapole operating in RF-only mode to allow the passage of all ions. The gas pressure used in the collision cell can be varied to alter the degree of fragmentation produced (higher gas pressures giving rise to a higher degree of fragmentation).

In practice many combinations of analyzer are possible and each combination lends specific qualities to the analysis, such as high resolution and/or the ability to make accurate mass measurements. MS/MS can also be achieved either by coupling multiple analyzers (of the same or different kind) or, with a single ion trap, by doing various experiments within the trap.

ESI mass spectrometry is very useful for obtaining molecular weight information of intact proteins and proteolytic peptides. Structural information however can be obtained from fragment ions. The process by which a precursor ion is characterized according to its fragments is known as tandem mass spectrometry (MS/MS) [33]. There are multiple fragmentation methods available including collision induced dissociation (CID) and electron transfer dissociation (ETD). One of the most commonly used MS/MS protocols is product ion scanning. In these experiments, a mass spectrum of the precursor ions is acquired. Ions of a particular m/z are then fragmented and then a second mass spectrum is acquired from which the m/z of the fragments can be determined [1].

There are a number of techniques available for fragmenting ions; the most commonly used technique is collision induced dissociation. CID is achieved by colliding ions with an inert gas such as helium or argon in the mass spectrometer [34]. Increasing the kinetic energy of the ions will lead to higher energy collisions and this kinetic energy is converted to internal energy in the collision. In proteomics, research is focused on peptide fragmentation. The mechanism behind CID peptide fragmentation has been explained by the mobile proton model [35]. The model assumes that the protons on a charged peptide are retained on the side chains of basic amino acid residues or the N terminus of the peptide and that fragmentation takes place when the proton is transferred to the peptide backbone. Transfer of the proton requires energy, so CID increases the internal energy of ions through collisions as they pass through the mass spectrometer. One of the downsides of CID is that the fragmentation pattern is nonspecific: the molecule breaks at the weakest or most labile bond [36]. This can make CID difficult for identifying labile post translational modifications, and certain amino acids such as proline fragment very differently to others. The presence of proline in a peptide tends to favor fragmentation at the N-terminal bond upstream of the proline residue [37]. There are a number of positions where a peptide can fragment and a system of nomenclature has been described by

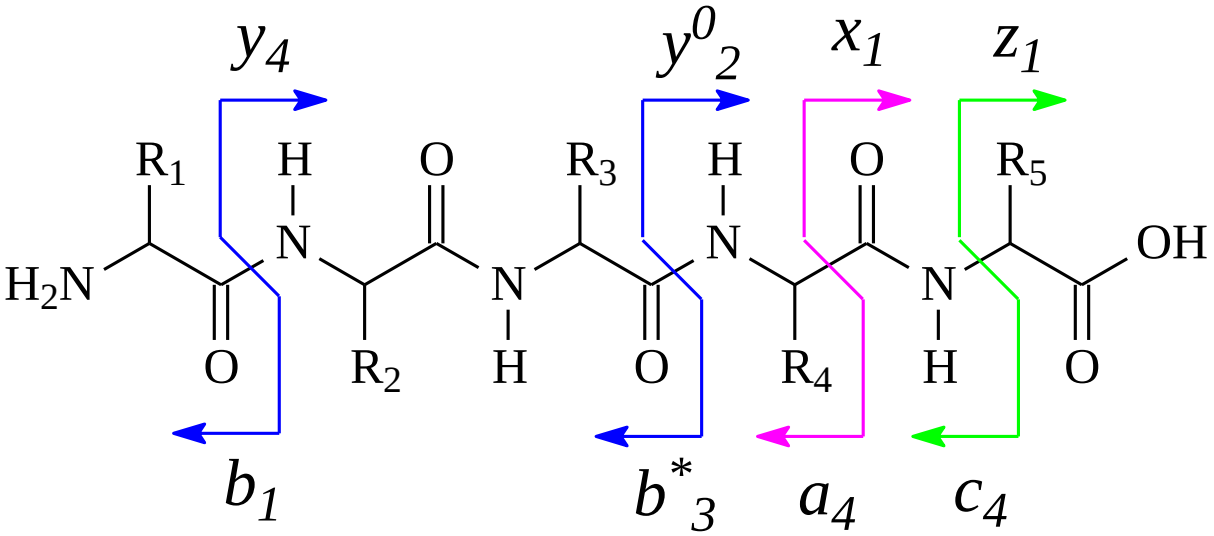
Roepstorff et al [38]. Table 1 shows how to derive the mass of the fragment ions with respect to the molecular mass values of the neutral amino acid residues. The notation M_r represents the neutral mass value of the target ion. M is molecular mass of the neutral amino acid residues.

Table 1. Neutral mass values of different ion types.

Ion Type	**Neutral Mass (*Mr*)**	**Ion Type**	**Neutral Mass (*Mr*)**
a	*H+[M]-CHO*	*c*	*H+[M]+NH₂*
*a**	*a-NH₃*	*x*	*OH+[M]+CO-H*
a°	*a-H₂O*	*y*	*OH+[M]+ H*
b	*[M]*	*y**	*y-NH₃*
*b**	*b-NH₃*	*y°*	*y-H₂O*
b°	*b-H₂O*	*z*	*OH+[M]-NH₂*
Note: to obtain *m/z* values, add or subtract protons as required to obtain the required charge and divide by the number of charges. For example, to get *y+*, add 1 proton to the neutral mass value for *y*, then the actual mass value of the *y − ion* with charge one is *OH* + [*M*] + *H* + *H*. Where *Mr* represents the neutral mass value of the target ion, and [*M*] is molecular mass of the neutral amino acid residues.			

CID cleaves peptides at the weakest bonds, which are usually the peptide bond [39]. These fragment ions are termed either *b* or *y* ions (Figure 3). In a *y* ion the charge remains on the fragment which contains the C terminus of the peptide. The opposite is known as a *b* ion, in which the charge is retained within the N terminal fragment. A population of ions will undergo fragmentation at various different points along the peptide, leaving a series of *b* and *y* fragments containing different number of residues. By finding the difference in mass between the peaks in a fragmentation spectrum, it is possible to deduce which residues were lost from each fragment and determine the sequence of the peptide. Each ion is given a number which counts how many amino acid residues from either the C or N-terminus the fragment contains. For example a C terminal fragment retaining 3 amino acid residues will be referred to as a y_3 ion and a fragment with the N terminus and 3 residues will be referred to as a b_3 ion [39].

Mass spectrometry is a highly complex analytical technique and mass spectrometry based proteomics experiments can be subject to a large variability, which forms an obstacle to obtaining accurate and reproducible results. Therefore, a comprehensive and systematic approach to quality control is an essential requirement to inspire confidence in the generated results. A typical mass spectrometry experiment consists of multiple different phases including the sample preparation, liquid chromatography, mass spectrometry, and bioinformatics stages [40]. LC-MS/MS based proteomics, often called shotgun proteomics, has been the leading proteomic technology of the 21st century. It is mostly used in two formats i.e. (i) two-dimensional (2D)-LC-MS/MS and (ii) SDS-PAGELC-MS/MS also termed 1D-gel-LC-MS/MS.



C-terminal ion series or *y*-ion series and N-terminal ion series or *b*-ion series ions are formed during Collision induced dissociation (CID) mode (*x*-ion and *a*-ion series are generated less frequently). Alternative fragmentation

method to CID which is called Electron capture dissociation (ETD) produces mainly *c* and *z* fragment ions. Subscript digits indicate number of aminoacid residues. Superscript symbol ^{O} indicates loss of H_2O, and superscript symbol * indicates loss of NH_3.

Fig. 3. Nomenclature of peptide fragmentation and possible cleavage points

The 2D-LC-MS/MS approach, also called MudPIT (multidimensional protein identification technology), was popularized in the early years of this decade by Yates et al [41]. MudPIT was initially performed in a single on-line 2D-LC-MS/MS routine, where capillary columns packed with strong cation exchange (SCX) material and a RP material is arranged in series in a single column. The sample, often tryptic peptides from a cell lysate, is loaded on the 2D-LC system and peptides are separated according to acidity in the first dimension (SCX) and hydrophobicity in the second dimension (RP). The eluted peptides are then analyzed by MS/MS. Alternatively, the ion-exchange separation can be performed off-line, fractions collected, de-salted and analyzed by RP-LC-MS/MS. This is the preferred 2D-LC-MS/MS methodology performed by Griffiths group [42].

An alternative LC-MS/MS based technology uses SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) as a first dimension of protein separation. The resulting 1D-gel-LC-MS/MS method involves protein separation according to molecular weight (by SDS-PAGE), followed by in-gel tryptic digestion and peptide analysis by LC-MS/MS, followed by protein identification by database searching. This methodology is extensively used by Mann's group in Germany [43].

Bioinformatic analysis of LC-MS/MS data

Possessing high resolution, sensitivity and accuracy, current mass spectrometers can produce thousands of MS/MS spectra in a single run. The huge volumes of data collected in an MS experiment require efficient computers and sophisticated software to automate the process of spectra interpretation.

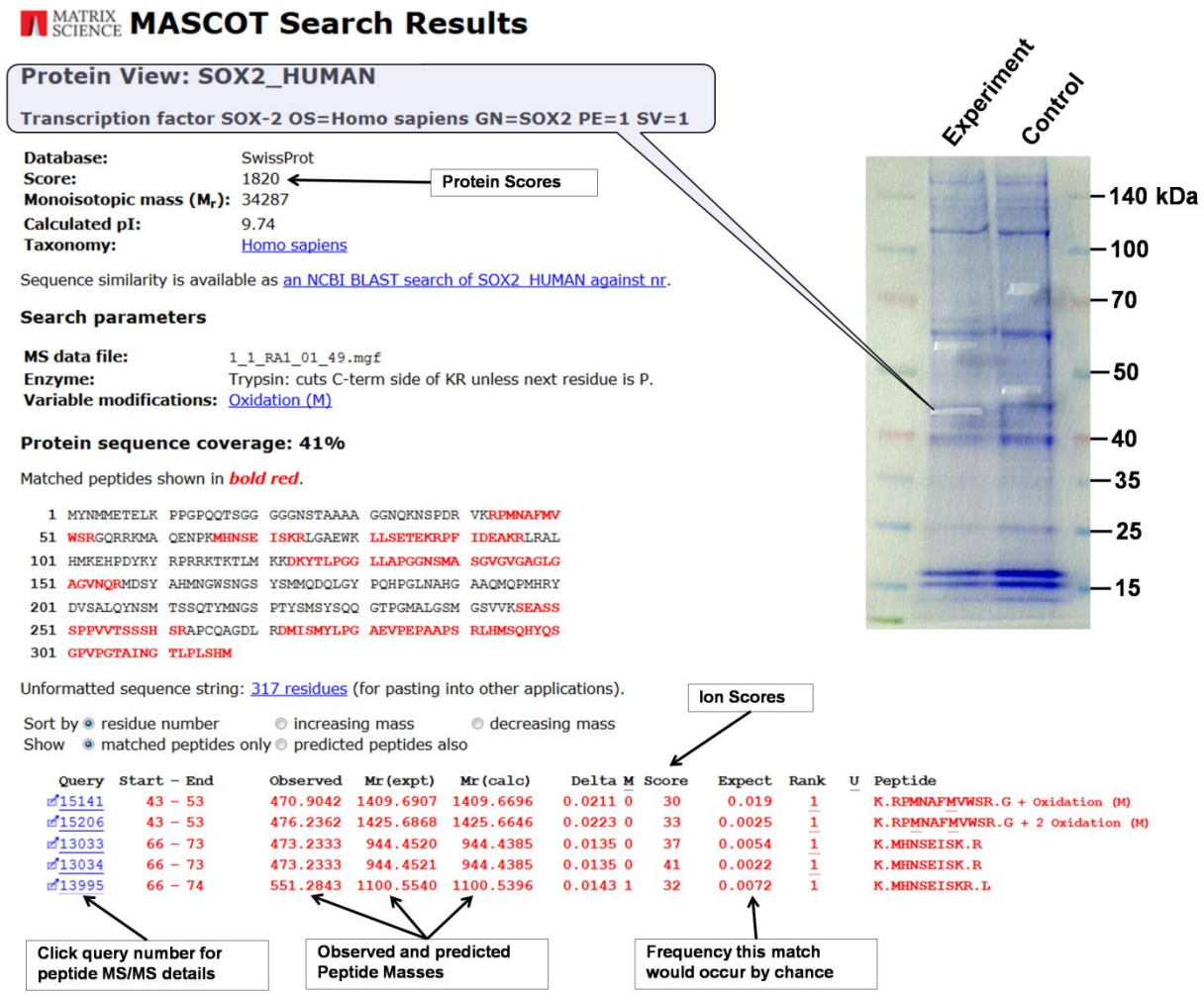
Currently, much effort has been made to develop approaches for the computational analysis of mass spectrometry based proteomics data [44]. Generally, the mainstream computational methods for this purpose fall into two categories: database search and *de novo* sequencing. The database search method has been extensively studied, in which the identification of MS/MS spectra is assisted with a protein sequence database, and the primary task is to correctly relate the collected spectra to amino acid sequences in the protein database. Many software packages are available for this purpose, including Mascot [45], PEAKS DB [46], SEQUEST [47], and OMSSA [48]. Usually, methods taking this approach make an assumption that all the sequences in the database are accurate and the proteins in the sample are included in the database. However, the aforementioned prerequisite that the targeted sequence is contained in the database is often not satisfied due to many reasons, such as incomplete genome sequencing, inferior gene prediction from the genome, and the existence of mutations and polymorphisms in the sample. Under this circumstance, *de novo* sequencing will serve as a complementary approach for peptide identification. In the *de novo* sequencing, the computation of peptide sequence does not rely on the protein database, the algorithm directly constructs the peptide sequence that best matches the spectra [49].

In Mascot, the ions score for an MS/MS match is based on the calculated probability, P, that the observed match between the experimental data and the database sequence is a random event. The reported score is -10Log(P). The empirically corrected identity threshold is 32 (http://www.matrixscience.com/help/interpretation_help.html). Protein Score is the sum of the ion scores of all peptides that were identified (Figure 4). PSM's is the number of peptide spectrum matches. The number of PSM's is the total number of identified peptide spectra matched for the protein. The PSM value may be higher than the number of peptides identified for high-scoring proteins because peptides may be identified repeatedly. Sequence Coverage means

the percentage of the protein sequence covered by identified peptides, for example for Sox2 protein matched (red colored) peptides corresponds to 41% of identified sequences (Figure 4).

Although much effort has been made to develop new computational approaches for the analysis of mass spectrometry data, there are still several unsolved problems that are challenging [50]. One specific challenge is that in a high throughput MS/MS experiment, usually only a fraction of the acquired spectra can be confidently interpreted by the existing computational methods. Many factors may contribute to this situation including: low precursor intensity, poor fragmentation of the selected precursor, or the presense of modified residues. Moreover, the gas phase fragmentation may result in MS/MS spectra with unconventional fragment ions that are not considered by the mainstream computational methods, and the sequenced peptides may not be present in the database or may have unanticipated post-translational modifications (PTMs).

Even coupled with High Performance Liquid Chromatography (HPLC), it is still not guaranteed that the peptides in the sample are completely separated. There is a chance that peptides with similar m/z values co-elute, generating a single spectrum that contains a mixture of spectra. The mixture spectra are induced by the isolation and simultaneous fragmentation of two or more distinct molecular ions within the same isolation window. Fragments from multiple precursors will be present in a single MS/MS spectrum, increasing the number of unidentified fragments in database search engines.



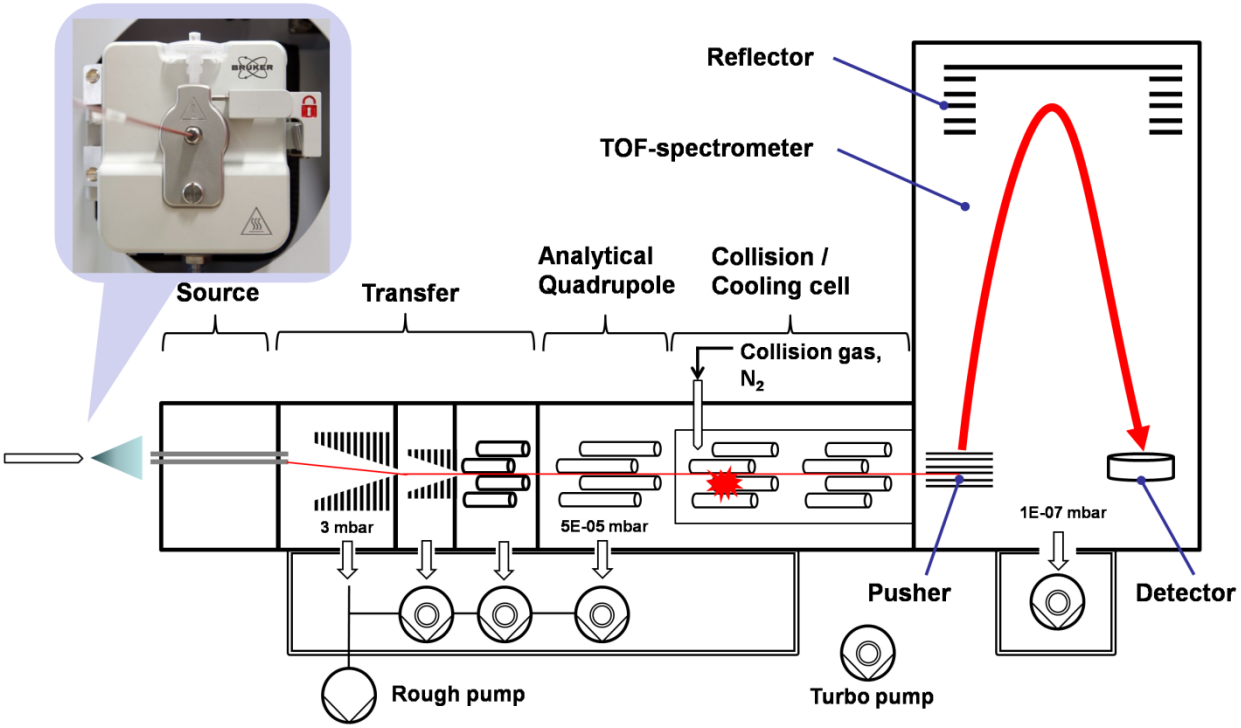
Protein excised from gel was identified as human Sox2 transcription factor. For each protein match, Mascot calculates an overall Protein Score. This number reflects the combined scores of all observed mass spectra that can be matched to amino acid sequences within that protein. A higher score indicates a more confident match. The "bottom line" is that a good match should have a high Score and contain multiple Query matches listed in bold red type.

Fig. 4. Protein identification using Mascot

The Impact II, a very high resolution QTOF tandem mass spectrometer

Among different instruments hybrid quadrupole time-of-flight (Q-ToF) is one of the most prevalent of mass spectrometers used in proteomics. Based on web-sites of manufacturers, time-of-flight (ToF) models, including hybrid and tandem models constitute a 37% of the total number of models of mass spectrometers on offer in 2014 [28]. While based on simple fundamentals, it has significantly evolved over the last decades in terms of achievable mass accuracy, resolution and dynamic range. The last generation of Bruker impact platform of Q-ToF instruments takes advantage of these developments with development of the impact II for shotgun proteomics applications [51].

The Bruker impact II is a Q-ToF in a benchtop format, with several improvements incorporated in its design. In short, ions are produced in the CaptiveSpray (Figure 5), which is in an encased nanoelectrospray source that features a well-defined gas flow to guide the ions into the vacuum via a capillary inlet. A double ion funnel, based on principles described by Smith and co-workers [52], is positioned off axis, which prevents neutral species from further transmission along the ion path. The pressure drops by several orders of magnitude from the capillary exit to the post-funnel stage (3 mbar to 3×10^{-4} mbar), while the ion current is virtually undiminished. Additionally, the funnel allows for soft transfer based on low electric field strength independent of the mass (typically 10 V/cm, much lower than in nozzle-skimmer designs). There is an analytical quadrupole mass filter, which has a monolithic design based on high precision glass. Precursor ions can be isolated by this quadrupole for subsequent fragmentation in the collision cell (Figure 5). Intact ions or fragments can be stored and extracted from the collision cell and enter the orthogonal deflection region as a very narrowly focused ion beam. Here they are accelerated into a field-free drift region. A newly designed, two-stage reflectron further compensates the velocity distribution orthogonal to the beam direction. Finally, the ions impinge on an MCP detector coupled to a 10-bit, very high frequency (50 Gbit/s), and zero noise digitizer. Data collection is coordinated by the Bruker Compass data system.



Source (CaptiveSpray, chamber and capillary), ion transfer stage (funnel 1, funnel 2, multipole), quadrupole, collision/cooling cell and TOF spectrometer (orthogonal accelerator (pusher), dual stage reflector, detector)

Fig. 5. Schematic diagram of a Bruker Impact II Q-ToF mass spectrometer

Improvements of Impact II instrument include the following:

1. Optimization of the collision cell. Precise geometrical alignment allows focusing of the ions along the axis of the collision cell, directly translates into well-defined starting conditions for the orthogonal accelerator and is therefore obligatory for high mass resolution. Engineers from Bruker Company also introduced a radial ejection step between any two MS or MS/MS experiments, in order to reduce the dead time. Most importantly, they optimized the time of ion fragmentation and extraction within the fragment spectra to ensure efficient high frequency MS/MS by implementing an electrical axial field gradient.
2. High transfer efficiency to the orthogonal acceleration unit. The ions travel through the flight tube and require as much time as the largest m/z species needs to reach the detector, before the HV pulser can send the next ion package towards the detector (typically between 100 and 150 μs). Together, they improvements led to an overall transmission efficiency of > 60% into the orthogonal acceleration unit. This compares favorably to a recent report, in which ion mobility was performed on fragment ions and their arrival times were synchronized with orthogonal extraction, which led to an up to 10-fold improvement of detection sensitivities in standard operation [51].
3. Sensitivity and ion transfer. The numbers of ions that successfully pass through the instrument and are finally recorded determine the mass spectrometer's sensitivity. Engineers from Bruker Company analyzed the ion current from the inlet capillary and found very high transmission (>80%) up to the collision cell. Simulation and measurement indicated 60% transfer into the flight tube.
4. Resolution and mass accuracy. For the Impact II several improvements were implemented: symmetrical shielding for better ion focusing; line grids to increase the transmission; low temperature coefficient ceramic spacers to decrease temperature related mass drift and improved axel bearings for precise alignment. Together this led to about 35% increase in resolution over the full proteomics mass range. In summary, the resolving power of the TOF analyzer is expected to increase by about 70 to 80% by the introduction of the new collision cell, reflectron and detector. The new reflectron and detector improve resolving power compared to the previous model up to 80%, i.e. to 40,000 at m/z 1222.
5. Reproducibility and accuracy of quantification. Software designers adapted MaxQuant for Q-ToF data, improving absolute average mass deviations to better than 1.45 ppm [51]. More than 4,800 proteins can be identified in a single run of HeLa digest in a 90 min gradient. The workflow achieved high technical reproducibility (R2>0.99) and accurate fold change determination in spike-in experiments in complex mixtures.

In laboratory of proteomics and mass-spectrometry of National Center for Biotechnology we are working on projects related to cellular proteomics (focusing on *in vivo* detection of protein-protein interactions in human cells, e.g. HEK293T or HeLa), protein biomarkers in blood plasma (laboratory animals, e.g. rats and rabbits).

Many cellular processes are carried out by physically interacting proteins and about 80% of them form multi-protein complexes. Identification of protein-protein interactions (PPI) is therefore a critical step in understanding of cellular function.

We have developed a systematic proteomics-based approach to study PPI which uses Proximity Utilizing Biotinylation (PUB) [53]. The coexpression of a protein of interest A, fused to BirA ligase with the fusion of a protein B with BAP (Biotin Acceptor Peptide, specifically biotinylated by BirA) leads to biotinylation of proteins interacting with (or else in proximity to) the BirA-fusion *in vivo*. Currently we are working to apply the PUB method for study of *in vivo* interactions of pluripotency transcription factors Sox2 and Oct4 in frame of the project AP05132131. We detected strong biotinylation signal of recombinant proteins BAP-Sox2 as result of interaction with BirA-Oct4. The corresponding band from nuclear lysate was identified by shotgun analysis on Impact II as recombinant protein BAP-Sox2 (figure 4).

One of the perspective directions of research for application in medicine is study of protein biomarkers in blood. The concept of preventive medicine is based on the monitoring and management of early stage diseases rather than from treatment of late stage disease. Among the strategies that have the highest potential to realize the expectations of preventive medicine is the detection of diagnostic and prognostic protein biomarkers in blood plasma. Mammalian blood is easily accessible for sampling and contains signaling molecules from all organs. Therefore, the quantitative protein composition of blood plasma contains information about the state of organs and the whole organism in health and disease. The mapping of this informational network requires robust, reproducible and sensitive measurements of single protein markers.

In contrast to enzymatic and antibody-based methods which are usually used in clinical practice, mass spectrometry (MS)-based proteomics measures the highly accurate mass and fragmentation spectra of peptides derived from sequence specific digestion of proteins. Because the masses and sequences of these peptides are unique, proteomics is inherently specific, which is in contrast to colorimetric enzyme tests and immunoassays a constant problem.

We identified and analyzed sequences of four most abundant proteins of blood plasma from *Homo sapiens* (human), *Oryctolagus cuniculus* (rabbit) and *Rattus Norvegicus* (rat) which include albumin, immunoglobulin gamma, serotransferrin and C3 complement protein. These proteins have crucial roles in lipid transport, homeostasis and the innate immune response. Some diseases are caused by mutations in protein sequence. For example Familial dysalbuminemic Hyperthyroxinemia, (FDAH) in albumin sequence (L90P, R242H, R242P), atransferrinemia (ATRAF) in Serotransferrin sequence (D77N, A477P) or Macular degeneration in C3 complement sequence (R592Q, R592W) (https://www.uniprot.org/).

Blood plasma was obtained by centrifugation at 1500 rpm for 10 minutes. Electrophoresis was performed in a gradient polyacrylamide gel (Novex NuPAGE). The proteins were visualized with Coomasie Brilliant Blue R-250 dye, then reduced and alkylated with a solution of dithiothreitol and iodoacetamide. Samples were subjected to trypsinolysis, desalted using a micro-Zip-Tip kit and analyzed on a Bruker Q-ToF Impact II mass spectrometer (laboratory of proteomics and mass spectrometry of the NCB MES RK). Mascot software was used to perform searches against the SwissProt 2014_08 database (546,238 sequences; 194,363,168 residues).

We also analyze samples to control and confirm aminoacid sequences and post-translational modifications of recombinant proteins provided by other laboratories. For example, we identified proteins originated from different species, for example, Alpha-amylase (*Bacillus licheniformis*), Maltose-binding periplasmic protein (*Escherichia coli*), Matrix protein and Nucleoprotein (*Rabies virus*), POLG_FMDVI 2966 Genome polyprotein (*Foot-and-mouth disease virus*), Red fluorescent protein drFP583 (*Discosoma*), PARK7_HUMAN, Protein deglycase DJ-1 (*Homo sapiens*), Antigen GM6 (*Trypanosoma brucei*), 13S globulin seed storage protein (*Fagopyrum esculentum*) and others.

Comparison of the typical performance characteristics of several types of commonly used mass analyzers

Several types of mass analyzers have been developed, including the Quadrupole mass analyzers [21], Ion trap analyzers [24,54], Time-of-flight (ToF) analyzers [27,28], Fourier transform ion cyclotron resonance (FT-ICR) [55], and Orbitrap [26]. Each type of mass analyzer has different capabilities in terms of sensitivity, accuracy, resolution, m/z range and some other properties. Table 2 provides a summary of the performance characteristics for each mass analyzer.

There are a wide variety of mass spectrometers each with different resolution, sensitivity, mass range, mass accuracy characteristics and dynamic range. The table 2 presents typical values of these parameters for selected mass analyzers. When performing quantitative analyses it is especially important to know the limits of detection of the mass analyzer being used as well as the linear range where sample concentrations are expected to vary over a wide range. It is also

important to know the dynamic range, which can be defined as the ratio of the largest to smallest detectable signal with the instrument operating under the same conditions. According to table 2 FT-ICR and Orbitrap mass spectrometers have the top resolving power, but this characteristic is a function of m/z which decreases rapidly over higher masses [26]. Unlike these Fourier transform instruments resolution of Q-ToF mass spectrometers linearly increases over higher m/z values [51]. For example, at masses around 1000 Da Impact II Q-ToF demonstrates comparable resolution with standard Orbitrap (table 2) and can outperform it at higher m/z values.

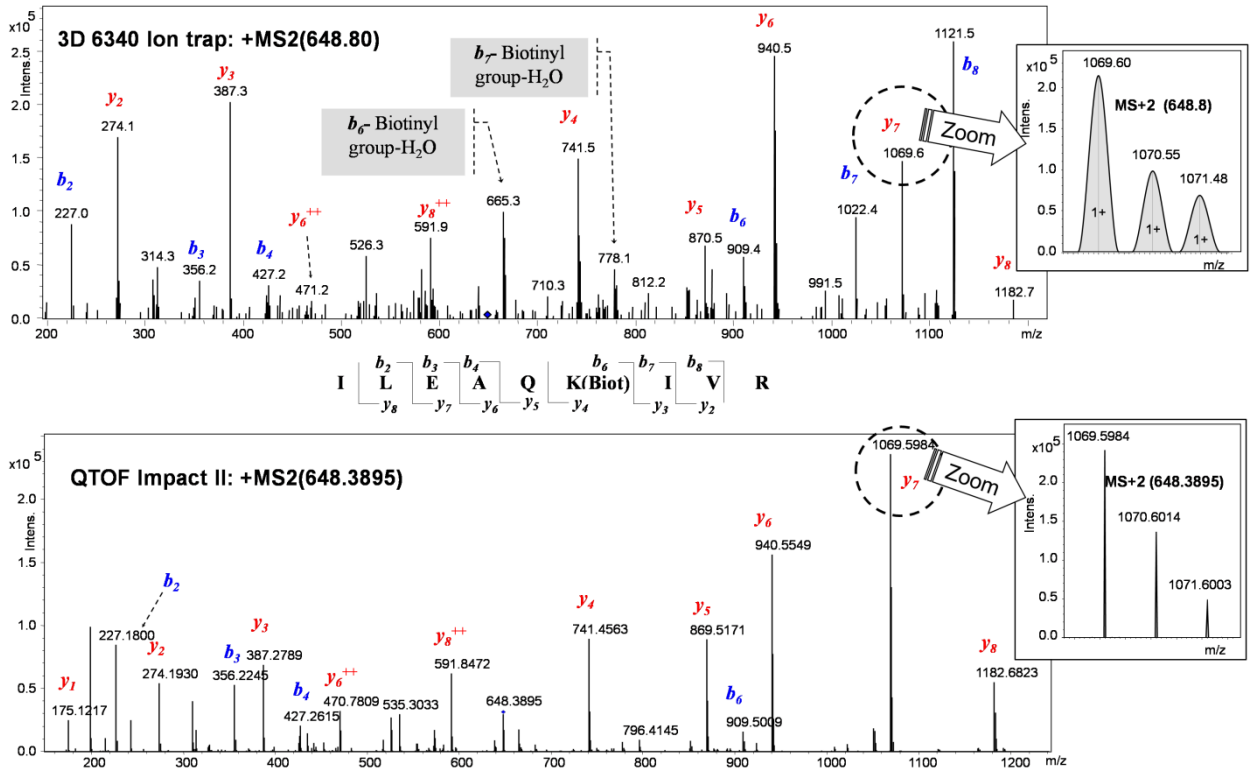
Resolution of quadrupole and ion-traps are much lower than ToF instruments. For example, Figure 6 demonstrates difference of Resolving power of 3D 6340 Ion trap and QToF Impact II. The same sample containing BAP peptide [53] was run on both instruments and resulted MS/MS fragmentation spectra look similar (see m/z values of *y* and *b*-ions on Figure 6). Zooming the peaks (e.g. y_7=1069.6) allows to see difference in width of peaks. Isotopic peaks 1069.5984, 1070.6014 and 1071.6003 Da are more narrow for spectrum obtained from QToF Impact II than isotopic peaks 1069.50, 1070.55 and 1071.48 Da from 3D ion trap. Resolution of the first instrument is equal to 40000 and of the second instrument 2000. Thus, Bruker QToF Impact II has 20 times more of resolving power in comparison with 3D 6340 Ion trap.

Table 2. Comparison of the typical performance characteristics of several types of commonly used mass analyzers including Resolution (represented by Resolving Power), Accuracy, m /z Range, Optimal Detection Level and Dynamic Range

Mass Analyzer	Resolving Power (Resolution)	Accuracy (ppm)	m/z Range	Optimal Detection Level	Dynamic Range
Quadrupole	500-2000	100-1000	50-4000	50-500 pg (scanning) 500fg-5pg (SIM)*	10^5
Ion trap	500-2000	100-1000	10-4000	1-10 pg	10^4
ToF	500-12000	10-100	50-1×10^6**	1-10 pg	10^4
QToF (Impact II)	40000 at m/z 1222	≤1.45	20-20000[$]	50fg-10ng[¶]	1.7×10^5 [§]
FT-ICR	100000-750000	≤ 2	50-4000	0.3-30 pg	10^4
Orbitrap	30000-100000[⊥]	2-5	50-4000	0.3-30 pg	10^4

Notes: *SIM - Selected Ion Monitoring mode
** No theoretical upper limit
[$] http://core.tmmu.edu.cn/attachments/193/maXis_User_Manual.pdf
[¶] CaptiveSpray nanoBooster increases the ionization efficiency of peptides significantly, leading to a much higher sensitivity than even conventional nanospray (https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/Literature/Brochures/1820273_nanoBooster_brochure_06-2013_eBook.pdf)
[§] https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/Literature/Brochures/1829433_impact_II_brochure_06-2014_ebook.pdf
[⊥] - 40 000 at m/z 1000 for Standard Orbitrap[26]

(https://application.wiley-vch.de/books/sample/3527329242_c01.pdf or http://www.usp.br/massa/2014/qfl2144/pdf/MassSpectrometry.pdf).



Fragmentation pattern of biotinylated BAP peptide (ILEAQK(Biotin)IVR) are similar. In case of spectrum from QToF Impact m/z values of *y*- and *b*-fragment ions are more precise and narrower in comparison with 3D ion trap spectrum (approximately 20 times more narrow peaks, see y_7 fragments ions zoomed in the right part of the figure).

Fig. 6. Comparison of MS/MS spectra of BAP peptide obtained from runs on two instruments 3D 6340 Ion trap and Q-ToF Impact II

Improvement of other parameters for Impact II such as accuracy, optimal detection level and dynamic range (table 2) was possible due to design of new collision cell with broad mass transfer, new ToF with enhanced resolving power, dual ion funnel, IonBooster Source, CaptiveSpray nanoBooster, 50 Gbit/sec data sampling technology and other technological innovations from Bruker company (https://www.bruker.com/products/mass-spectrometry-and-separations/lc-ms/o-tof.html).

CONCLUSION

The advent of nano-ESI has had a profound impact on the field of biological mass spectrometry and its application to proteomics. Currently, by using sophisticated sample preparation protocols and compatible orthogonal fractionation strategies, it is possible to identify thousands of proteins in a single experiment using ESI-MS methodologies. Indeed, state-of-the-art ESI-MS instrumentation in combination with advanced MS/MS techniques, have helped to advance the concept of “top-down” and “bottom-up” proteomics. For example, the recent introduction of hybrid Quadrupole Time-of-Flight Impact II mass spectrometer offers unrivaled mass accuracy (better than 1.45 ppm), high resolving power (40 000 at m/z 1222) and a high dynamic range (1.7×10^5), without the need for a superconducting magnet and its associated maintenance requirements. Moreover, ion trap technology can now be used in conjunction with CID (or ETD), which facilitates the acquisition of highly comprehensive sequence-specific information on electrosprayed peptides.

Mass spectrometry has a demonstrated capability to detect peptides and proteins in a specific manner. Combined with appropriate sample preparation and/or enrichment, sensitivity is high enough to quantify peptides and proteins for clinical applications. In particular, the detection of iso-forms and different post-translational modifications is of highest interest for

clinical applications for example when antibody cross-reactivity is observed or sensitivity of immunoassays is insufficient.

To conclude, a unique advantage of MS is the fact that the analysis is specific to the entire analyte molecule, which is total mass and structure. In comparison, other analytical techniques use non-specific physicochemical proprieties such as retention time or isoelectric point. Similarly, antibodies recognize only an epitope, not the entire molecule. MS is thus in a unique position to distinguish between proteoforms with subtle differences allowing to obtain a wealth of data not accessible by any other method.

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