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High-resolution mass-spectrometry analysis of peptides and proteins

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Abstract. Modern approaches to investigation of biomacromolecules using mass spectrometry techniques, which have crucially changed during the last decade, are considered. The attention is focused on the peptide and protein identification methods based on Fourier transform ion cyclotron resonance mass spectrometry, which provide high resolution and high accuracy and which are being developed with active participation of the authors. The ultimate possibilities of mass-spectrometric techniques regarding the sensitivity and uniqueness of identification of protein molecules are analyzed. The bottom-up and top-down identification approaches are described, their advantages and shortcomings for determination of post-translational modifications of proteins are estimated. Various methods for quantitative analysis of mixtures of biomolecules using isotope labels and isotope dilution are considered. The bibliography includes 192 references.

I. Introduction

Proteins and peptides are biopolymers that play an important role in the functioning of organisms. They are synthesized from amino acid molecules according to the genetic information read from chromosomes and undergo various chemical transformations after the synthesis. The protein structures and functions constitute the subject area of a new field of science called proteomics. Due to the chemical (so-called post-translational) modifications, the diversity of proteins in a particular organism is several orders of magnitude higher than the number of genes encoding them. For this reason, identification of proteins and peptides is a much more complicated task than identification of genes. It is also complicated by the fact that, unlike genomics in which various procedures for DNA multiplication exist, there is still no such possibility in proteomics. Therefore, it is necessary to use the most sensitive methods for molecular analysis, among which there is mass spectrometry.

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1. Modern mass spectrometry methods

Mass spectrometry (MS) is a physical method used since long ago for the analysis of substances. In the latest decades, mass spectrometry got a new life owing to the discovery of new non-destructive ionization methods for large biomolecules including DNA, RNA, proteins and peptides; consequently, mass spectrometry became a key tool of molecular biology and biochemistry.

The most useful methods for ionization of biomolecules are electrospray ionization (ESI)[1,2] and matrix-assisted laser desorption ionization (MALDI);[3–6] J Fenn and K Tanaka, who invented these techniques, were awarded the Nobel Prize in chemistry of 2002. Pioneering works dealing with electrospray ionization were performed by Russian researchers.[7,8] According to the most popular ESI implementation, solutions of proteins and peptides are sprayed in the atmosphere under the action of electric field to enter, as charged drops, a desolvating capillary connected to a mass analyzer *via* a vacuum and ion interfaces. Biopolymers are ionized upon vacuum vaporization of solution drops and deposition of charged species (for example, H^+, K^+ and HO^-) on the molecules. Thus, the molecules are ionized without destruction. It is noteworthy that ESI ionization of biomacromolecules affords multicharged ions.

In the MALDI method, ionization and desorption of molecules are induced by laser radiation, which is absorbed by a specially selected matrix, the molecules to be analyzed being co-crystallized together with the matrix. The MALDI ionization affords mainly low-charged ions; however, multicharged ions, as in the ESI ionization, can also be obtained under certain conditions.[9, 10]

Two procedures are mainly used to analyze proteins by means of a mass spectrometer. In one procedure, a mass spectrometer serves for the analysis of peptides and proteins that have been separated by 2D gel electrophoresis. Peptides and proteins are extracted from the gel and are either dissolved for ESI analysis or co-crystallized with the matrix substance for MALDI. A number of extraction techniques have been developed. In the second procedure, capillary electrophoresis or high-performance liquid capillary chromatography serves for preliminary separation of peptides and proteins, while a mass spectrometer with a ESI source serves for analysis and identification of the separation products on the real time scale.

In recent years, ideas of the development of direct (without sample preparation) atmospheric ionization have been put forward. Considerable attention of researchers was attracted by desorption electrospray ionization (DESI), which was invented by Cooks' group[11] in 2004. The method was based on the formation of secondary ions, which are formed upon extraction of molecules located on the surface to be studied into microscopic ionized solution drops obtained by electrospray from a capillary (Fig. 1). This ionization method is of interest because it is simple and convenient to use, as there is no need for sample preparation.

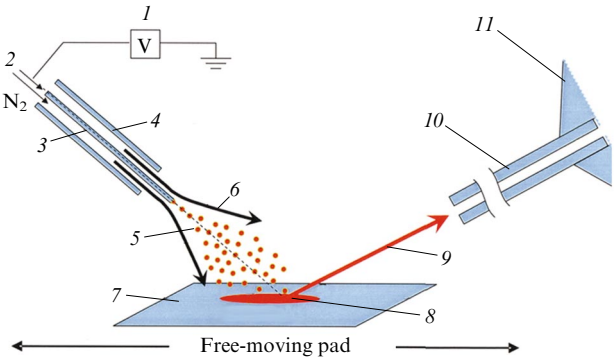


Figure 1. Scheme of the desorption electrospray ionization source.[11]
(*1*) Power-supply source, (*2*) solvent, (*3*) solvent supply capillary, (*4*) nebulizer, (*5*) spray drops, (*6*) gas jet, (*7*) substrate, (*8*) sample, (*9*) desorbed ions, (*10*) heated capillary, (*11*) atmosphere interface of the mass spectrometer.

One more recent method of direct ionization[12] is based on laser desorption combined with electrospray ionization (ELDI). In the ELDI method, the desorption and ionization processes are separated. The sample molecules are desorbed from the substrate on exposure to laser radiation. Then the desorbed molecules get into the electrospray jet where they ionize upon interaction with charged drops of the sprayed solvent (Fig. 2).[13]

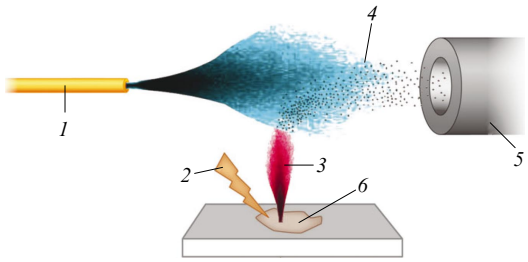


Figure 2. Schematic view of the operation of the ELDI source.[13]
(*1*) Electrospray, (*2*) laser exposure of the surface, (*3*) desorption of sample molecules, (*4*) electrospray ionization, (*5*) atmosphere interface of the mass spectrometer, (6) sample.

The principal advantage of ELDI over MALDI is the fact that, similarly to DESI, no additional sample preparation is required. One more advantage of the method is that ionization results in multicharged ions, as in electrospray ionization.

The scope of above-listed methods can be extended if the experimental conditions are optimized.[14, 15]

The highest mass resolving power and mass accuracy with fairly high sensitivity are provided by Fourier transform ion cyclotron resonance (FT ICR) mass spectrometry.[16] The role of this method in biological and biochemical research is constantly increasing despite the high instrument purchase and operation costs.[17] A new turn in the development of this method was stimulated by the Nikolaev's group discovery of the dynamic harmonization of Penning traps.[18, 19] These traps are suitable for achieving an unrestricted mass resolving power. The progress in the design and manufacture of more advanced mass analyzers is largely associated with the use of modelling to predict the motion of ion ensembles.[20]

The FT ICR method was proposed in 1974 by Comisarow and Marshall.[21, 22] It is based on measurement of frequencies of the cyclotron movement of ions in a magnetic field by measuring the signal induced by ions between the capacitor sheets and the Fourier transform analysis of this signal. Numerous original papers and reviews exhaustively describe the potential of this method.[23–38] Another method having similar potential was implemented several years ago by the Thermo Fisher Scientific company, which designed the instrument Orbitrap[39–41] based on the idea of Kingdon ion trap[42] where ions are held by electrostatic fields of cylindrical symmetry without application of a magnetic field. This mass spectrometer measures axial vibrational frequencies and uses the Fourier transform method for the frequency analysis of spectra. The instrument demonstrates high mass accuracy and high resolving power comparable to those obtained in the FT ICR method at magnetic fields of about 7 T by means of conventional Penning traps. Orbitrap gains popularity and successfully competes with instruments based on ion cyclotron resonance principles and with time-of-flight mass spectrometers (TOF MS).[43] The instruments that use ions accelerated to equal energy for mass measurement are currently the second most popular in proteomic research after radio frequency ion traps. They are manufactured by almost all large companies that produce mass spectrometers. By using reflectron technique,[44] it is possible to avoid the scatter of ions in energy and thus to achieve the mass accuracy of nearly 1 ppm (5 ppm in

routine measurements) and a resolving power of about 20 000 in the mass range up to one megadalton when special ion detectors are used and 200 kDa in routine measurements, which cannot be attained with mass spectrometers of other types.

In proteomic research, radio frequency ion traps (three-dimensional and linear ones) and quadrupole mass spectrometers are employed most often. Mass spectra are measured with these instruments by creating ion mass-dependent conditions for ion retention and by measurement of amounts of ions ejected from the trap or quadrupole filter in the time sweep of one of the parameters that determine the stable motion conditions (most often, the radio frequency field amplitude). These instruments have high sensitivity (as the detection system allows one to measure the signal from single ions) and high spectrum recording rate (tens milliseconds per spectrum). Furthermore, they are able to record the spectra of products formed upon repeated MS/MS analysis, which makes them unique for determination of the primary structure of ions. However, their resolving power (several thousand) does not resolve the isotope structure in the mass spectra, while the low mass accuracy (< 100 ppm) precludes their use in the accurate mass tag procedures. In view of the advent of relatively cheap precise mass spectrometers such as time-of-flight instruments, ion traps retreat to the background in proteomic research as mass analyzers, while functioning more and more often as accumulation devices and preliminary mass filters.

2. Mass spectrometry in proteomic research

Proteomics is among the most rapidly developing areas of biochemistry. Analysis of the proteomes of various organisms, single organs and physiological fluids becomes a routine task if the appropriate genetic information is available. This task can be accomlished by mass spectrometry using enzymes to cleave proteins into shorter peptides and various methods of peptide separation into several fractions.[45] The understanding of the biological functions of particular proteins should facilitate the development of new effective and systematic approaches to the creation and evaluation of new drugs and pharmaceutical products and could lead to the advent of new diagnosis and therapy methods.

The proteome is the combination of the proteins present in a body cell, tissue or fluid in a given time instant, in the existing physiological condition. The proteomes of particular organisms, even of the same species, differ for different reasons, genetic polymorphism being the most important among them. The human genome comprises $\sim 20\,000$ genes. The human proteome is much more diverse than the genome. The diversity of proteins is caused by alternative splicing, which results in that one gene encodes several different proteins, and post-translational modifications of amino acids in the proteins. The protein concentrations in the cells of one human organ can differ by more than six orders of magnitude. A very interesting object for proteomic research is blood plasma in which the dynamic range of protein concentrations exceeds 10^{10}. Albumin is the major (60%) protein of the blood plasma, its concentration being $40-50$ g litre^{-1}. Methods for the removal of more than 20 major blood plasma proteins, which totally account for 99.99% of the total plasma proteins, have now been developed and are actively used. The blood plasma proteins vary considerably in the solubility, molecular mass and other parameters. These facts call for the use of potent analytical methods for the investigation of proteomes.

Mass spectrometry is suitable for protein identification with a high degree of reliability and for protein quantification in complex protein mixtures.[46] Therefore, world proteomic laboratories are engaged in the search for approaches to apply the developed methods of proteome analysis for detecting biomarkers of various diseases, which are suitable for early disease detection, by comparing the proteomes of sick and conventionally healthy people.[47–49] The major difficulty is the detectable variability in the proteome composition caused by both adaptive changes and the molecular polymorphism of the studied subjects. These difficulties cannot be overcome without reliable methods for quantification of the protein compositions of specimens. The key identification methods of proteins and peptides using high- and low-resolution mass spectrometry are subdivided into bottom-up and top-down approaches.

The trends in the development of proteomics include both the extension of the range of proteomic research subjects and further progress of the methods aimed at increasing the information content, the sensitivity, the resolving power and reproducibility upon increase in the speed of analysis. These goals can be attained by further development of sample pretreatment, chromatographic separation and mass spectrometry methods and approaches to experimental data processing. It was shown[50] that if the peptide mass has been measured to an accuracy sufficient for its unambiguous identification among all peptides that have been predicted from the given genome sequence, the mass can serve as the biomarker for determining the parent protein. The required accuracy of measurements (ppm) is achieved by Fourier transform mass spectrometry, which comprises ion cyclotron resonance instruments, other Fourier transform instruments and time-of-flight mass spectrometry.

This enables the development of high-performance quantitative methods using gas chromatography/mass spectrometry and eliminates the long-term procedure of gaining MS/MS data. These methods are based on a two-stage approach using liquid chromatography and ion cyclotron resonance mass spectrometry.

II. Peptide identification: bottom-up approach

An important task of mass spectrometry in the proteomics is protein identification. Proteins are mainly identified based on the primary structure. After mapping of the genome, comparison of the experimental and genome-derived primary structures has become the main approach to identification. This approach is ambiguous due to alternative splicing and post-translational modifications and, hence, in detailed studies it is supplemented by *de novo* sequencing.

1. Identification using search engines and databases

The main approach to determining the primary structure using mass spectrometry is comparison of the experimental mass spectra of the products of dissociation (chemical or physical) of proteins with the *in silico* dissociation products obtained upon the appropriate software processing of amino acid sequences from proteomic databases, and thus probability judgments about the identity can be made. This

is called the bottom-up strategy. The simplest and most available approach to protein identification is the hydrolysis of the initial protein and subsequent analysis of the obtained peptides by a mass spectrometer (see Ref. 51).†

This procedure was proposed independently by five research groups, in particular, by Henzel and co-workers,[52] James' group,[53] Roepstorff's group,[54] Pappin *et al.*[55] and Yates' group.[56] The sample representing a mixture of proteins and peptides purified from nucleic acids, fats and sugars is cut by an enzyme at pre-specified amino acids (if the case in point is trypsinolysis, the peptide bonds at the lysine and arginine residues are cleaved in the protein). After enzymatic hydrolysis, the resulting peptide mixture can be analyzed using MALDI or ESI mass spectrometers coupled with a high-performance liquid chromatograph (LC-MS). Using the resulting mass spectrum of peptides, database search is performed by various search systems (see below), which compare the measured masses with the masses of theoretically hydrolyzed peptides (if the known enzyme is specified beforehand)

The search systems for the peptide mass fingerprinting (PMF) method are as follows:

Aldente (Phenyx)
— http://www.expasy.org/tools/aldente/
Mascot
— http://www.matrixscience.com/search_form_select.html
MassSearch
— http://cbrg.inf.ethz.ch/Server/MassSearch.html
Mowse
— http://srs.hgmp.mrc.ac.uk/cgi-bin/mowse
MS-Fit (Protein Prospector)
— http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm
PepMAPPER
— http://wolf.bms.umist.ac.uk/mapper/
PeptideSearch
— http://www.mann.emblheidelberg.de/GroupPages/PageLink/peptidesearchpage.html
Profound (Prowl)
— http://bioinformatics.genomicsolutions.com/service/prowl/profound.html
XProteo
— http://xproteo.com:2698/

The search system produces several possible candidates and the probabilities that exactly this protein or peptide is present in the sample. The probability is characterized by the so-called confidence index (score). Obviously, high scores can be obtained if the mass accuracy is high. Usually the score is higher if the spectrum contains numerous peaks with relatively low mass accuracy than for one or two peaks with high accuracy. This approach is usually called peptide mass fingerprinting method. Hydrolysis results in a set of peptide fragments that is unique for each protein, and their masses are used as fingerprints to identify the parent protein. The peptide mass fingerprinting method has been widely used in combination with conventional MC equipment but its productivity is limited because the method requires primary isolation of a single protein (or a simple mixture of two or three proteins in approximately equal concentrations). An advantage of this method is high speed, simplicity of interpretation of the analysis results and high sensitivity. The drawbacks include the need to have the primary sequence of the target protein in the database; the method cannot be used for complex mixtures of proteins and peptides, especially if the protein for which the cleavage products are to be identified is not the major protein.

The second method for determination of the protein primary structure is based on the information gained upon dissociation (caused by collisions with neutral atoms or molecules or other activation methods) of one or several polypeptides, which should be isolated in the first stage of mass spectrometric separation. The use for protein identification of the fragment ions of the peptides that are formed upon hydrolysis is a further development of the fingerprinting method. This approach was used by Yates and coworkers.[57] According to this methods, proteins are identified by means of tandem mass spectrometry: the search is carried out by peptide parent ion masses and the fragment ion masses. This can be done using the Mascot and XProteo systems (see above) and a number of special systems.

The search systems for the analysis of MS/MS fragmentation products are as follows:

MS-Tag (Protein Prospector)
— http://prospector.ucsf.edu/ucsfhtml4.0/mstagfd.htm
Omssa
— http://pubchem.ncbi.nlm.nih.gov/omssa/index.htm
PepFrag (Prowl)
— http://prowl.rockefeller.edu/PROWL/pepfragch.html
Phenyx
— http://www.phenyx-ms.com/index.html
Sequest
— commercial system, the web address is inaccessible
Sonar (Knexus)
— http://bioinformatics.genomicsolutions.com/service/prowl/sonar.html
X!Tandem (The GPM)
— http://thegpm.org/TANDEM/index.html

This procedure is usually combined with chromatographic separation (LC-MS/MS). In this approach, the number of input data that can be used for the search of compliance in the existing databases increases.[58, 59] This approach, which underlies the methods discussed below, considerably reduces the number of polypeptides needed for reliable identification of proteins by conventional tools. The bottom-up approach in the proteomic analysis usually provides no information about the whole protein sequence and, hence, it is incomplete. This is due to the fact that, as noted above, peptides can be derived from different forms of the parent protein encoded by one gene due to the alternative splicing and due to the possible partial degradation and various covalent modifications. Therefore, they are usually indistinguishable without additional information (such as the parent protein mass). An advantage of the method is the possibility to derive a satisfactory result from a minor set of data; the method can be easily automated for high-throughput analysis. A drawback is that in some cases, hydrolysis gives a moderate yield of peptides because some of the obtained products may be insoluble or some protein fragments may fail to be cleaved due to specific features of the tertiary or secondary structure. In addition, a large set

† See also J Cottrell *The 53rd Conference of the American Society for Mass Spectrometry, San Antonio, TX, 2005* Tutorial Lecture.

of modifications, large number of input conditions and the database size increase the retrieval time.

2. *De novo* sequencing

De novo sequencing [50] is a procedure for identification of peptides and proteins alternative to the database search of the primary sequence. There are several reasons that necessitate the use of *de novo* search, in particular, the desired protein sequence may be absent in the database (the database may be incomplete or the given protein may be new and still unknown); there are errors in determination programmes of the gene encoding this protein; the encoding gene may be subjected to alternative splicing, giving rise to new proteins; post-translational protein modifications are possible; and database search may provide ambiguous result, which may be corrected by using *de novo* sequencing.

De novo sequencing is a procedure in which the amino acid sequence is determined only based on the mass spectrum without resorting to databases. If peptide is assumed to consist of 15–20 amino acids, about 40 fragments are expected to arise upon cleavage at one type of bond without taking account of internal ions. If the mass spectrum of the fragmentation products contains few fragment peaks, the information does not suffice to reconstitute the amino acid sequence. In this case, only partial elucidation of the amino acid sequence in the peptide is possible.

3. Peptide fragmentation methods

The peptide fragmentation methods‡ using diverse procedures for exciting their internal degrees of freedom aimed at identification of the peptides and subsequent identification of proteins have been comprehensively studied and developed. Most of them were borrowed from molecular physics where they are used to study the structure and statistic properties of few-atom molecules. The most general procedures of peptide fragmentation used in FT ICR mass spectrometry are collision-induced dissociation (CID),[60] infrared multiphoton dissociation (IRMPD),[61] blackbody infrared radiative dissociation (BIRD)[62] and (used much less frequently) surface-induced dissociation (SID).[63–65]

a. Collision-induced dissociation

Among the above-listed methods, CID ensures the most complete coverage of the amino acid sequence. The practical implementation of the collision-induced dissociation depends on the mass spectrometer used. In any case, dissociation is a result of energy transfer to the vibrational degrees of freedom of ions upon collisions with residual gas molecules or atoms (for example, in the ion transport system). When the energy transferred to the ion exceeds the energy level needed for dissociation, bond cleavage takes place. Energy redistribution is faster than dissociation; therefore, the bond that is cleaved is most often the weakest bond in the peptide backbone, *i.e.*, the peptide bond. The shortcomings of this type of fragmentation are the difficulties arising in the determination of post-translational modifications and the problem of peptide bond cleavage in the regions where disulfide bridges are formed in the secondary structure.

‡ Induced decomposition of ions formed from peptides upon primary ionization is implied.

The IRMPD method does not differ, in essence, from CID but the energy transfer to the internal degrees of freedom occurs not upon collisions with inert gas molecules in the transport system, but upon absorption of IR radiation in an ion trap (the method is applicable in FT ICR and radio frequency ion traps). In both cases, the same b- and y-ions are mainly formed (Fig. 3).[66,67]

b. Dissociation upon electron capture and transfer

An alternative fragmentation method is the electron capture dissociation (ECD) based on dissociative recombination of doubly or more protonated polypeptide molecules and slow electrons. Since the discovery by McLafferty and co-workers[68] in 1998 electron capture dissociation has long been used only in the FT ICR method. The mechanism of ECD is still little studied. In general, it can be conceived as follows: the polypeptide polycation captures an electron to a high Rydberg orbit, and this is followed by a sequence of processes that ends in charge neutralization and formation of a radical, which reacts with the rest of the molecule to cause, as a rule, bond cleavage. The fragmentation depends appreciably on the type of bond. The disulfide and some other bonds dissociate with a higher probability than upon collision-induced dissociation. This backbone fragmentation method is more suitable for long polypeptides. The $N-C_\alpha$ bond is cleaved most often giving rise to N-terminal c-fragments and C-terminal z-fragments (see Fig. 3), unlike the $C(O)-N$ peptide bond cleavage in the case of CID. A specific feature of the ECD method is the fact that labile groups arising upon post-translational modifications are not split off during dissociation, as is often the case in CID and IRMPD. All these specific features of this method are associated with the lack of ergodicity of the process. It is believed that energy evolution upon recombination of ions and electrons is so fast that there is no enough time for energy redistribution between the bonds, *i.e.*, the energy evolves locally and there is enough energy to cleave a bond at the recombination site.

We have a different view on the ECD mechanism. In our opinion, the Rydberg state relaxation of an ion is accompanied by heating *via* energy transfer from the Rydberg electron to the internal degrees of freedom of the ion. This process lasts for about a microsecond, as known from Rydberg spectroscopy data for polyatomic molecules. It is obscure how protons of a multicharged ion behave in the Rydberg electron field. The orbit of high-lying Rydberg states embraces the whole ion and all of the protons interact with the Rydberg electron. Protons can migrate over the molecule, which can be interpreted as migration of radicals or recombination products. It is obvious that the ECD process duration is not several picoseconds, as was considered, but hundreds of nanoseconds. Quantum chemical approaches to analysis of Rydberg states have not been developed, and no experimental approaches are present either, except for the methods for determination of lifetimes of the states formed upon excitation of electrons present in the molecule (central states) rather than captured from the outside. At some moment of relaxation, electron transition can occur with $N-C_\alpha$ bond dissociation, thus producing the c- and z-fragments. This transition should take place during one vibration, and this part of the whole ECD may be regarded as non-adiabatic. This type of mechanism is supported by the data on the difference between ECD and ETD mass spectra (see below) and by the fact that doubly

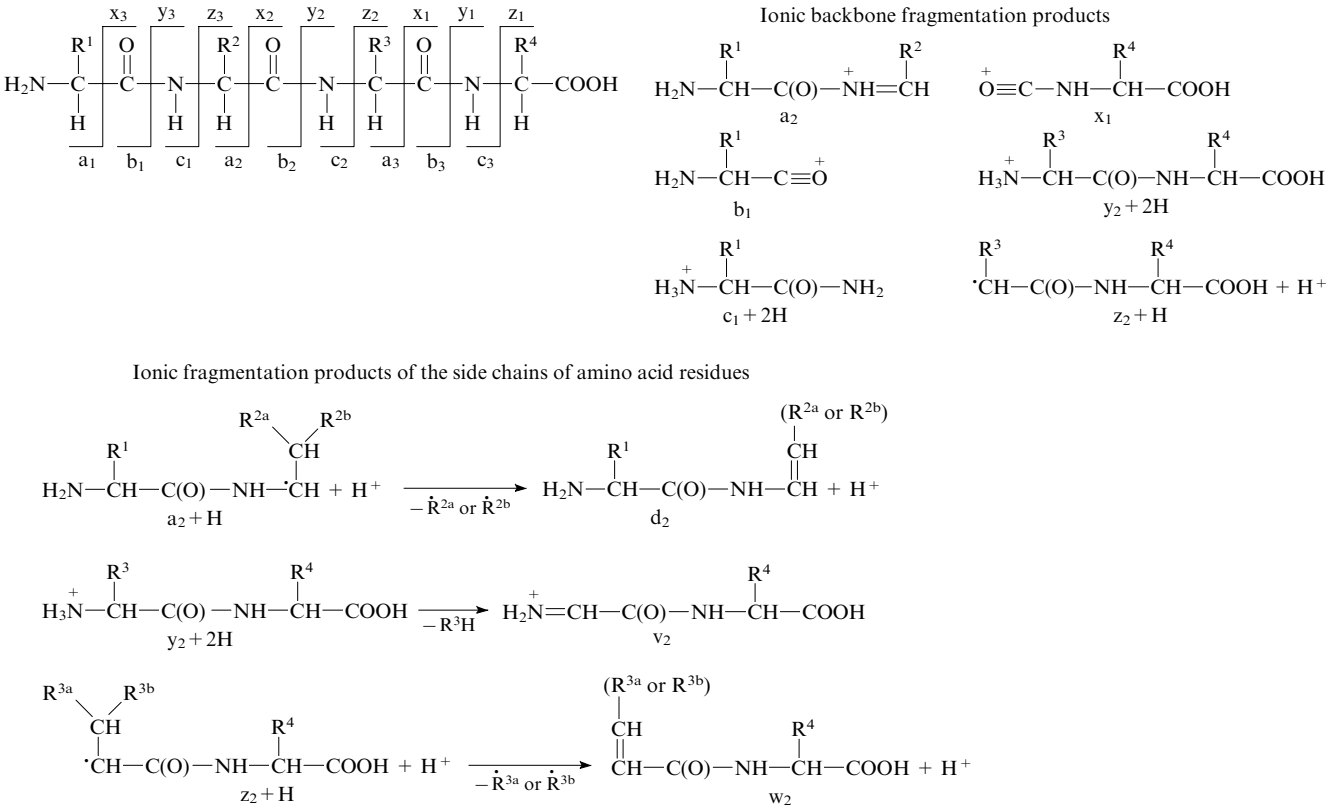


Figure 3. Generally accepted international nomenclature of fragments in mass spectrometric studies.[66, 67]

charged ions are not, as a rule, fragmented during ETD if they are not preheated by either collision or radiation.

The theory of the ECD method is unlikely to be subsequently developed, as it is being successfully displaced by a more facile electron transfer dissociation (ETD) method (see below). The ECD method, which was initially applied only to multicharged protein molecules, has been lately applied also to fragmentation of peptide ions with the charge of at least two. The capture of low-energy (< 1 eV) electrons by the $[M + n\,H]^{n+}$ ions gives rise to $[M + n\,H]^{(n-1)+}$ ions. These ions decompose faster (and more randomly) than, for example, during CID (due to recombination of low-energy electrons in various protonated sites of the peptide or protein). The unique feature of ECD that is useful for identification of proteins and peptides is that the fragments retain the post-translational modifications, which are unstable upon other fragmentation procedures, and this often precludes the unambiguous determination of the modification site (see Section II.5). Furthermore, ECD leads to cleavage of disulfide bonds. Note that measurement of the spectra of peptide fragments requires higher analyte concentration than recording the mass spectrum of the primary ions. Håkansson and coworkers[69, 70] recorded high-grade spectra for 10 nM solutions of substance P and neurotensin. However, the time required for achieving a high signal-to-noise (S/N) ratio is too long for ECD to be efficiently used together with high performance liquid chromatography (HPLC) or for high-throughput analysis. However, even with these limitations, the ECD method still can be coupled with either HPLC or capillary electrophoresis.[71, 72]

Upgrading of the design of electron sources for ECD resulted in the possibility of recording spectra of fragment ions sufficient for identification by using 10–100 ms pulses of electron current, this duration being much shorter than the retention time for a chromatographic peak. However, the sensitivity of ECD is still low; therefore, identification requires acquisition of at least 4 spectra to increase the S/N ratio to a required level. Therefore, the ECD time scale and the typical width of the chromatographic peak are of the same order of magnitude (2–4 s). Owing to its advantages, ECD can be successfully used in a number of studies such as identification of proteins and peptides according to the top-down approach (see below), *de novo* sequencing, analysis of disulfide bonds and mapping of post-translational modifications. In most cases, data on ECD fragmentation supplement the knowledge gained from other fragmentation techniques.

The major application of ECD is ion cyclotron resonance mass spectrometry where it was discovered. Numerous research groups were able to implement the method using other types of instruments, first of all, ion traps.

In recent years, considerable attention has been paid to another dissociation technique based on electron transfer to a multicharged peptide from negative ions (electron transfer dissociation, ETD), which are selected in such a way that the evolved energy is sufficient for bond cleavage. In this process, as in the ECD, the $N-C_\alpha$ bond is mainly cleaved and the c- and z-ions are formed (see Fig. 3). An advantage of this procedure is that it can be implemented in radio frequency devices such as three-dimensional ion traps and quadrupole linear traps. In the case of FT ICR mass

spectrometry, ETD allows the fragmentation procedure to be removed away from the spectrometer cell and, hence, simplifies the design and reduces the measurement time, which is important for high-throughput analysis. In all modern FT ICR instruments, collision-induced dissociation is performed outside the measuring cell, so that the resulting mass spectra of fragmentation products are similar to those recorded using more conventional instrumentation.[73]

Peptides are usually fragmented to ions of types b and y with slow multistage activation processes (all of those listed above except for ECD and ETD). The efficiency of peptide fragmentation and relative intensities of fragment ion peaks depend strongly on the amino acid sequences of peptides.

To date, most of studies dealing with FT ICR MS peptide fragmentation have been carried out in the positive ion mode; however, a lot of biologically significant proteins and peptides contain several amino acid residues with acidic properties (*e.g.* glutamic and aspartic acid residues) or post-translational modifications (*e.g.*, phosphate or sulfonate groups).[74] Such specimens are difficult to analyze in the positive ion mode (for example, at low pH of the solution). Switching to the negative ion mode may result in more intense peaks for the fragments of parent ions. Ewing and Cassady[75] were among the first to perform detailed investigations of the dissociation of negative multicharged peptides containing numerous amino acid residues with acidic properties. They used sustained off-resonance irradiation collision-induced dissociation (SORI-CID). The mass spectra of dissociation products mainly exhibited c- and y-fragment peaks although a-, b-, x- and z-fragments were also formed. A substantial part of the fragments were formed upon bond cleavage on both sides of acidic residues (aspartic, glutamic and cysteic acids) and on the C-terminus. The loss of small neutral molecules (*e.g.*, NH_3, H_2O, CO_2) and radicals (*e.g.*, CH_3) from both parent ions and fragments was also observed. For some of the studied systems, partial information about the primary structure was obtained. The measurement of one SORI-CID spectrum usually takes 2 s but even in this case, depending on the amino acid sequence, problems may arise due to low fragmentation efficiency (low signal-to-noise ratio for fragment ions) and high fragmentation selectivity (fragmentation follows only the pathway with the lowest energy of bond cleavage).[76]

The best sensitivity of the MS/MS method was achieved by Hunt's group.[77] They attained the sensitivity limit of <1 fmol by using the IRMPD MS/MS technique and demonstrated that the limit practically attainable by liquid chromatography is 5 fmol (the amount injected into the column). Futrell's and Nikolaev's groups[64,65] designed a FT ICR mass spectrometer with the option of performing SID directly in the measuring cell at high spectrum recording speed (0.3 s per spectrum), which allowed them to efficiently retain the fragment ions and to accurately measure the kinetic energy of parent ions and which had high fragmentation efficiency. This time scale makes SID a potentially useful method for high-throughput MS/MS experiments.

4. The use of various methods of peptide fragmentation for identification of aspartic acid isomers

Isomerization of aspartic acid is among the most significant modifications from the biological and medical standpoints. According to the amyloid hypothesis, modification of this type is a key molecular event leading to Alzheimer's disease. The first works on differentiating the isomerized and non-isomerized aspartic acid residues in peptides were carried out back in the late 1980s–1990s. In these studies, some indirect indications useful for identifying the protein isoform were elucidated.[78,79] The first studies demonstrated that replacement of aspartate by its β-form in small peptides induces characteristic changes in the collision-induced fragmentation spectra at high energies of the $[M+H]^+$ ions. For example, it was found that aspartate-containing proteins give rise to w_n and v_n ions (see Fig. 3), whereas the mass spectra of the peptides containing isoaspartate residues do not show such ions. The v_n ion is formed in the former case upon elimination of a neutral methyl formate molecule from the y_n ion.[66] When CID fragmentation is employed, b- and y-fragments are mainly formed at low energies, while w- or v-fragments are not formed.[80]

It was shown that when CID is used at low energies, the mass spectra of the ion fragments containing different aspartic acid isomers differ in the relative intensities of the peaks corresponding to the b_n, y_m and b_{n+1}, y_{m-1} fragments and to the aspartic acid immonium ion (m/z 88, m/z 70). A method was proposed for quantitative estimation of the relative contents of the two isoforms (aspartate and isoaspartate) in binary mixtures based on these criteria using synthetic peptides for construction of calibration curves.[81] However, further investigations demonstrated that this method is not sufficiently versatile, as the fragmentation of many peptides does not yield the corresponding ions or ion pairs, or they are formed in unreliably low amounts, because at a certain protein structure the formation of such ions may be energetically unfavourable.[82]

The most reliable way for identification of the aspartic acid isoform in a peptide molecule is detection of isoform-peculiar ions that are not formed in the case of α-analogues. Thus the high-energy CID MS/MS spectra of β-Asp-containing peptides with a basic amino acid (lysine, arginine, histidine) at the N-terminus, were found to exhibit a $[b_{n-1}+H_2O]$ ion, whereas this ion was absent in the spectra of the α-isomer.[80,83]

In subsequent studies, the $[b_{n-1}+H_2O]$ ion was shown to occur also in the low-energy CID MS/MS spectra of β-Asp-containing peptides where a different characteristic ion, $[y''_{l-n+1}-46]$ (n is the aspartate position, l is the number of amino acid residues in the peptide), was found to form with this fragmentation technique. These ions are believed to result from the internal rearrangement in the β-aspartate. Two different structures of the $[y''_{l-n+1}-46]$ ion were proposed to interpret the specificity of fragmentation. The formation of an intermediate complex containing a five-membered ring is assumed for β-Asp-containing peptides. This complex is readily converted to the $[y''_{l-n+1}-46]$ ion, which resembles the structure proposed by Carr *et al.*[66] for the v_n ion,[67] resulting from high-energy fragmentation of CID. In the case of α-aspartate, the intermediate complex includes a six-membered ring, and the subsequent rearrangement furnishes the $[y''_{l-n+1}-46]$ ion, which has a different structure and includes a three-membered ring (Fig. 4). Presumably, this structure is less preferable, which may be responsible for the absence of $[y''_{l-n+1}-46]$ ions in the spectra of peptides containing α-aspartate. However, the possibility of detecting these specific ions depends on the positions of basic amino acids in the peptide. It is assumed that the interaction between the side chains of the basic

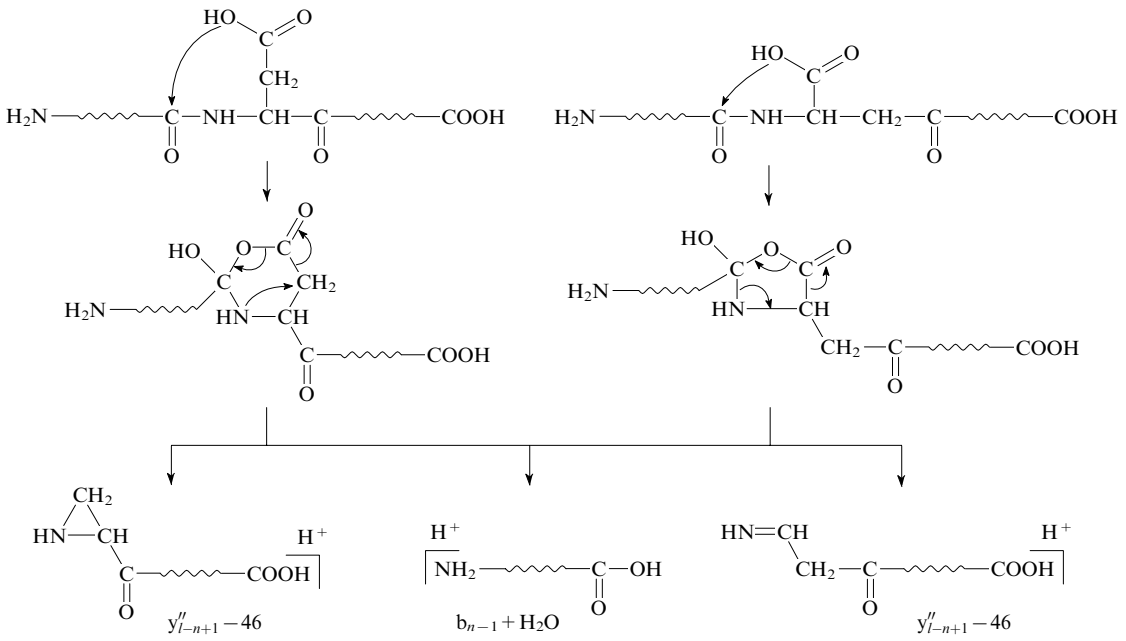


Figure 4. Scheme of formation of the $[b_{n-1}+H_2O]$ and $[y''_{l-n+1}-46]$ ions upon CID fragmentation of peptides.[82]

amino acid and β-aspartate makes the formation of $[b_{n-1}+H_2O]$ ion more preferred. However, the possibility of using these characteristic ions for quantitative analysis has not been demonstrated.

The $c^{\bullet}_n+58$ and $z_{l-n}-57$ ions, which arise upon electron capture dissociation (ECD) of isoaspartate-containing proteins, can serve as an alternative ion pair for determination of isoaspartate in proteins. The fragmentation of analogous α-Asp-containing peptides by this method is accompanied by abstraction of the aspartate side chain to give the neutral $C_2H_4O_2$ molecule and the corresponding ion (Fig. 5). Presumably, the positively charged side chain of the basic amino acid captures an electron, while the hydrogen atom thus formed is bonded to the carboxyl oxygen atom of the aspartate side chain, resulting in electron redistribution and $C_\alpha-C_\beta$ bond cleavage (see Fig. 5). Thus, the ECD fragmentation separates the side chain of aspartate, whereas in the case of isoaspartate, the peptide backbone is cleaved and the side chain is not detached.[84]

The possibility of quantitative estimation of the relative content of the normal and isomerized aspartate in proteins relying on these differences in the spectra of peptides

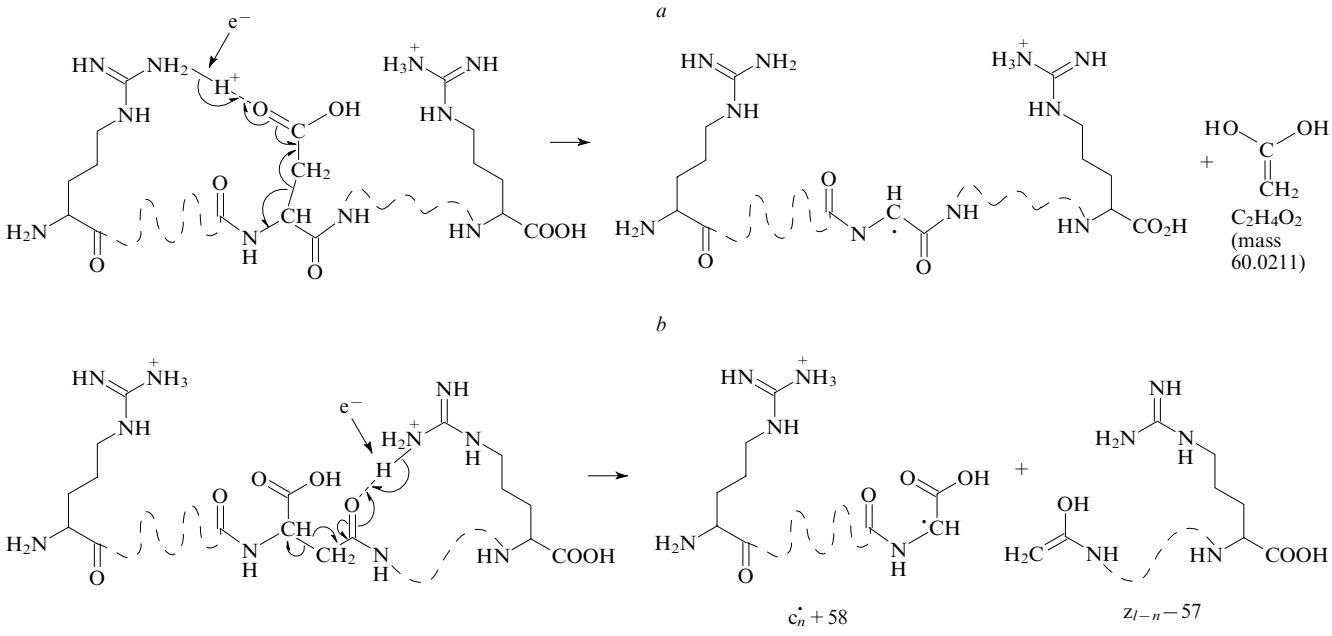


Figure 5. Presumptive mechanism of ECD fragmentation of peptides containing normal (*a*) and isomerized (*b*) aspartic acid forms.[84]

containing different isoforms was demonstrated.[85] However, no detailed analysis of the scope of applicability of this method was performed, and, hence, it is impossible to make unambiguous conclusion about its applicability to the studies of real biological specimens. All investigations were carried out at the overall content of both peptide forms of 5–10 μmol; however, since the volume of a standard blood sample is 5 ml and the β-amyloid (Aβ) concentration in the blood is about 10 nmol litre^{-1} (Refs 86 and 87), for this method to be used to analyze biological specimens, the method sensitivity should be at least 500 nmol (the standard volume of the ESI-MS sample is 100 μl). In reality, considering the unavoidable loss (up to 90%–99%) associated with peptide isolation from blood, the required sensitivity of the method should be about 5–50 nmol. Therefore, a detailed investigation is required to define the analytical potential of mass spectrometry in determining the relative amounts of Asp7 isoforms in Aβ isolated from biological specimens.

Thus, the research carried out for many years by a number of research groups revealed the marker ions for the aspartate isoforms in synthetic proteins and also showed the applicability of mass spectrometry for the quantitative estimation of the relative contents of the two forms in binary mixtures.[88, 89]

5. Identification of post-translational modifications by mass-spectrometric techniques

The biological activity of many proteins depends on the occurrence and positions of post-translational modifications. The post-translational modifications increase the ensemble of proteins by several orders of magnitude as compared with the ensemble predicted by the genome. An important and abundant post-translational modification is phosphorylation of serine, threonine and tyrosine, which regulates the cellular metabolism, growth and multiplication. Other important post-translational modifications are glycosylation, sulfonation and carboxylation (of glutamic acid). Characterization of the post-translational modifications of proteins is a challenge for proteomic methods of analysis, since the concentration of the modified proteins in complex mixtures of highly diverse modifications is usually very low. Among the multitude of modifications, phosphorylation and glycosylation of proteins that are involved in various biological processes are most important.[90–93] They cannot be observed at the transcription level and, hence, development of a method suitable for their selective separation and characterization is in high demand.[94–99] Usually, SORI-CID fragmentation of ions aimed at the subsequent identification of their primary structures results in the loss of modifications, and their spectra can be very complex.[100] The ECD method often preserves the modification, which allows one to identify its position. McLafferty, Zubarev and co-workers[101–103] studied several model phosphopeptides using SORI-CID and ECD fragmentations. As expected, SORI-CID led to the loss of H_3PO_4 and HPO_3 groups, while in the case of ECD, almost no loss of this type was observed in the spectra. Both techniques led to bond cleavage in the peptide backbone to give complementary ions, and this was used to determine the site of modification.

No successful use of chromatographic separation coupled with ECD for identification of post-translational modifications in real biological specimens has been accomplished as yet; however, apparently, the possibility of utilization of ECD for high-performance LC-FT ICR MS/MS analysis cannot be ruled out.

Flora and Muddiman[104–106] developed a method based on ESI/FT ICR MS with IRMPD fragmentation for identification of phosphopeptides in a mixture resulting from enzymatic cleavage. The fragmentation in the negative ion mode yielded fragment ions from which either H_3PO_4 (98 Da) and H_2O molecules (phosphorylated serine and threonine) or HPO_3 and H_2O molecules (phosphorylated tyrosine) were detached. At an equimolar concentration (100 nmol litre^{-1}) of the peptides HGLDNYR (M) and HGLDN(pY)R (Mp), a selective increase in the phosphopeptide signal was attained at pH 11.8 (in the presence of 20 mmol of piperidine). The average Mp to M peak ratio was from 3.28 to 0.33. The application of a similar method to β-casein tryptic hydrolysate provided a sensitivity limit of 500 pmol for phosphopeptide. The infrared laser irradiation at 11.1 W for 4 s resulted in detachment of H_3PO_4 and $H_2O + HPO_3$ from phosphorylated peptides, whereas non-modified peptides did not dissociate. This difference in the behaviour is caused by lower activation energy for decomposition of phosphorylated peptides. In the β-casein tryptic hydrolysate, various phosphorylated peptides were identified at 10 nmol level based on a single spectrum.

Zubarev and co-workers[107, 108] showed that high-energy HECD[§] is suitable for differentiation of leucine and isoleucine in a peptide sequence. For these amino acid residues, HECD fragmentation gives characteristic w-fragments formed upon elimination of C_3H_7 or C_2H_5 radicals, respectively. Twenty out of twenty-five Ile/Leu (Xle) residues were exactly identified in this way. The absence of characteristic peaks of w-fragments in the mass spectrum or their overlap with peaks of other fragments, *e.g.*, b-fragments, makes the differentiation impossible.

6. The use of accurate mass and time tags (AMT tag) for the identification of peptides

The bottom-up approach using the accurate mass tags for the identification of peptides is suitable for fast characterization of a large number of proteins directly from the cell lysate or a biological fluid (without preliminary separation by 1D or 2D electrophoresis). This approach implies the use of polypeptide accurate mass tags and is based on the assumption that the molecular masses of many peptides (including modified ones) of the protein hydrolysate are unique among all peptides from all proteins predicted based on the known genome (for rather high accuracy of mass determination). Therefore, this approach resembles the peptide mass fingerprinting method when applied to complex protein mixtures. In practice, the specificity of the method can be enhanced by using also time resolution (*e.g.*, retention time in the chromatographic column in liquid chromatography can be used). The combination of the exact peptide mass and the exact peptide retention time in the chromatographic column form a unique mass and time tag of the peptide, which identifies the peptide almost unambiguously. The algorithm of gaining and utilization of the accurate mass and time tags is shown in Fig. 6.

§ Hot ECD (HECD) differs from the conventional ECD in that the average electron energy is 3–13 eV instead of 0–1 eV.

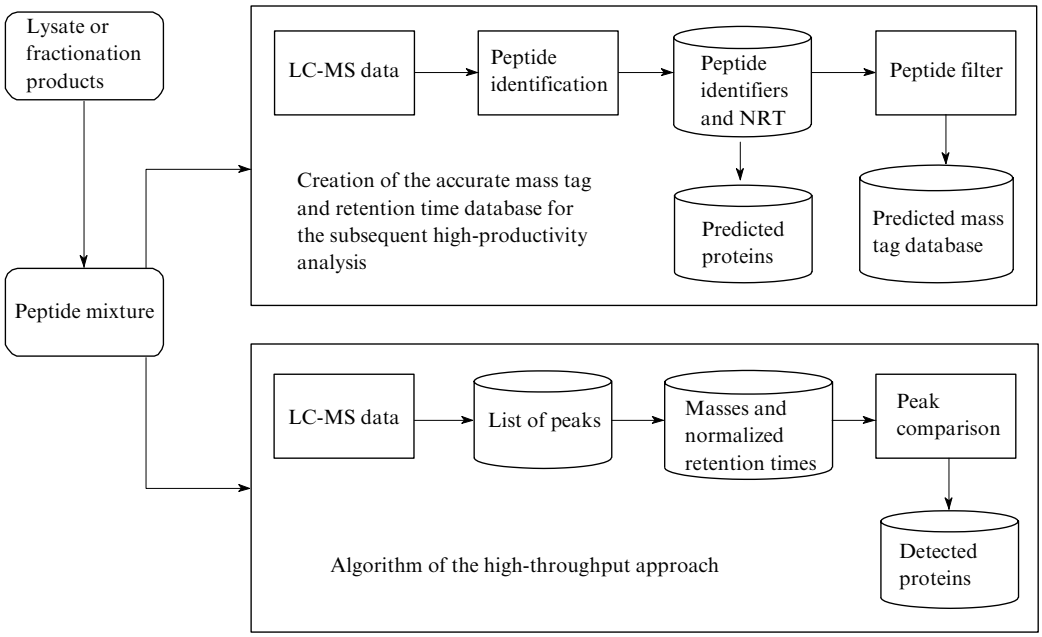


Figure 6. Chart of the algorithm of gaining and utilization of the accurate mass and time tag. NRT is the normalized retention time of the peptides in the chromatographic column.

Since the FT ICR mass spectrometry can have an accuracy of measurement of less than 1 ppm, it is among the most potent tools in this proteomic approach providing high reliability of protein identification. Owing to the enhanced sensitivity of these mass spectrometers, it is possible to detect proteins that are present in very low relative concentrations, which provides a better proteome coverage (*i.e.*, increase in the fraction of identified proteins in the proteome).

A considerable benefit of the mass and time tag procedure is the high throughput, which is achieved due to the absence of tandem mass spectrometry stage. In this approach, MS/MS is used only to construct the initial set of potential mass and time tags. These tags are accurate masses of the protein peptides identified in an MS/MS experiment, which can be performed on almost any mass spectrometer (*e.g.*, quadrupole mass spectrometer), usually with a moderate accuracy of mass determination. During the identification, the trypsin hydrolysate of a proteome protein mixture, which is a mixture of peptides, is analyzed by tandem gas chromatography/mass spectrometry. The mass spectra thus obtained are analyzed most often using MASCOT search system.[109] The masses and the chromatographic retention times of these identified peptides constitute the set of presumptive mass and time tags. The mass and time tag approach was first used in the analysis of microbial proteomes by the Smith's group[110] at the USA Pacific North-West National Laboratory (PNNL). The accurate mass and time tags serve as reliable indications of the presence of the corresponding protein in the specimen. This approach eliminates the need for further long-term measurements and, hence, the analysis productivity rate markedly increases. The critical aspect, however, is the preliminary time it takes to compile the tag database. This can also include a number of stages of preliminary separation of peptides, which differs little if at all from those used in MS/MS experiments. These stages are also needed to characterize peptides present in low concentrations that can be detected by LC-FT ICR MS experiments but not LC-MS/MS experiments (if no preliminary separation is performed). However, the result of these additional efforts is that tandem mass spectrometry is no longer needed for the subsequent analyses.[111]

The approach using accurate mass and time tags potentially requires more extensive separation of the peptides, *e.g.*, an additional chromatographic measurement (the use of multidimensional chromatography) or sample fractionation when the procedure is applied to analysis of large genomes. A version where stricter criteria are imposed on the protein identification such as the validation of the presence of a protein only provided that at least two mass and time tags have been detected. However, in most cases, apparently, it would be better to increase the accuracy of determination of the mass and chromatographic retention time using, in particular, the approaches to prediction of the retention times in the chromatographic column, which make it possible to increase the chromatogram normalization accuracy.[72, 112, 113] Since most of results of genome sequencing have not been fully announced and contain errors, numerous problems arise. For instance, as the proteome increases and if modifications are present, a mass determination accuracy even higher than that provided by FT ICR mass spectrometry is required. Currently, a mass resolving power of about 50 000 – 100 000 is used in LC-FT ICR investigations. This resolving power is sufficient almost in all cases for the separation of peptides that emerge from column in each particular instant of time. Therefore, the mass accuracy is, in principle, a more important parameter in these studies and considerable effort is being spent to overcome the obstacles that restrict the mass accuracy (for example, removal of the excessive volume charge in the measuring cell of an FT ICR spectrometer and utilization of upgraded calibration methods). Some of these problems can be solved by more efficient

preliminary separation of peptides. Actually, a large benefit of coupling FT ICR mass spectrometry with high-performance chromatographic separation is that this alleviates the requirements to resolution of mass spectra. However, in some cases, a resolving power of 1 000 000 or even more may be insufficient. For example, two isobaric peptides, RVMRGMR and RSHRGHR, were distinguished only when a 3 300 000 resolving power was attained.[114] The difference between the compositions of these peptides is that the former contains N_4O group and the latter contains S_2H_8 group (the difference is 0.00045 Da, which is less than the mass of electron 0.00055 Da). This superhigh resolving power was achieved owing to numerous factors: strong magnetic field, large diameter of the Penning trap, digital quadrature detection, axial cooling of ions by stepwise lowering of the trapping potential, low pressure in the cell, narrowband detection and co-addition of numerous spectra. Another similar experiment carried out by Marshall's group[115] demonstrated a 552 000 resolving power at high mass accuracy (0.032 and 0.053 ppm, respectively) for unambiguous identification of two peptides, ($[DY(SO_3H)MGWMDFNH_2\text{-}2\,H]_2$ and $[DY(PO_3H_2).MGWMDFNH_2\text{-}2\,H]_2$) in the broadband mode (the measured mass to charge ratio was 400 – 1400 Da). However, such approaches are not suitable for high-performance proteomic analysis. The use of 32-megabyte digitized time signal and co-addition of 64 spectra results in that acquisition of one spectrum requires 30 min, which is too long for real-time chromatography needed for the analysis of protein and peptide hydrolysates.

The accurate mass and time tag approach is used in proteomic research carried out by many research groups all over the world, in particular, in Russia.[116–134] Note that this method can be used not only in combination with FT-ICR mass spectrometers. For example, a procedure called ion mapping, which has been implemented in the Waters corporation, employs the accurate masses determined from TOF mass spectra coupled with the peptide retention times for unambiguous identification of chromatographic peaks and mass spectral peaks. If the peak positions in both sets of data strongly change, the ions corresponding to these peaks are subjected to one more MS/MS analysis.

III. Quantitative mass spectrometry in the proteomic studies

The knowledge of changes in the absolute and relative protein concentrations of a certain proteome under particular conditions is important for the understanding of the biological cycles and protein functions in these cycles. There exist several approaches used in the quantitative analysis of the proteome composition. According to a simple approach, all peptides and, hence, the proteins are detected and identified and it is determined which of the proteins are present in the mixture and which are absent. Then their amounts are compared based on the signal intensities in the mass spectra of the corresponding ions. However, in this procedure it is difficult to determine the peptide contents due to the pronounced difference in the efficiencies of their ionization and due to the ion suppression effect of peptides with respect to one another if, for example, they simultaneously emerge from the chromatographic column at comparable high concentrations.[135–138] For this reason, from the very beginning of using the bottom-up approach in proteomics, isotope labels for comparative quantitative analysis of peptides in different samples have been developed.[139, 140] Chemical labels are so designed that they do not affect the peptide ionization, mobility or chromatographic retention time. The selection of the labelling technique depends on the particular experiment and is directly related to the type of mass spectrometer to be used. After labelling, the sample mixture is analyzed on a mass spectrometer, the intensity ratios of peptide or fragment peaks are calculated and conclusions about the relative and absolute contents of the corresponding proteins in the samples are made.

In this Section, we consider the key procedures for the comparative quantitative analysis of proteomes.

1. Isotope-coded affinity tags (ICAT)

Protein hydrolysates are extremely complex mixtures comprising hundreds of peptides. A less complex fraction of the hydrolysate can be labelled and thus isolated from the main bulk. According to one frequently used approach, relatively low-abundance amino acids in peptides are labelled. This underlies the method of isotope-coded affinity tags (ICAT) in which the isotope tag is incorporated in the side chain of the cysteine residue.[141, 142] The ICAT consists of three principal elements: (i) the marker group able to react at a definite site of the peptide amino acid sequence (at the cysteine side group), (ii) reagents either enriched or not enriched in deuterium (d_0 or d_8) and (iii) the end group (biotin), which is necessary for separation of the labelled peptides by affinity chromatography. For quantitative comparison of two proteomes, one sample is labelled by the light-isotope (d_0) and the other is labelled by the heavy-isotope (d_8) ICAT reagent. By labelling the reference sample by the light isotope and the modified sample (*i.e.*, bacteria grown under different conditions) by the heavy isotope and then by mixing the samples, one can gain qualitative information about the relative concentrations of proteins in the proteomes.

Smith's group[143] attempted to apply the ICAT method in combination with the accurate mass and time tag approach to identify variations in the protein expressions in control and treated mouse cortical neurons. By combination of two methods, quantitative measurements for more than 125 proteins were performed with high accuracy and sensitivity over a broad dynamic range. The relative concentrations of the proteins present in minor quantities were determined. The greater part of the proteins involved in glycolysis and the Krebs cycle were determined. The quantitative measurements in this method are complicated due to the fact that light (ICAT-d_0) and heavy (ICAT-d_8) reagent-labelled peptides can have different retention times in a chromatographic column. In order to find the relative concentrations most accurately, it is necessary to use averaging over all spectra that comprise the peptides of interest. More recently, a similar method (cICAT) has been developed using the ^{13}C isotope instead of deuterium, so that the peptide retention times in the chromatographic column (with the C18 phase) no longer depend on whether the peptide is labelled with the light or heavy reagent.[144]

2. Peptide labelling by stable ^{13}C and ^{15}N isotopes

Peptide labelling by stable isotopes (^{13}C or ^{15}N) is one of the principal methods in quantitative proteomics.[145–147] Proteins and peptides are labelled directly in the cells when they

are grown in a special culture medium. The most successful abbreviation for this approach was proposed by Mann and co-workers,[145] namely, SILAC (stable isotope labelling with amino acids in cell culture). According to the SILAC method, one cell pool is grown in a culture medium containing intact amino acids, while the other cell pool is cultured in the medium containing amino acids labelled by stable heavy isotopes. For example, the medium can contain arginine labelled by six ^{13}C atoms. The cells that grow in this medium have heavy arginine in all of their proteins. After protein hydrolysis, all peptides containing one arginine residue will be 6 Da heavier than the non-labelled forms. For quantitative analysis, the labelled and non-labelled samples are mixed in equal ratios and pairs of chemically identical peptides are identified by a mass spectrometer. From the peak intensity ratio for these pairs of peptides in the mass spectra, it is possible to determine the concentration ratio of the corresponding proteins.

As in the cICAT method, here, it is convenient to label amino acids by ^{13}C isotope because in this case, the peptide retention time in the chromatographic column (C18) does not depend on the reagent (light or heavy) by which the peptide has been labelled. The use of this method is exemplified by the work of Smith's group[147] where they studied a complex peptide mixture obtained by total cleavage of water-soluble proteins isolated from yeast. The proteins were hydrolyzed by the endoproteinase Lys-C. The yeast was grown in an artificial medium containing normal lysine and ^{13}C-labelled lysine (6 carbon atoms). The peptides were identified by MS/MS analysis. The peptide pairs composed of labelled and non-labelled forms were identified based on the pairs of peaks with m/z difference of 6.2 (singly charged ion), 3.1 (doubly charged ions) and 2.0 Da (triply charged ion) to an accuracy of 0.1 Da. Using the SWIFT procedure,¶ a peptide pair was isolated and dissociated by means of SORI-CID excitation at a frequency corresponding to $\Delta m/z = -3$ Da for the ^{12}C peptide and $\Delta m/z = 4$ Da for ^{13}C peptide. The MS/MS spectra of the chosen pairs exhibited peaks for N- and C-terminal fragments of the peptides. Only the C-terminal fragments (y-ions) contained information about the lysine isotope, and the spectrum exhibited two sets of peaks (from the labelled and non-labelled forms), whereas for N-terminal fragments (b-ions) only one set of peaks was present (due to the non-labelled form).

The following benefits ensured by hydrolysis by the enzyme Lys-C are noteworthy: the resulting peptides are larger than in the case of trypsinolysis; therefore, ionization and fragmentation of peptides is more efficient, because numerous sites for protonation are present. In addition, since fewer peptides are formed, chromatographic separation becomes simpler and the number of MS/MS experiments is reduced. Altogether 17 857 different peptides were detected (unique mass and time tags). It was found that 48% of the total number of peptides form labelled/non-labelled pairs. Out of all pairs, 89.4% corresponded to fully cleaved (at Lys residues) peptides ($\Delta m = 6$ Da), 10.1% were peptides containing one non-cleaved Lys bond ($\Delta m = 12$ Da) and 0.5% were peptides with several non-cleaved bonds. The chromatographic retention time was found to be the same for labelled and non-labelled peptide forms. Further, the average intensity ratio for the peaks corresponding to peptide pairs with mass difference of 6 Da was 1.0 ± 0.2. Note also that ^{13}C-lysine labelling leads to easy detection of the replacement of one lysine residue by glutamine residue (K/Q) in two peptides, (NVPLYKHLADLSK and NVPLYQHLADLSK) with very similar masses ($\Delta m = 0.0036$ Da) and, hence, one can find out which protein is present in the sample (enolase 1 or 2, respectively). If the mass spectrum exhibits only a pair of peaks (labelled/non-labelled) corresponding to 6 Da difference between the masses, this implies that only one lysine is present in the peptide, and the type of protein can be exactly determined (enolase 2).

A modification of the SILAC approach similar to the ICAT method was proposed by Conrads *et al.*[148] The tag comprised ^{15}N-cysteine and a biotin derivative containing an *N*-iodoacetyl-3,6-dioxaoctane-1,8-diamine fragment interacting with ^{5}N-cysteine. The tag resembled the ICAT reagent while being less expensive. The proposed procedure was employed to isolate and measure cysteine-containing peptides from *Deinococcus radiodurans* and from B16 mouse melanoma cells. A mixture of peptides (in 1 : 1 ratio) was analyzed by LC-FT ICR MS. The high resolution of this method made it possible to identify $^{14}N/^{15}N$-peptide pairs. More than 600 pairs of cysteine-containing peptides from *D. radiodurans* samples with an average intensity ratio of 1.12 were detected. Analysis of the isotope distribution in the mass spectra of peptides from samples of B16 mouse melanoma cells demonstrated that the label has been fully introduced into the proteins grown in an ^{15}N-enriched medium. Peptide labelling did not change considerably the chromatographic retention time, which is important for quantitative analysis. It was thus demonstrated that ^{15}N-labelling can be used in investigations of mammal cell cultures.

3. Enzymatic labelling of peptides by the ^{18}O isotope

The use of ^{18}O as a label has been known since the works of Spirson and Rittenberg,[149] who studied the mechanism of inhibition of amide bond hydrolysis by chymotrypsin. They found that incubation of benzyloxycarbonylphenylalanine (inhibitor of this enzyme) with chymotrypsin in heavy water ($H_2{}^{18}O$) leads to incorporation of ^{18}O into the carboxy group of CbzPhe. Later, Mirgorodskaya *et al.*[150] adapted this method for quantitative mass spectrometry. The method is underlain by proteolysis (trypsinolysis) of two samples that is carried out in parallel in light and heavy water; the ^{18}O label replaces ^{16}O in the C-terminal carboxyl group of the peptides. The problem arising during labelling is variable inclusion of either one or two heavy oxygen atoms into one peptide.[151] This occurs because the first oxygen atom is replaced during the enzymatic reaction, while the second one can be replaced in the subsequent exchange reaction. Procedures for single or double ^{18}O-labelling were developed. In the former case, the exchange reaction is inhibited. The incorporation of the second ^{18}O atom was found to be pH-dependent. For complete double substitution, the reaction is carried out in weakly acidic medium (pH $\sim$4.5).[152] After the reaction has been stopped, the samples are mixed and analyzed. The quantitative comparison is performed for pairs of peaks

¶ Stored waveform inversed Fourier transform is a method of signal excitation in which the cyclotron motion is excited by a signal obtained by Fourier transform from a rectangular function that shows which ions are to excited and to which amplitude.

with 2 or 4 Da difference between the masses depending on the number of exchanged oxygen atoms (one or two). Owing to the uncomplicated chemistry and the lack of site specificity, the peptide labelling with ^{18}O has become a popular procedure in the quantitative proteomics. However, because of the minor mass difference between the labelled and non-labelled peptides (2 or 4 Da), this approach is used only when high resolution mass spectrometers are at hand.

As noted above, the proteolytic cleavage of proteins in the presence of $H_2{}^{18}O$ is suitable for peptide labelling to be performed simultaneously with the proteolysis. Using this method, Yao *et al.*[153] carried out comparative quantitative analysis of tryptic peptides of two viral proteins from the adenovirus serotypes Ad2 and Ad5. Separate fractions were collected by HPLC, and each fraction was investigated by MALDI/FT ICR mass spectrometry. Owing to high resolving power of this method, numerous pairs of peaks with 4 Da difference between the masses were observed in the spectrum. The average intensity ratio for the peaks of labelled and non-labelled peptides was found to be 3.3 ± 0.6, which coincides with the ratio of isotope components in the mixture (3 : 1). Although the whole dynamic range of adenovirus protein concentrations (up to 600) is easily covered by FT ICR MS, not all of the proteins were identified due to the low signal intensity for some peptides.

Subsequently, the same authors[154] proposed splitting the labelling into two stages by carrying out, first, proteolysis in usual water and then, after freeze-drying, labelling of the peptide mixture in an $H_2{}^{18}O$ buffer. The conditions of each of the two stages can be optimized independently of one another.

4. Isobaric tags for relative and absolute quantitation (iTRAQ)

The further development of the above quantitative methods of protein analysis was aimed at extension of the proteome coverage, increase in the statistic significance and rate productivity of the analysis by comparing several samples in one experiment (multiplexing) and development of methods for not only relative but also absolute quantitative analysis. These objectives are solved by the method of isobaric tags for relative and absolute quantitation (iTRAQ).[155, 156]

The reagents used for iTRAQ contain several isobaric (having the same mass) tags which consist of the reporter group (based on *N*-methylpiperazine), balance (carbonyl) group and reactive group (*N*-hydroxysuccinimide). The reactive group, which has affinity for the amino group, is attached by a covalent bond, together with the isobaric tag, to the lysine side chain or to the N-terminal group of each peptide. The balance group makes the masses of all labelled peptides equal irrespective of the reporter mass (*e.g.*, 114, 115, 116 and 117 Da for four isobaric tags). In order to analyze quantitatively four different samples in one experiment (provided that the samples contain the same peptides), each sample is labelled by a peculiar tag (with a unique reporter group). All labelled samples are mixed and analyzed by mass spectrometry. During MS/MS analysis, the fragmentation of the peptide is accompanied by elimination of the isobaric group. Upon fragmentation, the neutral balance group is lost and ions of the iTRAQ reporter groups are formed; they are responsible for diagnostic peaks in the range of 114–117 Da. The low mass region around the reporter ion peaks is free from other ions; therefore, the area under these peaks can be used to determine the relative content of the initial peptides in the sample. Usual series of peptide y- and b-ions are also present and are used for peptide identification. Thus, it is possible to quantitatively compare the concentrations of peptides in four samples in a single experiment. The information about post-translational modifications is not lost.

The iTRAQ reagents applied currently for quantitative analysis contain up to eight isobaric tags. Using these reagents, quantitative comparison of the proteomes under eight different conditions can be performed in a single LC-MS/MS experiment.[157] Note that since iTRAQ requires the use of MS/MS analysis, it is not applied in the accurate mass and time tag technique.

IV. Protein identification. Top-down approach

The bottom-up approach rarely provides information on the complete sequence of the protein and, hence, restricts the possibility to study site-specific mutations and post-translational modifications of some proteins, which can be significant for biological processes. Due to these limitations, the top-down approach in which particular proteins are analyzed with a mass spectrometer without chemical or enzymatic proteolysis has lately attracted increased attention in the proteomic research. The top-down method is based on the ionization of whole native proteins that have not been chemically treated in any way.

1. Top-down analysis

Several research groups proposed, at the same time, working with native proteins without trypsinolysis or any other treatment before ionization and injection into a mass spectrometer.[100, 158–164] According to this method, the molecules of the initial proteins are injected into a mass spectrometer where they are fragmented in the transport system, ion trap or the cell of a FT ICR spectrometer. After that, the fragment masses are measured.[165–167] The recorded mass spectra can be used for database search or for *de novo* sequencing.[50, 168]

Several procedures for the analysis of whole proteins in terms of the FT ICR method have been proposed. For instance, Kelleher's group[165] described a top-down procedure for proteome characterization making use of two-dimensional gel electrophoresis of whole proteins followed by IRMPD-MS/MS analysis by means of a FT ICR tandem quadrupole mass spectrometer.[93] By using the FT ICR MS and ECD-MS/MS techiques, Ge *et al.*[169] identified numerous covalent and non-covalent post-translational modifications of the proteins isolated from *Escherichia coli*.

The recently proposed electron transfer dissociation method for peptides (see Section II.3.b) can considerably accelerate the top-down research due to simplicity and efficiency of this dissociation technique.[170] The identification of proteins without fragmentation is still less effective than identification based on peptide analysis; however, this approach is attractive due to some possibilities that are missing when dealing with peptides. In the top-down approach, the separation of proteins before MS analysis is more complicated because they are more chemically diverse than the fragment peptides. The procedures of the proper MS analysis are not so well developed as in the bottom-up approach but it is possible to directly observe the diversity of proteins caused by post-translational modifications and

splicing. In the bottom-up approach, hydrolysis increases the system complexity 20–100-fold. The methods for peptide separation and analysis are well-developed; however, only indirect analysis of post-translational modifications and splicing versions is possible.

2. Determination of the protein mass and characterization of proteins

The protein identification by exact mass measurements was among the objectives of the research in FT ICR mass spectrometry during the last decade.

a. Mass restrictions

The greatest mass of the molecule for which the exact monoisotope mass had been measured did not change during the last 14 years. This mass belongs to chondroitinase I, a protein composed of 997 amino acids with the molecular mass of 112 508 Da (according to the measurements, 112 509 Da). When the mass of this molecule was measured, a resolving power of 170 000 was achieved.[171] The invention of the new Penning ion traps with dynamic harmonization allowed for surpassing this record by using a solenoid with a relatively low magnetic field (7 T).[18, 19, 172] The isotope resolution was thus obtained for the enolase tetramer with the molecular mass ~186 000 Da. As stronger magnetic fields are becoming accessible (now it is 15.5 T) to commercial FT-ICR mass spectrometers, it becomes more probable that this new record will be surpassed in the next years. The high resolution at the level of one atomic mass unit is useful for identification of disulfide bridges (S–S instead of 2 SH corresponds to 2 Da difference), deamination (NH_2 instead of OH implies a 1 Da mass difference) and other post-translational modifications of proteins and adduct formation. Furthermore, if ^{13}C, ^{15}N-depleted proteins are used (double depletion), even more pronounced increase in the ultimate mass, perhaps up to >400 000, may be expected.

b. Mass determination accuracy

The FT ICR mass spectrometry has a high potential for superaccurate mass determination but even after thorough calibration, pronounced errors may be present for several reasons. First of all, these errors are often caused by frequency shifts induced by the spatial charge effect, as in the analysis of light ion masses. The magnitude of this effect depends on the number of ions in the ICR cell. For quantitative estimation of this effect, Amster's group[173] used a modification of the MALDI/FT ICR method for demonstrating that there is a linear correlation between the observed frequency and the number of ions. They used the ion capture method based on the pulse gas injection into a FT-ICR cell, which decreased the ion vibration amplituide in the trapping potential, while axialization by means of quadrupole excitation suppressed the magnetron motion, and the ions were accumulated at the centre along the magnetic field axis. It was found that the frequency shift induced by the spatial charge barely depends on the ion mass distribution over the mass spectrum. This means that the spatial charge effect on the observed frequency shift is a combined effect of the total charge in the cell. Thus, high mass determination accuracy may be achieved provided that (i) the calibration curve used was derived from the mass spectrum that was recorded at the same total number of ions in the cell as in the case of real sample measurement or (ii) the number of ions is taken into account directly in the calibration procedure. Using both these methods and a PEG-3400 sample (PEG is polyethylene glycol) as the external calibrant, the authors obtained average errors of 1.6 and 2.3 ppm, respectively, for the mass of the insulin B-chain. They arrived at the conclusion that by using large-diameter cells, it is possible to reduce the spatial charge effect but simultaneously an increase in the cell diameter may have negative consequences due to non-uniformity of the magnetic field. Later, Amster's group[174] used a similar approach in order to demonstrate that the frequency shift does not change upon increase in the ion charge. By applying internal calibration against doubly and triply charged B-insulin ions, the researchers measured the mass of 8- and 13-charged ions of haemoglobin A to an accuracy of ~1 ppm, mainly owing to the fact that the peaks of the calibrant and the test compounds did not differ much in intensity, and, hence, they had similar frequency shifts. For large proteins, the use of external calibration was less successful than for peptides.

Chan *et al.*[175] recorded a broadband MALDI/FT ICR mass spectrum with a baseline resolution covering the masses from 1000 to 5700 Da. They showed that using FT ICR with a variable time gap between the ionization pulse and ion trapping in the spectrometer trap, successive injection of ions into the FT ICR cell and slow change in the trapping potential provide higher accuracy of measurement of peptide and protein masses. They used the parabolic calibration curve and the above-mentioned methods to identify bovine insulin (molecular mass of 5734 Da) in dehydrated (or deaminated) form. The attained resolving power was 14 000 (half-width at half-height).

Lee *et al.*[167] used the internal calibration for LC-FT-ICR analysis of a set of whole proteins from a yeast large ribosomal subunit. They used a dual electrospray ionization source: one electrospray capillary was connected to the chromatographic column and the other capillary was connected to a syringe pump that contained the calibrant. This procedure provided rather small errors in the mass measurement. γ-Endorphine was used as the internal standard and the $[M+2H]^{2+}$ ion (m/z 929.9664) was the reference mass for data processing.

Hannis and Muddiman[176] designed a dual electrospray ionization source for the internal calibration combined with hexapole ion accumulation. The analyte and the internal calibrant were successively electorsprayed (both ESI emitters were sequentially positioned in front of the desolvating capillary), collected, transported and trapped in a FT-ICR cell and then simultaneously detected. The application of this method for determination of the PCR (polymerase chain reaction) products with an average mass of 50 849.20 Da gave an average absolute error of 8.9 ppm when thymidine 5-phosphate $d(pT)_{18}$ oligomer was used as the internal standard. In the case of external calibration, the error was 44 ppm (5 measurements). The research along this line resulted in the design of a dual electrospray ionization source with dual-channel ion funnel in a FT ICR instrument with the internal calibration option.[133]

The internal calibration can be included in MS/MS experiments, as was demonstrated by Flora, Hannis, Muddiman[104] and Kruppa *et al.*[177] For enhancing the accuracy of the product ion mass determination, Smith and coworkers[178, 179] developed the technique DeCaL for correct-

ing the frequency shift caused by the spatial charge, which eliminated the need of internal calibration.† This calibration utilizes the multicharged states of the same neutral mass for calculating the frequency shift and for precise aligning at least two restored isotope distributions. This process is repeated for all of the charge pairs present in the mass spectrum. The final frequency shift, which is applied to all peaks in the spectrum, is based on the weighed-average frequency shift over all charge states, the signal intensity being highly important. The calibration DeCAL was also applied with success to peptides. For proteins with a multitude of possible charge states, it was demonstrated that the errors in determination of the exact molecular mass increase for lower charge states (*i.e.*, higher m/z).

Williams' group [180] demonstrated that proteins and peptides can form higher charge states if *m*-nitrobenzyl alcohol is added to the electrosprayed solution. The addition of the alcohol resulted in the shift of mass spectral peaks towards smaller m/z (the region where higher resolution is attained) due to the greater number of protons that remain on the molecule (this number dictates the value of z). The high charge states are expected to considerably reduce the errors in determination of exact molecular masses. However, the high charge density can result in peak coalescence.

High accuracy of mass determination is very important for proteome investigation. In LC-FT ICR MS studies, 30% of the observed peaks are not unambiguously resolved by using the accurate mass and time tag approach, *i.e.*, one peak corresponds to several tags. If a 1 ppm accuracy is reached in high-throughput analysis, the potential of the accurate mass tag technique will considerably increase. A key approach to increase in accuracy is calibration using constant masses (*i.e.*, internal calibrants with exactly known masses) during the recording of every mass spectrum. An accuracy of 2 ppm was achieved with the use of such calibrants.[181]

The listed approaches usually effectively operate only for model systems. When working with real specimens, one cannot reach a mass determination accuracy of 1 ppm over the whole range of masses. To reduce the error of measurements, the number of ions in the cell is decreased, which narrows down the dynamic range of the instrument.

3. Elemental composition

The knowledge of the elemental composition is very useful and, in some cases, even necessary for proteomic analysis. The works of Spengler's group [50] demonstrate that the elemental composition of relatively short peptides can be determined, most often, from exact masses with a high degree of reliability. Known elemental composition substantially simplifies the peptide sequencing procedure. The superhigh resolution and accurate measurement of the relative intensities — these are factors required for determination of the elemental composition. The potential of FT ICR mass spectrometry in the determination of the elemental composition of complex bioorganic compounds is illustrated most vividly by works of Marshall's group.[182, 183] The authors observed the isotope fine structure of ubiquitin at a resolving power of 8 000 000 and in the absence of peak coalescence. This was achieved by using a combination of several approaches: FT ICR mass spectrometry in strong magnetic fields (9.4 T), electrospray ionization, digitized heterodyne detection, minimized number of retained ions, summation of frequency signal and the use of large-diameter FT ICR cells (10.2 nm). The number of ions was controlled by SWIFT isolation of one of the charged states and by decreasing the cell trapping potential from 2 to 0.12 – 0.20 V over a period of 60 s. The use of strong magnetic fields and large-diameter FT ICR cells reduces the spatial charge and coalescence effects. The application of this strategy to the ^{13}C,^{15}N-depleted (doubly depleted) protein p16, a tumour suppressor (15.8 kDa mass), made it possible to find out from the resolved fine isotopic structure that the protein molecule contains 5 sulfur atoms. This experiment consumed only $\sim$1 μg of the protein.

The use of a new measuring trap with dynamic harmonization provides the possibility of determining the elemental composition from the fine structures of the peaks of isotope peptide clusters.[172] This trap provides fine structure resolution even for relatively low fields of the superconducting magnets of ion cyclotron resonance mass spectrometers (Fig. 7).

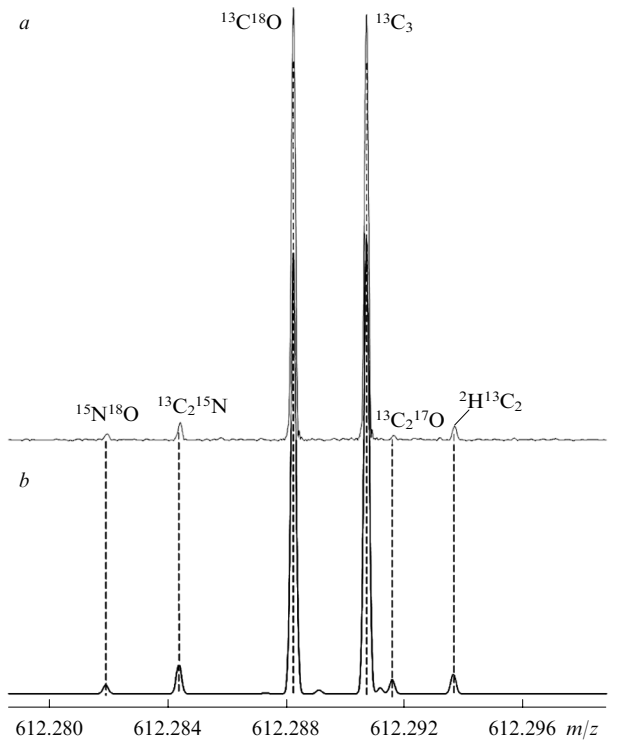


Figure 7. Isotope distribution fine structure of reserpine around the $^{13}C_3$ peak.[172]
(*a*) Experiment, (*b*) calculation

4. Detection sensitivity

The high sensitivity demonstrated by FT ICR mass spectrometry is in some sense surprising, because for a detectable signal to be generated, at least 20 ions must be present

† Deconvolution of Coulombic affected linearity (DeCal) is the transformation process of charge states with allowance for Coulombic effects under the assumption of their linearity.

in the spectrometer cell, whereas in a conventional ion trap or a time-of-flight mass spectrometer, a detectable signal can be created by a single ion. Among other factors, the high sensitivity is caused by the efficiency of using the ions in a FT-ICR spectrometer. Unlike ion traps or time-of-flight mass spectrometers, this method suits for the work with ion ensembles containing up to million ions, and hence, a much greater fraction of ions generated in the ion source in each particular moment of chromatographic separation is used for recording the mass spectra. The quality of the spectrum in the case of weak signal is also higher, as the peak intensity in isotopic clusters does not stochastically fluctuate due to a small number of ions in the ensemble, while the high resolving power creates the possibility to eliminate the chemical noise. Owing to these features, FT ICR mass spectrometry can be used to study the protein composition of cells or small volumes of biological fluids or to monitor the contents of proteins present in ultrasmall amounts in the analyzed samples.

Up to now, the minimum amount of a protein used in a top-down study by FT ICR mass spectrometry (*i.e.*, the lower detection limit for whole proteins) was 0.7–3.0 attomol for a protein of 8–29 kDa mass (McLafferty's group [184]). In this case, the samples were injected by capillary electrophoresis, and the indicated amount corresponded to the amount applied onto the column.

Baykut *et al.*[185] used MALDI / FT ICR mass spectrometry (4.7 T) with hexapole ion accumulation to record the mass spectra of samples containing substance P (> 200 attomol) with the ratio S/N = 133 at the resolving power of 300 000. They showed that the mass spectra with S/N = 16 can be recorded for 10 attomol of a compound. The use of FT ICR mass spectrometry in a 7-T field gives the mass spectrum of insulin B-chain with a resolving power of 900 000 from two laser flashes in one signal recording cycle.

Belov *et al.*,[186] who used an electrodynamic ion funnel on a FT ICR mass spectrometer with a 3.5-T field, reached the lower detection limit of 30 zeptomol when working with 8–20 kDa proteins by optimizing ionization and ion transport, retention and accumulation in the quadrupole ahead of FT ICR. The value of 30 zeptomol was based on the estimated total amount of protein used in the experiment. Probably, most of the *E. coli* proteins detected using isoelectric focusing and ion cyclotron resonance mass spectrometry by Smith's group [187–189] were present in attomol amounts. Note that the use of strong fields (*e.g.*, 12–15 T) in FT ICR mass spectrometry should considerably increase the sensitivity of the method.

For the biomedical research based on proteomic methods, procedures suitable for analyzing protein samples in amounts equivalent to their content in 1000 blood cells are required. The sensitivity and the dynamic range of proteomic analysis are especially important if the absolute amounts of the sample are limited. Smith's group developed a mass spectrometric procedure based on a nanoflow LC system, which allows identification of proteins present in complex biological mixtures in amounts of about a nanogramme.[122, 190–192] The authors of these works used a microextraction device in order to extract the peptides as fully as possible and a high-performance capillary LC column with an internal diameter of 15 μm (20 nl min^{-1}, 680 atm), which was connected to a FT ICR mass spectrometer through an electrospray emitter of 5 μm diameter. This combination was applied to identification of the proteins from samples containing totally from 50 to 0.50 ng of the protein, which is an order of magnitude lower than the amount of the protein present in 1000 blood cells. The high sensitivity and simultaneously so low consumption of the sample became possible owing to optimization of sample injection procedures, the use of a chromatographic capillary with the ultimately small internal diameter and the use of electrodynamic funnel for effective accumulation of ions and highly sensitive FT ICR mass analysis.

V. Conclusion

Among the mass spectrometric techniques available now, FT ICR provides most information for characterization of peptides and proteins. However, this method is not as routine and widely used as other mass spectrometric methods. The situation changes as FT ICR instruments with new possibilities appear and sweep into the market. For example, the use of DREAMS‡ for transport to the trap of the FT ICR spectrometer and the application of data-dependent adjustment of ion accumulation times (called automated gain control, AGC) resulted in extension of the dynamic range and mass measurement accuracy.[27] A decrease in the cooling time of ions would also increase the information part of the measuring cycle and contribute to increase in the sensitivity and the dynamic range of the method. The advent in the market of LTQ FT MS (Thermo) instrument where AGC is used in both methods (MS and MS/MS) was the key point in enhancing the analytical potential of commercial instruments. Other upgrading of the method such as enhanced identification of peptides present in low concentrations by multiplex MS/MS will increase the productivity rate and, finally, the performance of this approach. It appears probable that in the near future, FT ICR will continue to play an important role in proteomics.

Whereas the future FT ICR is associated with increase in the magnetic field of superconducting magnets used in this mass spectrometry technique, which markedly increases the instrument cost, in other mass spectrometry techniques such as orbital traps and TOF MS, further increase in the mass determination accuracy and in the resolving power is expected, while the instrument cost will be maintained at the current level, and this will undoubtedly make this class of instruments most competitive. The TOF MS method has an important advantage over FT ICR, namely, an order of magnitude higher rate of spectrum recording, which is crucial when chromatographic and capillary-electrophoretic separation methods are used. It cannot be ruled out that an instrument combining a FT ICR spectrometer, a time-of-flight mass spectrometer and an ion mobility spectrometer§ or some other new combinations will appear in the future. It is desirable that the future new automated multipurpose

‡ Dynamic range enhancement applied to mass spectrometry is a procedure for the removal of ions predominating in the mass spectrum upon their resonance excitation in the accumulation quadrupole of the ion transport system from the source to the ICR trap.

§ Ionic mobility is an analytical method in which ions are separated upon movement in an electric field in buffer gases (at a ~1 Torr pressure) according to their collision cross-sections with gas molecules.

FT ICR instrument have a control and data acquisition system to provide more effective control of ion dissociation processes and to make decisions for cases of high-throughput analysis of various specimens. Further development of the theory and technical implementation of FT ICR is the crucial factor for the future analytical use of this potent method for solving new problems.

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