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Review

Methods for samples preparation in proteomic research ☆

Anna Bodzon-Kulakowska [a], Anna Bierczynska-Krzysik [a], Tomasz Dylag [a], Anna Drabik [a,b], Piotr Suder [a,c], Marek Noga [a], Justyna Jarzebinska [a], Jerzy Silberring [a,d,*]

[a] *Department of Neurobiochemistry, Faculty of Chemistry, Jagiellonian University, Ingardena St. 3, 30-060 Krakow, Poland*
[b] *Institute of Medical Biochemistry, Medical School, Jagiellonian University, Krakow, Poland*
[c] *Regional Laboratory, Jagiellonian University, Krakow, Poland*
[d] *Centre for Polymer Chemistry, Polish Academy of Sciences, Zabrze, Poland*

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Abstract

Sample preparation is one of the most crucial processes in proteomics research. The results of the experiment depend on the condition of the starting material. Therefore, the proper experimental model and careful sample preparation is vital to obtain significant and trustworthy results, particularly in comparative proteomics, where we are usually looking for minor differences between experimental-, and control samples. In this review we discuss problems associated with general strategies of samples preparation, and experimental demands for these processes.

Keywords: Sample; Proteomics; Analysis; Proteins; Peptides; Identification; Sequence

Contents

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* Corresponding author at: Department of Neurobiochemistry, Faculty of Chemistry, Jagiellonian University, Ingardena St. 3, 30-060 Krakow, Poland. Tel.: +48 12 2927949; fax: +48 12 6340515.
E-mail address: silber@chemia.uj.edu.pl (J. Silberring).

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1. The source of samples in proteomic research

Proteomics examines all proteins expressed in a cell, tissue or organism. Proteins carry out different functions and are responsible for maintaining homeostasis in organisms. Changes in their composition could lead to pathological processes; so there is an extensive interest in applying proteomics to the identification of disease markers. Tissues, cell lines, primary cell cultures and body fluids such as plasma or cerebrospinal fluid are used as a source of proteins. Another branch of proteomics is devoted to plants, bacteria and viruses.

The results of any experiment are dependent on the condition of the starting material. Therefore, choosing the proper experimental model and preparing the sample carefully is crucial for obtaining significant and trustworthy results. Sample preparation is a matter of great importance, especially in comparative proteomics, where we are usually looking for minor differences between experimental and control samples [1].

One of the major obstacles associated with analyzing such complex material as a biological sample is the dynamic range of protein abundance. In a single cell there could be only 10 copies of transcription factor at the bottom of this range, but at the other end we may expect up to 1,000,000 copies of a more abundant protein. To deal with this problem, the most abundant proteins could be removed or the complexity of the entire sample could be reduced. Several methods of samples fractionation and techniques of proteins enrichment could be used to achieve this goal [2].

It has to be remembered that protein content in contrast to genome, which is stable and identical in all cells of one organism (apart from germ cells) is not even similar in various cell types. In fact, this difference is responsible for such great diversity of the cells. Apart from this, changes in protein composition could also occur in response to different stimuli and in different timepoints and space (cellular compartments). Thus, the aim of the experiment and an appropriate model has to be carefully considered to obtain reliable results. Below, strategies and methodologies for samples preparation used in proteomics are reviewed and their advantages and drawbacks are discussed.

1.1. Animal models

Recent advances in medical sciences would not be possible without animal research. Animal models for human diseases are indispensable in understanding the background and biology of a disease and in finding out the methods of its treatment. Mice and rats, for several reasons, are the models of choice. These animals have relatively short lifespan, which enables us to study the progression of a disease. Well-characterized strains of animals are available. Physiological processes that occur in humans are often (but not always!) similar to those in rodents. Thus, studies on the pathophysiology of various diseases at the proteome level are possible due to a relatively high similarity between the rats/mice and human proteins. Transgenic animals seem to be another great promise for obtaining appropriate and useful models [3].

It has to be remembered that these models cannot be considered as a complete equivalent of human disorders, as they present only some of their aspects. We have to be aware that, apart from similarities, some systems could be different (for example, the rat steroid system is different from the human one). Aspects of gender, weight, feeding, etc., also need to be taken into consideration before establishing a model for a particular experiment [4].

1.2. Animal tissue

When a particular animal model has been established, usually the tissue connected with the disease is chosen for detailed analysis. Every tissue has its own characteristics. For example, lipids are particularly abundant in the brain and have to be eliminated together with nucleic acids, in order to obtain results of high quality. The most common method used here is selective precipitation of the proteins with acetone and trichloroacetic acid (TCA) [1].

During tissue preparation for proteomic analysis, it is important to diminish its heterogeneity, as much as it is possible. The sample should be pure and relevant. For example, in case of cancer proteome analysis, it should be free of stroma, blood, serum, etc., and whenever possible, should represent only tumor cells [5]. When a specific part of the whole organ is isolated (for example, striatum from the brain), it is important to preserve its regional and cellular specificity and not extract too much of the surrounding tissue [1].

Fresh tissue should be freed from connective tissue and fat and preferably perfused with an ice cold saline prior to excision or at least rinsed right after it. It should be well minced with surgical scissors in the freshly prepared lysis buffer containing chaotropes, detergents, reductants and protease inhibitors. If a tissue contains a large proportion of connective tissue, it should be frozen in liquid nitrogen, ground to fine powder in a mortar and placed in the lysis buffer.

Such prepared tissue should be placed in ground glass tissue grinder and homogenized until a uniform homogenate, without any visible tissue particles is formed. Then the sample should be left for some time at room temperature and in darkness to allow each constituent of the sample to solubilize. The homogenate should then be centrifuged to remove nucleic acids and insoluble material. As a result, a clear supernatant should be obtained, followed by its division into aliquots and freezing [6].

There is also a possibility of performing some of the research on human post-mortem tissue, but apart from the ethical considerations, a question remains as to whether pathophysiological mechanism could indeed be evaluated in such material. Here, data such as age, gender, ethnicity, medical history, agonal state, post-mortem and post-autopsy intervals have to be taken into consideration. In case of brain tissue, many proteins are remarkably stable post-mortem or undergo degradation only to a minor degree; thus valid and practical measures of some of the parameters may be performed in human brain [7].

Biopsy might be another source of the tissue for proteomics analysis. It seems to reflect the state of living organism, and sometimes, collected material could be cultured for further experiments. Such samples, usually obtained during surgery, have to be frozen in liquid nitrogen and must be stored at $-80\,^{\circ}C$ prior analysis [8].

1.3. Cell cultures

Simplification of the sample is one of the greatest advantages of cell culture serving as an experimental model, especially in proteomics. Tissue samples are invariably heterogeneous and, thus, more complex. It is assumed that about 1×10^4 proteins are expressed in one cell. In a tissue composed of different types of the cells this number is much higher.

In contrast, selective pressure of the culture conditions, after one or two passages, tends to produce a homogenous culture of the most vigorous cell type. It means that this model allows for studying the behavior of a single type of cell in the absence of the complexity of the entire tissue. This may help to reveal changes in low abundance proteins, which could be impossible in the whole tissue study.

Experiments involving cell cultures very often test the influence of potential medicines or toxic substances. Owing to the homogeneity of the culture cells in each dish, they are virtually identical; therefore, examination of the influences and comparison with the control could be highly relevant and trustworthy. Reagents, to which cells are exposed, could be administered directly at a defined concentration, which is almost impossible during in vivo experiments. This technique also ensures for significant control of the environmental conditions [9].

Primary cell culture is derived either from enzymatic or mechanical dispersal of the tissue, or by outgrowth of migrating cells from a tissue fragment. Cells capable of proliferation under particular conditions, after appropriate time, reach the confluence and form a monolayer. At this stage the culture shows the closest morphological resemblance to the parent tissue. A primary cell culture becomes a cell line after the first passage.

Continuous cell line may be obtained after transformation of normal cell line either spontaneously or by chemical or viral induction. Those cells are usually aneuploid and could have unlimited culture lifespan. A number of the properties of continuous cell lines, such as reduced serum requirement and reduced density limitation of growth, are associated with malignant transformations [10].

Preparation of cell culture for 2D gel electrophoresis (2-DE), one of the major methods of proteins separation in proteomic, is not a trivial task. Witzmann et al. [11], using primary hepatocyte cell culture, showed that the recovery of the cells from monolayer cell culture by scraping, washing and centrifugal pelleting of the cells, followed by solubilization, results in the introduction of significant variability between the samples. Proteins localized in cytosolic, cytoskeletal or external compartments lost over half of their abundance during this procedure.

According to their research, direct solubilization of cells in the cell culture dish in lysis buffer, after removal of medium, is a best way of preparing the sample for proteomics analysis. During this procedure, lysis buffer should be added directly to the cell culture dish and left in the incubator for 1 h with intermittent manual agitation. After solubilization, the entire volume of liquid should be placed in a tube and sonicated. Sonication should be carried out every 15 min for 1 h and the obtained solution should be stored at −80 °C until analysis.

In samples prepared in this way, 2-DE gel analysis detected and matched an average of 1388 proteins compared to an average of 899 proteins in washed/scraped/pelleted cell sample.

It has to be remembered that limited complexity, lack of homeostatic regulation from nervous and endocrine system, loss of three-dimensional organization of the tissue and specific cell interaction characteristic of its histology may lead to some differences in cell behavior between cultured cells and their counterparts in vivo. It means that discoveries done on this kind of models demand further confirmation by referring back to the original tissue.

1.3.1. Between tissue and cell culture—laser capture microdissection

Laser capture microdissection (LCM) is a technology that permits the isolation of selected cells or groups of cells from a thin tissue section mounted on the glass slide. We can say that samples obtained by this method are somewhere between tissue and cell culture. During this process a narrow infrared laser beam is shone through a heat-sensitive transparent polymer film (thermoplastic membrane), which contacts the tissue section. Laser causes local melting of the polymer and adhesion of the selected cells to it. Cells can then be removed from the section, together with the polymer [12].

This method of reduction of the sample complexity is crucial in the analysis of heterogeneous samples such as solid tumor tissue. For the study of cancer, it is very important to isolate malignant cells away from their surroundings, including normal, inflammatory or reactive cells [13], and this method permits obtaining homogenous populations of those cells. LCM allows for microscopic verification of the material and then, for selective transfer and recovery of the cells from histological tissue sections with greater speed and precision than the manual dissection methods [14].

LCM increases anatomical specificity of the sample, but with this method only a very small amount of the sample could be obtained. One LCM experiment usually consists of 3000 laser shots, which involves approximately 15,000 epithelial cells, while for 2-DE, 100,000 or more cells are essential. So far, only the most abundant cellular proteins were detected with this method; so its sensitivity needs further improvement [1].

1.4. Body fluids

Body fluids are an important source providing vital information on the function of living organisms. For example, cerebrospinal fluid (CSF), being in a direct contact with nervous tissue, has long been considered as reflecting dynamic changes in the Central Nervous System (CNS). Body fluids such as blood, CSF and saliva are relatively easily available; thus they are commonly used for clinical diagnosis. In particular, the use of saliva does not raise any serious ethical questions and can be taken even by a non-trained personnel for fast analysis of, e.g. narcotics. Moreover, sensitivity of the nowadays methods is so high that only a small amount of these samples is required for analysis.

As they are a great potential source of diagnostic data, proper preparation of such samples for analysis is important. The first difficulty is associated with the broad dynamic range of components present in body fluids [15]. One of the major challenges is the reproducibility of the two-dimensional gel electrophoresis, which is still the main method of proteome analysis, as this procedure requires several steps including gel transfer, strict temperature control, appropriate number of replicates, selection of additives and an experienced operator. Such studies were described by Terry and Desiderio [16], who demonstrated that the analysis of human CSF by 2D gels can achieve a high level of within-sample and between-sample reproducibility.

1.4.1. Blood, serum and plasma

A simple Medline search for “serum” and “proteomics” reveals ca. 460 papers describing various approaches for the identification of blood proteins. Each strategy seems to be individually developed or modified by a particular research group.

Preparation of the blood samples differs depending on a method chosen and a purpose of the analysis. Serum is a fraction of blood, obtained after clotting and centrifugation of the whole blood, without addition of anticoagulants. Plasma is a fraction obtained after collection of blood using various coagulants. The major drawbacks of blood samples are its complexity and the presence of a huge amount of major protein “contaminants” such as albumin, immunoglobulins and haptoglobin. These proteins comprise a major fraction of the blood and therefore, special care should be taken to remove them before analysis [17].

The complexity of blood and methodological problems, with its analysis at a reproducible and reliable level, initiated a HUPO (The Human Proteome Organisation) recommendation for preparation of such samples prior to proteome analysis. HUPO-initiated a pilot phase, which evaluated advantages and limitations of many depletion, fractionation and MS (mass spectrometry) technology platforms. Reference specimens of human serum and EDTA (ethylene diamine tetraacetic acid), heparin and citrate-anticoagulated plasma were compared in laboratories. The panel recommends use of plasma instead of serum, with EDTA (or citrate) for anticoagulation. To improve resolution, sensitivity and reproducibility of peptide identifications

and protein matches, a combination of depletion, fractionation and MS/MS (tandem mass spectrometry) technologies is recommended, with explicit criteria for evaluation of spectra, use of search algorithms and integration of homologous protein matches [18].

As it was said before, abundant proteins present in a blood sample sometimes need to be removed prior further analysis. The work described by Zolotarjova et al. [19] addresses some of the potential problems in depleting proteins in typical biomarker studies. The authors conclude that significant differences were noted between the depletion techniques employed, and this should be considered based on the expectations set during experimental design. Some of those techniques are described below.

One of the strategies involves depletion of albumin on the dye-based columns or removal of immunoglobulin G (IgG) on a protein A-immobilized column [20]. Another reasonable solution to the above mentioned problems might be retentate chromatography and surface-enhanced laser desorption/ionisation (SELDI) concept. This method based on a MALDI-TOF (Matrix-assisted laser desorption/ionization – time of flight) methodology applies chips with modified surfaces. Samples are bound to the chip surface and the unbound part of the biological material is washed out. Using various surfaces, one can perform a more selective analysis, thus substantially decreasing the complexity of samples [21,22].

A method that allows for the reduction of the protein concentration range within a complex mixture, such as neat serum, through the simultaneous dilution of high-abundance proteins and the concentration of low abundance ones in a single simple step was given by Guerrier et al. [23]. This methodology utilizes solid-phase ligand libraries of large diversity. With a controlled sample-to-ligand ratio, it was possible to modulate the relative concentration of proteins such that a large number of peptides or proteins that are normally not detectable by classical analytical methods were found.

Recent developments suggest several other approaches in serum analysis, including antibody-based microarrays and other affinity-based agents such as aptamers [24]. A similar approach has been applied by Ahmed et al. for tracking of serum proteins isoforms as biomarkers of ovarian cancer [25]. Serum samples were preseparated on the Affigel Blue, prior to the IEF (isoelectric focusing) separation. The combination of stepwise IgG and albumin depletion by affinity chromatography and ultrahigh-efficiency capillary liquid chromatography separations coupled to ion trap-tandem mass spectrometry enabled identification of 2392 proteins from a single plasma sample with an estimated confidence level of >94% and an additional 2198 proteins with an estimated confidence level of 80% [26]. The authors reported that more than 80% of the observed proteins demonstrated interactions with IgG and/or albumin. This result is consistent with another report where the investigators concluded that though serum depletion of highly abundant proteins significantly increased the number of proteins identified, both the degree of sample complexity and this depletion method resulted in a non-selective loss of other proteins [27], and with that of Granger et al. [28], who found that albumin depletion removes low-abundance proteins, including cytokines. Detailed information about abundant protein depletion could be find in Section 2.3.

Application of derivatized cellulose for generating protein profiles of human serum samples was demonstrated by Feuerstein et al. [29]. The technique allows for high enrichment of sample without depletion of albumin and immunoglobulin, and sample elution prior to MS analysis.

Wasinger et al. described preseparation of human plasma samples, performed with the aid of the membrane-based preparative electrophoresis technology platform [30], which served for albumin depletion. Various anticoagulants were tested here, including EDTA, citrate and heparin. Another strategy, for comprehensive profiling of human plasma and serum proteomes, termed as protein array pixelation, was described by Tang et al. The approach consists of three sequential high-resolution protein prefractionation methods (major protein depletion, solution isoelectrofocusing and one dimensional electrophoresis (1-DE)), followed by nanocapillary reversed phase (RP) tryptic peptide separation prior to MS/MS analysis [31].

A multidimensional and non-denaturing proteome-separation procedure using microplate technology was also presented [32]. In the first dimension, the sample under study was separated into 96 fractions by size-exclusion chromatography (SEC). In the second dimension, the fractions of the first dimension were transferred by the liquid-handling device to 96 parallel anion exchange chromatography columns. In this way, the proteins were conserved in their native states and were distributed in 2400 liquid fractions. The fractions were subjected to MALDI-MS, and their tryptic digests to both MALDI- and LC–ESI-MS/MS. The method was applied to separate normal human serum proteome. Within 255 fractions exhibiting the highest protein concentrations, 742 proteins were identified by LC–ESI-MS/MS peptide sequence tags.

Peptidomics in blood is another subject of special interest, as in neuroscience, where particular peptides/neuropeptides are measured predominantly by RIA (Radio Immuno Assay) or ELISA (Enzyme-Linked ImmunoSorbent Assay). There are also several strategies that allow for such studies. One of them is peptidomics platform, coupling magnetic-based, automated solid-phase extraction of small peptides with a high-resolution MALDI-TOF mass spectrometric readout [33,34]. Another way of profiling of blood peptides was described utilizing preseparation with various physical methods, followed by Differential Peptide Display strategy for a semi-quantitative peptides, profiling [35]. A “reversed” strategy was applied by Lowenthal et al. [36] to investigate blood peptides bound to albumin. First, albumin was removed by a solid-phase affinity captured under native binding and washing conditions. Captured albumin-associated proteins and peptides were separated by gel electrophoresis and subjected to iterative MS sequencing by microcapillary reversed-phase tandem MS.

Another aspect is analysis of cell components in the body fluids. As this topic is beyond the scope of our review, we will only mention an example given by Pasini et al. [37], who described the identification of the membrane and cytosolic proteome of red blood cells. A total of 340 membrane proteins and 252 soluble

proteins were identified, validated and categorized in terms of subcellular localization, protein family and function. Splice isoforms of proteins were identified and polypeptides that migrated with anomalously high or low apparent molecular weights could be grouped into either ubiquitinylated, partially degraded and ester-linked complexes.

1.4.2. Cerebrospinal fluid

The CSF shows a similar protein content as blood plasma. The major difference is their lower concentration in CSF [38]. As the brain is in direct contact with CSF, the biochemical changes in the nervous system might be reflected in the fluid, which implies CSF analysis as a potential diagnostic tool. Sample preparation is a challenge, similar to the above described blood plasma and begins already at the moment of collection, a procedure often affected by blood contamination [39].

The difference between blood and CSF withdrawal is that the latter is done under aseptical conditions, most often without participation of laboratory personnel, and under certain pressure (surgery, etc.). Therefore, the CSF sample might be out of a strict control (e.g. temperature and time of storage), thus gaining many fluctuations in protein/peptide content.

Numerous excellent reviews on CSF analysis were recently published. Yuan and Desiderio [40] compared several sample preparation methods and also discussed an improvement in confidence level for determining differential spots in comparative proteomics. Another work [41] describes in details the procedure for CSF preparation and analysis, including sample handling, separation, analysis and data interpretation. The authors were able to identify more than 480 spots separated on the 2-D gels using MALDI-TOF and ESI (electrospray ionization) linked to nanochromatography. In general, the 2-D gels are clearer when the fluid is preseparated and more spots are detected. These authors also utilized the advantage of the different hydrophobic properties of CSF proteins, and a reversed-phase solid-phase extraction (SPE) cartridge was used to prefractionate human lumbar CSF proteins into three separate fractions prior to the two-dimensional gel electrophoresis [42]. Davidsson et al. [43] applied liquid-phase isoelectric focussing for CSF preseparation, prior to 2-DE, thus leading to the detection of low-abundant proteins. Several proteins, including cystatin C, IgM-kappa, hemopexin, acetyl-coenzyme A carboxylase-alpha and alpha-1-acid glycoprotein, were identified in prefractionated CSF but not in unfractionated CSF. Low-abundant forms of post-translationally modified proteins, e.g. alpha-1-acid glycoprotein and alpha-2-HS glycoprotein, can be enriched, and thus are better resolved and detected on the 2D gel. Liquid-phase IEF, as a prefractionation step prior to 2-DE, reduces sample complexity, facilitates detection of less abundant protein components, and increases the protein loads and the protein amount in each gel spot for MALDI-MS analysis.

Several prefractionation methods, involving ethanol, TCA, and TCA–acetone precipitation were compared to direct 2D-PAGE by Hansson et al. [44] in CSF analysis. The results suggest that, with respect to protein recovery and purification potential, ethanol precipitation was found to be most efficient.

Bearing in mind the low abundance of many CSF components, preconcentration of the fluid is often necessary. Such work has already been reported in 1960s by Kaplan and Johnstone [45], where earlier papers on this topic are also cited.

An interesting approach utilizing bottom–up proteomics and two-dimensional LC–MS/MS for the analysis of human ventricular CSF was given by Wenner et al. [46]. The neat fluid samples withdrawn from neurologically normal elderly persons were treated with trypsin, followed by C_{18} solid-phase extraction. Tryptic CSF peptides were separated by 2D-LC–MS/MS, and individual samples were compared to one another. Using this strategy, it was possible to identify 249 CSF proteins from 10 subjects. Of these proteins, 38% were unique to individual patients, whereas only 6% were common to all 10 subjects. The results clearly suggest substantial subject-to-subject variability in the CSF proteome. Another thorough analysis of individual human samples was performed by Finehout et al. [47]. The applied procedure included lumbar puncture, storage of CSF at −70 °C and ethanol precipitation of proteins from the 250 μl aliquots. The obtained 2D gels contained 600 identified spots representing 82 different proteins. Of these 82 proteins identified, 25 have not appeared in any previously published 2-DE map of CSF and 11 have not been previously reported to exist in CSF. This paper shows the potential of such approach, utilizing small CSF aliquots and simple preconcentration of the sample.

1.4.3. Saliva

Saliva is one of the most easily available human body fluid. It may be easily, safely and non-invasively collected. As a proteomic sample, saliva does not need any special preparation, like in the case of blood or serum. Usually ultrafiltration and initial purification on the RP column or microcolumn are sufficient to obtain proteins and peptides of interest. Depending on a diagnosed population and a goal of analyses, operating personnel should remember that saliva, like almost every human-derived fluid, is potentially biohazardous material and following the safety rules during the entire procedure is a must.

Saliva contains well-known proteins such as lysozyme and alpha-amylase in comparably huge amounts. Besides, it seems to contain other proteins and peptides that may be used for diagnostics of the condition of the human organism while applying fast, proteomic assays. Even based on the major proteins, applying the proteomic method such as 2D electrophoresis supported by high throughput MALDI-MS, on saliva sample shows a great complication. Hirtz et al. detected that about 140 electrophoretic spots correspond to alpha-amylase isoforms. About 90 of them correspond to full-length post-translationally modified protein; the rest are probably products of truncation of amylase before secretion [48]. To interesting proteins from the immunological point of view, we should include defensins found in this fluid. Defensins may play an important role in the protection against microorganism infections caused by food or drinks [49].

In the recent years, we observe a rapid increase of interest in saliva as a potential diagnostic fluid. On the basis of the novel techniques, scientists from the University of Minnesota created a catalog of 437 saliva proteins, which is a good reference source

for further analyses [50]. Moreover, saliva seems to be the most convenient source of markers for cancer and other diseases [51]. Nowadays, a few biomarkers of tumors have been found. One of the good examples of the usefulness of this fluid may be the salivary c-erbB-2 protein, which proved to be a reliable marker of the breast malignant cancer [52]. Other diagnostic targets using saliva as the source of markers may be the following: osteoporosis [53], preterm labor [54,55], exposure to organophosphate pesticides [56], drug testing [57,58] and various periodontal diseases [59,60].

1.4.4. Synovial fluid

This type of fluid is rather not used in proteomics. Procedure of synovial biopsy is not very difficult, but its application in detection of a disease based on the proteins and peptides content is limited. At present, synovial fluid is used in diagnostics of joint's infectious diseases after detection of bacterial DNA [61]. Sometimes, analysis of synovial fluid can be useful in sarcoidoses identification, but diagnostic success depends on the detection of CD 4(+) lymphocytes and other cells during histological investigations [62]. Synovial fluid is also useful in the diagnosis of, e.g. rheumatoid arthritis and other types of inflammatory processes [63]. So far this human-derived fluid is not involved in strictly proteomic investigations.

1.5. Plants

Characteristic property of plant cell is its cell wall, mostly made up of cellulose and its derivatives. Young plant cells are surrounded with primary cell wall; in some plants and cell types, a rigid secondary cell wall is present between the plant cell and the primary wall after the phase of development. Generally, disruption of a cell wall and protein release is crucial for analytical success. Various chemical and physical techniques are used to destroy cell wall, for example, lysing buffer, sonication, freeze–thawing and high-speed blending [64]. In particular, mature plants need special treatment. Islam et al. presented a procedure of extracting proteins from mature rice leaves for two-dimensional gel electrophoresis with superior resolution [64].

The plant cell wall is a dynamic structure and plays a key-role in the plant life cycle. About 10% of the cell wall mass consists of cell wall proteins (CWP) [65] involved in signaling, interactions with plasma membrane proteins and modifications of the cell wall components [66]. Extraction of CWP is a challenge for proteomics: to date available cell wall proteomes include only labile and loosely bound proteins [67,68] and there is no efficient procedure for the extraction of the strongly bound CWP [68].

Most of the research is conducted on Thale cress (*Arabidopsis thaliana*) and rice (*Oryza sativa*), which are the model plants of a relatively small genome, which have been sequenced for *A. thaliana* and the sequencing of the genome of rice is in progress.

Another specific feature of plant proteome analysis is the presence of non-proteinaceous contaminants specific to the plant, such as polyphenols, lipids, organic acids, terpenes or pigments [69] that can interfere with separation methods. Therefore, cleaning procedures are desirable; for instance, the most frequently used acetone or 10% trichloroacetic acid in acetone [64,70]. Islam et al. demonstrated that introduction of 10% TCA alone is not sufficient to remove contaminants and suggested TCA cleaning with sonication in the presence of glass beads, and brief grinding [64].

As in the other biological samples, variability of proteins in their p*I* range, abundance, solubility, hydrophobicity and other features mentioned above makes them difficult to separate by classical two-dimensional gel electrophoresis. An alternative separation method can be the liquid chromatography technique connected on-line to mass spectrometry (LC–MS/MS).

Typically, analysis of the protein complement proceeds through the phases of extraction, prefractionation, separation, mass spectrometry and identification [71]. General procedure for sample preparation in proteomic research strongly depends on the plant type, its fragment being analyzed (leaf, fruit, sap, etc.) or even, as mentioned above, on stage of the plant development. To show one exemplary way of handling plant sample, a protocol for sample preparation of plant material from rice embryo (*O. sativa*) and its further analysis by 2D electrophoresis are briefly presented below. This procedure is described in details by Fukuda et al. [72]. The authors applied chemical homogenization with solution consisting of urea, thiourea, Ampholine pH 3–10, CHAPS (3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate), 2-mercaptoethanol and PVP (polyvinylopolypyrrolidone), followed by boiling at 100 °C, and centrifugation. After discarding the supernatant, hexane was added to remove lipids and this step was repeated three times. Samples prepared in this way were analyzed by 2-DE.

1.6. Bacterial samples

Pathogenic bacteria are an interesting object for proteomic study in search of proteins having vaccine and diagnostic significance, determining novel targets for drug design and elucidating the cause of antibiotic or chemical resistances of these organisms.

During sample preparation, problems can arise in disrupting bacterial cells, due to the presence of thick cell walls and polysaccharide capsule in certain bacterial groups [73]. Some bacteria could simply be lysed by the constituents of the lysis buffer, but others must be disrupted mechanically (by, e.g. sonication). Sometimes, removal of the cell wall by enzymatic digestion is necessary. It has to be remembered that 2-DE analysis or another technique, used to separate and analyze bacterial proteins, will reflect the proteome of the bacteria at the time when proteins were solubilized. It means that all manipulations, such as centrifugation, may stress the bacteria and thus, influence the protein pattern [74].

Another promise of bacterial proteomics, arises from the fact that the genomes of some bacteria, e.g. *Escherichia coli*, are now determined; so the complentary proteome analysis may bring some new interesting facts concerning cellular metabolism. The relatively small size of bacterial genomes makes it likely that we obtain a complete description of the free living organism from its genes to its complementary proteins and their functions, in the next few years [73].

1.7. Viral samples

Recently, some attempts have been made to examine the proteome of viruses. Yoder et al. [75] analyzed the proteome of *Vaccinia* virus. Two fractions of viral proteins were prepared: membrane fraction containing soluble proteins and a fraction enriched with the cores and insoluble proteins. Those two fractions were prepared via treatment with detergent and centrifugation. Sixty-three different proteins were identified during this study.

Such research could be crucial for our understaining of virus biology and should help to discover new antiviral drugs and vaccines.

2. Samples preparation—a general strategy

2.1. Methods of cell disruption

Homogenization is one of the steps allowing for preparation of any biological material as a sample for the proteomic analysis. The term "homogenization" covers many meanings such as mixing, stirring, dispersing, emulsifying, but in general, it means: receiving sample of the same composition and structure in the whole volume. By applying homogenization in the procedure of sample preparation, we assume that the sample should change its physical properties without any changes in the chemistry of components.

Homogenization methods used for the proteomics purposes can be divided into five major categories:

1. mechanical;
2. ultrasonic;
3. pressure;
4. freeze–thaw;
5. osmotic and detergent lysis.

2.1.1. Mechanical homogenization

Mechanical homogenization can be realized by at least two types of devices: so-called rotor–stator homogenizers and open blade mills.

Rotor–stator homogenizers are one of the best homogenizing tools applied in the laboratories. They can homogenize samples in the volumes from 0.01 ml to about 20 l, depending on the tip and power of a motor applied. Homogenizing tips can easily be cleaned and sterilized. Use of disposable tips completely eliminates cross-contamination of the series of samples. Heat transfer to the processed mixture is low to moderate but usually needs external cooling. Sample loss is minimal in comparison to pressure processors (French presses), and very small amounts of samples can easily be homogenized.

This kind of homogenization is widely used for various tissues and cells. Depending on the chemical resistance of a cutting tool, it is possible to homogenize samples under strongly acidic or basic conditions to prevent degradation by endogenous enzymes. A good example could be the investigation of high-energy phosphates in myocardial tissue where mechanical homogenization occurs in 0.4 M perchloric acid [76] and the tissue is much too tough for ultrasonic processing. Mechanical homogenizers are also chosen for processing of hard or filamentous tissues, such as bones, teeth or cartilages [77,78].

In some cases mechanical homogenization may result in loss of the activity of the investigated material, particularly when it is heat-sensitive and the cooling during processing is ineffective. In the case of dispersing of human breast tumor tissue and calf uterus, mechanical homogenization leads to the rapid denaturation of the estrogen and progesterone receptors [79]. In this case, detergent lysis at low temperature did not lead to any significant loss of the biological activity [79].

Rotor–stator homogenizers can effectively break up animal-derived, as well as plant tissues. In the case of plant tissues, where cells are covered with strong cell walls, mechanical homogenization seems to be one of the best methods of their disruption [80]. For special applications, such as releasing chromosomes from the plant cells, mechanical homogenization can be supported by addition of a lysis buffer [81].

Using rotor–stator homogenizers, we should remember that to gain optimal results, the tissue should be precut to slices, the size of which is slightly smaller than the diameter of the applied stator. Larger pieces of the sample, especially in the case of rough or fibrous tissues, may clog generator's inlet and make effective homogenization impossible.

To homogenize dry samples using mechanical processing, open blade homogenizers, also called as blenders, are used. Rotating blades are closed in glass or metal chamber according to the safety rules. Blenders can be used to dry or liquified samples, and in case of non-satisfactory results, sample usually needs to be processed by another apparatus, e.g. ultrasonic homogenizer, to receive optimal homogeneity [82]. This observation confirms the investigations of the homogenization method of the skeletal muscles to receive high enzymatic activity in the final solution. In this case, glass–glass homogenizer seems to be much more effective than other methods (blender + detergent, blender + sonicator or blender + teflon pestle) [83].

2.1.2. Ultrasonic homogenization

Ultrasonic homogenizers, also called as disintegrators or sonificators, are based on the piezoelectric effect while generating the high energy or ultrasonic wave, interacting with the sample. Energy, resolved after explosion/implosion of gas microbubbles, effectively destroys solid particles such as cells. This method is widely used in laboratories and manufacturing practices. Ultrasonic devices are used for homogenizing, emulsifying [84], dispersing [85], extracting or suspending of the mixtures [86], and even for cleaning small metal parts in electronics [87]. For preparation of the sample, ultrasonic disintegrators are successfully used to homogenize cells, after cell culturing or after isolation from the organism. For example, after detailed studies on the usefulness of ultrasonic homogenization on the leukocytes, Fauth et al. confirmed that this type of homogenization did not affect enzymatic activity of 13 investigated enzymes [88]. However, the procedure may lead to the disruption of non-covalently bound molecular clusters (like multienzyme complexes) [89].

Ultrasonic devices are mainly used to homogenize small pieces of soft tissues (brain, blood, liver). Plant cells and microorganisms can also be effectively homogenized by ultrasonic processors. One of the examples can be isolation of the ribonucleotide reductase from *Streptomyces aeurofaciens* where ultrasonic processor was very effective towards disruption of bacteria cells [90]. Tough and dense tissues are not recommended to homogenize using this equipment.

2.1.3. Pressure homogenization

Pressure homogenizer, also called as French press, is an effective system for homogenization of eukaryotic cells as well as microorganisms in suspension.

French press is often applied for the preparation of the cell membranes for further experiments, e.g. in case of *Leptospirosis pulmonary* [91], or *E. coli* K^+/H^+ transmembrane transport system [92]. Pressure homogenization is one of the most effective homogenizing system towards microbial and plant cells in suspension and is widely used in biological and biotechnological laboratories to fast purification of the desirable proteins from culture [93]. Some important molecules, such as mRNA, cannot be obtained from the homogenate after using French press or glass–glass milling [94].

Because of the construction, this type of homogenization is ineffective towards tissues or organs without previous preparation in another type of homogenizer.

2.1.4. Freeze–thaw homogenization

This type of homogenization uses effect of ice crystals formation in the tissue during freezing process. The method is relatively fast, effective and, what is also important, does not introduce any external impurities into the sample because freezed solution is isolated from the external environment. Freeze–thaw homogenization is effective towards most of the bacterial, plant and animal cells in water solution and may be used as an additional or final step after mechanical or ultrasonic homogenization.

Some microbial cells, preconditioned in starvation mediums, are resistant to homogenization by freeze–thaw method, as in the case of *Vibrio parahaemolyticus* [95]. Another inconvenience of this method is a possibility of causing the changes in activity or properties of bioactive molecules (enzymes, membrane proteins) after a few freeze–thaw cycles. Such changes were confirmed in the case of G-protein coupled receptor kinases, β-arrestins and other proteins [96].

2.1.5. Osmotic and detergent lysis

These methods of disruption of cells utilize osmotic pressure or detergent interactions to destroy cells' walls and membranes. They are also efficient for homogenization of nuclear and mitochondrial membranes in cell extracts. Osmotic lysis is often used to disrupt blood cells. It may be useful for RNA extraction, even from bacteria like *Brucella abortus* internalized in macrophages [97], or to determine survival of *Staphylococcus aureus* after phagosytosis by human granulocytes. After phagocytosis, granulocytes were osmotically lyzed, which led to the release of *staphyllococcal* cells. Conditions for osmotic lysis were efficient only towards granulocytes, which allowed the determination of bacteria viability [98]. Depending on the conditions, osmotic lysis can be used also for microbial cell disruption. In case of *staphylococci*, after addition of lysostaphin to hypertonic solution, lysis is as effective as ultrasonic homogenization [99]. According to another report, addition of lysozyme to the buffer supports osmotic lysis of *Pseudomonas* sp. [100] and other bacteria.

Detergent lysis is used for almost every type of cells, viral envelopes and subcellular structures. The most commonly applied detergents are Triton X-100, Tween 80, Nonidet P-40 (NP 40) and saponin. Similarly, freeze–thaw and osmotic lysis process, in proper buffer conditions, causes rapid permeabilization of cell membranes and does not change the native conformation of intracellular antigens. This feature is often useful during staining of the internal proteins of the cell. Among the typical applications of detergent lysis are, e.g. release of the endogenous substances from bacterial organelles [101], protein staining after cell permeabilization [102,103] and disruption of the cells [104].

2.2. Protein solubilization

Protein solubilization process is widely quoted among the protocols of special importance applied in each proteomic sample preparation procedure. Regardless of the further separation technique, this step strongly affects quality of the final results and thus determines the success of the entire experiment.

Once isolated, proteins in their native state are often insoluble. Breaking interactions involved in protein aggregation, e.g. disulfide/hydrogen bonds, van der Waals forces, ionic and hydrophobic interactions, enables disruption of proteins into a solution of individual polypeptides and thus promotes their solubilization [105,106].

Considering the great diversity of heterogeneity of proteins and sample-source related interfering contaminants in biological extracts, simultaneous solubilization of all proteins remains a great challenge. Integration of proteins into membranes and their association, and forming complexes with other proteins or nucleic acids hamper the process significantly. Numerous attempts undertaken during the past years flourished in the development of strategies enabling the identification of the so far "unreachable" proteins, e.g. membrane [107,108], acidic/basic [109–111], high/low molecular weight [112,113] or low-abundant [114] and thus allowed for a more complete proteome analysis. Despite the progress in the field, one needs to remember that there is no single approach that may be multiplied or copied. Each sample and conditions require a unique, experimentally determined treatment. To avoid protein modifications, aggregation or precipitation resulting in the occurrence of artifacts and subsequent protein loss, sample solubilization process implicates the use of chaotropes (e.g. urea and/or thiourea), detergents (e.g. 3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS) or Triton X-100), reducing agents (dithiothreitol/dithioerythritol (DTT/DTE) or tributylphosphine (TBP)) and protease inhibitors in a sample buffer [115]. Their proper use, together with the optimized cell disruption method,

dissolution and concentration techniques determines effectiveness of solubilization [116].

2.2.1. Chaotropes

Chaotropes disrupt hydrogen bonds and hydrophilic interactions enabling proteins to unfold with all ionizable groups exposed to solution. The reagents applied within this group are not as diversified as detergents. A neutral chaotropic agent, urea, is used at high concentrations ranging from 5 to 9 M to effectively disrupt secondary protein structure. As indicated by Rabilloud et al. [117], addition of thiourea to the denaturing solution containing urea, allows for substantial improvement of protein solubility, manifested in an increased number of protein traces visualized on 2D gels. These are, however, mostly water-soluble and several transmembrane proteins [118–120]. Inclusion of thiourea to the sample buffer decreases solubilization of urea. Therefore, when combined with 2 M thiourea, urea concentration should not exceed 5–7 M [121]. While performing 2-DE, thiourea should also be included in rehydration buffer to ensure solubility of all extracted proteins. Nevertheless, because the reagent may hinder binding SDS (sodium dodecyl sulfate) to proteins, it has to be omitted in the equilibration buffers prior to the separation in the second dimension [122].

Urea and thiourea may hydrolyze to cyanate and thiocyanate, respectively. This may result in modification of proteins and hence, evokes artifactual charge heterogeneity. The process is promoted by heat; therefore, samples containing the chaotropes should not exceed temperatures higher than 37 °C. Thus, carrier ampholytes, known to act as cyanate scavengers, are often included in the urea buffer [115].

Charged chaotropic agent, guanidine hydrochloride (GdnHCl), is another choice for the extraction medium for 2-DE however, as it interferes with IEF, it needs to be removed by dialysis against urea and thiourea. This may result in the loss of some classes of proteins.

2.2.2. Detergents

Detergents and amphipathic molecules disrupt hydrophobic interactions, thus enabling protein extraction and solubilization. With respect to the ionic character of the hydrophilic group, they are classified into several groups: ionic (e.g. anionic sodium dodecyl sulfate (SDS)), non-ionic (uncharged, e.g. octyl glucoside, dodecyl maltoside and Triton X-100) or zwitterionic (having both positively and negatively charged groups with a net charge of zero, e.g. CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), tetradecanoylamidopropyl-dimethylammoniobutanesulfonate (ASB-14)). Applicable concentrations of detergents range from 1 to 4%, and the exact content of solubilization solution needs to be verified in accordance to the method of choice for protein separation (some reagents may interfere with subsequent steps).

Ionic SDS, highly efficient in solubilizing hydrophobic and membrane proteins, interferes with non-denaturing electrophoresis and isoelectric focusing step and therefore, cannot be used for 2-DE unless diluted and replaced with, e.g. CHAPS, Triton X-100 or Nonidet P-40 (NP-40) [123,124]. Otherwise horizontal streaks may appear. To solve the problem associated with the presence of SDS, zwitterionic and non-ionic detergents became widespread alternatives [114,125,126].

Uncharged detergents, mild and relatively non-denaturing such as Triton X-100, NP-40 and dodecyl maltoside were among the most widely applied in the present day proteomics, to ensure protein solubilization and prevent aggregation [127,128]. Further studies demonstrated the generally better solubilizing power of zwitterionic detergents [129,130], although Luche recently proved the non-ionic detergents, dodecyl maltoside and decaethylene glycol mono hexadecyl ether to be more efficient [126]. Also Taylor and Pfeiffer found the non-ionic *n*-dodecyl-β-D-maltoside and the zwitterionic amidosulfobetaine ASB-14 to be more effective in solubilizing myelin proteins than the commonly used zwitterionic CHAPS [131]. In turn, Babu et al. reported a distinct detergent 1,2-diheptanoyl-*sn*-glycero-3-phosphatidyl choline (DHPC), to be even more potent in solubilizing integral membrane proteins than sulfobetaines or ASB-14 [132]. The non-charged reagent, stable in a wide pH range, may be useful in generating proteomic maps for most complex organelles including sarco(endo)plasmic reticulum.

The advantage of zwitterionic detergents is that they combine properties of detergents of other classes enabling efficient disruption of protein aggregates. Offering the low-denaturing and net-zero charge characteristics of non-ionic detergents, zwitterions also efficiently disrupt protein aggregation. Although Triton X-100 and NP-40 were less effective in solubilizing very hydrophobic proteins, zwitterions and sulfobetaines substituted them successfully [133,134], allowing combination with urea/thiourea and solubilization of membrane—but not integral proteins. The sulfobetaine CHAPS is most commonly applied in proteomic studies nowadays due to its high solubility and a relative lack of detergent-induced artifacts. Its concentration ranges between 2 and 8% in 8 M urea. Conventionally for 2-DE, 4% CHAPS is used. Other alkylsulfobetaines such as *N*-decyl-*N*-*N*′-dimethyl-3-ammonio-1-propane sulfonate (SB 3-10) have also been applied, however, these are relatively insoluble in high concentrations of urea [135]. On the contrary, 4-octylbenzol amidosulfobetaine and ASB-14 are well compatible with 7 M urea and thiourea and are reported to have superior properties [136].

Combining various detergents and chaotropes may also be beneficial. Chevallet et al. obtained best results with a denaturing solution containing urea, thiourea and synthesized zwitterionic detergents and amidosulfobetaines with an alkyl tail containing 14–16 carbons [129]. The amidosulfobetaine type ASB-14 and mixed alkyl-akryl tail C8ø allowed solubilization of multiple membrane, transmembrane and hydrophobic proteins from *A. thaliana*. Moreover, the reagents used for fractionation of membrane proteins followed by 2-DE and combined with 7 M urea and 2.5 M thiourea, allowed solubilization of integral membrane proteins of *E. coli* and *A. thaliana* by Santoni et al. [137] and Moloy et al. [138].

Solubilization of the proteins associated with membranes, existing in close contact with membrane lipids and forming membrane complexes, constitutes the greatest challenge for the researchers nowadays. Although the number of identified mem-

brane proteins is still minor, in comparison to water-soluble ones, the growing interest in the field and development of new detergents are promising for further research. Till date, there are several membrane complexes purified and crystallized. The detailed list of those has been presented by Kashino in his review, including the detergents used for protein solubilization [139]. The author points out that the choice of not only detergents but also their concentrations is significant. The paper describes the advantages, limitations and applications of SDS, CHAPS, Triton X-100, Tween 20, *n*-octyl-β-D-glucoside (OG), *n*-dodecyl-β-D-maltoside (DDM), and *n*-heptyl-β-D-thioglucoside (HTG), to name a few.

Hydrophobic proteins are not recovered with the use of CHAPS; therefore, other detergents need to be applied in 2D gels. An excellent comparison of various reagents used for separation of hydrophobic proteins from extrinsic ones (Triton X-100, Triton X-114, carbonate, chloroform/methanol), and the efficiency of new zwitterionic detergents (øC5-øC8, C8ø, ASB14) was reported by Santoni et al. [140]. In another report, this group demonstrates the advantages of membrane washing and the use of zwitterionic detergent C8ø against Triton X-114 fractionation combined with CHAPS solubilization [137]. Successful fractionation and improved recovery of hydrophobic proteins on gels are also reported after the protein pretreatment with alkaline solution containing sodium carbonate or Triton X-100/KBR [138,141].

Blue Native (BN) polyacrylamide gel electrophoresis developed by Schagger and von Jagov [142], based on the introduction of Coomassie dyes to induce a charge shift on the proteins, employed a combination of a mild detergent, lauryl maltoside, and a zwitterionic salt aminocaproic acid to improve solubilization and isolation of membrane complexes greater than 1 MDa under non-denaturing conditions. The approach was often implicated in proteomic research [143]. Henderson et al. successfully extended the technique to the analysis of pyruvate dehydrogenase complex [144]. As to the applied detergents, modifications considered replacing laurylmaltoside with Triton X-100, allowing solubilization of the membrane-bound complex without its dissociation and the use of lower concentration of aminocaproic acid. Triton X-100 and sodium deoxycholate (DOC) were previously successfully utilized in the native electrophoresis of hydrophobic proteins. The choice of detergents for BN electrophoresis is still limited as compared to IEF [145]. Detergents used for solubilization of membrane proteins include mainly *n*-dodecyl-maltoside, Triton X-100 and digitonin, used for, e.g. analysis of mitochondrial respiratory complexes. Eubel et al. [146,147] cite also other detergents suitable for the native solubilization of proteins, e.g. octyl glucoside, Brij 96, saponin, Big CHAPS, C12E5/8, *n*-decanoylsucrose and NP-40. Digitonin proved to be a very suitable detergent for the solubilization and stabilization of supercomplexes of *Arabidopsis* mitochondria. In combination with BN-PAGE, nine photosystem supercomplexes were resolved by Heinemeyer et al. [148] and incubation of membranes with sublytic amounts of digitonin improved separation of plasma membranes from other membranes [149]. Finally, protein separation using the cationic detergent cetyl trimethyl ammonium bromide (CTAB) and SDS in second dimension, performed by Navarre et al. [150], resulted in identification of intrinsic plasma membrane proteins from 1 to 12 transmembrane domains and positive GRAVY value. Another cationic detergent, benzyl hexadecyl ammonium chloride (16-BAC) in subsequent combination with SDS-PAGE, the system introduced by MacFarlane [151], was employed in numerous researches [107,108,152,153], improving separation, resolution and identification of integral membrane and basic proteins.

Regardless of recent advances and development of new detergents, there is still no simple procedure allowing simultaneous solubilization of the complex set of soluble, and membrane proteins. The most frequent combinations of reagents for protein solubilization are presented by Govorun and Archakov [154]. Seddon et al. [155] in turn, focus on the relevant molecular properties of detergents and lipids, and summarize different reconstitution and solubilization methods of membrane proteins, implicating the strengths and weaknesses of the chosen reagents. For more detailed information on basic aspects of detergent physical chemistry, see the review by Garavito and Ferguson-Miller [156].

Sonication may accelerate the protein solubilization process, which usually requires several hours. In order not to overheat the sample and prevent protein degradation and modifications in solutions containing urea, the burst should not last more than few seconds.

2.2.3. Reductants

Reductants disrupt disulfide bonds between cysteine residues, thus, promote unfolding of proteins and enable analysis of single subunits of proteins. Conventionally, sulfhydryl reducing agents: dithothreitol (DTT), dithioerythritol (DTE) are applied in the sample preparation protocol. DTT and DTE are used at concentrations ranging from 20 to 100 mM. The free-thiol-containing regents, week acids, are charged particularly at alkaline pH, therefore may migrate out of the pH gradient while performing isoelectric focusing. This results in the decrease in reductant's concentration and may cause reoxidation of sulfhydryl groups and loss of solubility for certain proteins [121].

More recently, phosphines, e.g. tributhylphosphine and tris-carboxyethylphosphine (TCEP) in concentration of 2 mM were introduced as remedies for the problems associated with the use of thiol reagents. Application of non-charged phosphines benefits when alkaline gradient is performed. The reagent significantly increases solubilization of proteins during IEF, including keratins, and unlike DTT, does not interact with the alkylating substrates such as 4-vinylpyridine and acrylamide [157]. Thus, reduction and alkylation may be performed in a single step. The reagents are discussed in details by Govorun and Archakov [154] and Herbert [121]. Previously, β-mercaptoethanol was used, however it needs to be used at higher concentrations and can produce artifacts [158].

2.2.4. Protection from proteolysis

Proteases regulate many essential biological functions including influencing physiological processes, cell cycle and apoptosis, to name a few. According to Rawlings et al. [159]

there are nearly 700 proteases and their homologs defined in the human genome that may be classified to one of the metallo-, serine-, cysteine-, or aspartyl proteases groups. If not inhibited, liberated/activated endogenous proteases during cell membrane disruption, are responsible for uncontrolled enzymatic proteins degradation. Such proteolysis may produce artifacts and hence complicate further analysis.

Olivieri et al. [160] showed major differences in 2D patterns of red blood cell membranes, with and without application of protease inhibitors. Substantial proteolytic action in untreated cells resulted in poor recovery of high molecular weight proteins and the peptide mixture barely extended a molecular mass of 50 kDa. As the problem arises in early stage of sample preparation, it concerns not only 2-DE but also other techniques involved in proteome analysis. Denaturants employed while performing sample preparation, tend to inhibit majority but the most resistant protease. Proteases, however, are more resistant to denaturation than most other proteins.

Protein degradation may be minimized by quick and small-scale tissue extraction [161], boiling the sample in SDS buffer with the high-pH Tris-base, or, on the contrary, lowering the pH and performing ice-cold precipitation in, e.g. 20% trichloroacetic acid. Alternatively, denaturation in boiling in water [162], focused microwave irradiation [163] and the use of organic solvents [164] may be applied to inhibit proteases activity as described by Ivanov and Yatskin [165]. While active in high concentration of urea, proteases may effectively be inhibited by addition of thiourea to a lysis solution. Moreover, concentration-dependent efficiency of thiourea in inhibition of the proteolysis of sensitive substrates and solubilizing proteins was underlined by Castellanos-Serra and Paz-Lago [166]. In another experiment, heat shock proteins (sHsps), were found to protect proteins in vitro from proteolytic degradation [167].

In general, addition of specific protease inhibitors (e.g. phenylmethylsulfonyl fluoride (PMSF), aminoethyl benzylsulfonyl fluoride (AEBSF), ethylene diamine tetraacetic acid (EDTA), pepstatin, benzamidine, leupeptin, aprotinin) or cocktails with a broader activity spectrum, is recommended during cell disruption and subsequent preparation [168–171].

As observed by Finnie and Svensson [172], protein degradation, minor during protein extraction, was considerably increased when isoelectric focusing (IEF) separation was performed. In this case proteolysis may be almost completely prevented by using cup loading to apply proteins to the IEF strip and inclusion of protease inhibitors in the IEF reswelling buffer. Protease inhibitors should be applied with precaution, as it was reported that they may modify proteins, introduce charge trains and adducts, and hence interfere with further peptide studies [173,174]. A variety of the most commonly applied protease inhibitors in 2-DE, including their advantages and limitations, have been described in details in [116].

For liquid chromatographic separation, protease inhibitors are advised to be added both in binding and elution buffers, maintained at 0–4 °C. Oh-Ishi and Maeda suggest GdnHCl as the efficient reagent for inhibiting protease activity and the endogenous proteases in cells [175].

2.3. Removal of contaminants

The pH and ionic strength of sample solutions considerably influence protein solubility. Therefore, buffers, salts and detergents are included in sample solutions. They often tend to interfere with further protein separation steps, inhibit the digestion process, collide with the mass spectrometry analysis, or complicate data analysis significantly, thus need to be removed at a proper time of analysis. An excellent review on sample preparation for peptides and proteins in biological matrices was recently presented by Visser et al. [176].

2.3.1. Salts

Salts naturally occur in body fluids such as plasma, cerebrospinal fluid and urine, or may be added into the sample buffer to prevent protein precipitation. Salts migrate away from proteins during isoelectric focusing, thus contributing to their precipitation and aggregation. Moreover, a high electrical current carried by the salt load, interferes with electrophoretic separation of proteins and reduces the efficiency of 2-DE [177]. Hence, if present in concentrations >100 mM, salts should be removed prior to IEF. Cup loading tolerates a slightly higher salt concentration [115]. It is also possible to dilute sample below the critical concentration and apply larger sample volume on the immobilized pH gradient (IPG) gel. Sample dilution is also advised prior to capillary electrophoresis (CE), provided that proteins of interest are present at detectable concentrations [178].

Most often, salt removal is being accomplished via (spin, micro) dialysis [179,180], ultrafiltration [181,182], gel filtration, precipitation with TCA or organic solvents [161] and solid-phase extraction. Other alternative is the use of commercially available clean-up kits [183].

Dialysis is an effective method enabling extraction of peptides/proteins from biological matrices. This procedure has however, some drawbacks: is time consuming, difficult to automate, requires large volumes of solutions, and may result in sample degradation or loss. Dialysis is usually followed by protein precipitation or concentration in vacuum. Spin dialysis is faster, no extra sample volume is needed, but also protein loss may occur due to the adsorption on a dialysis membrane. The technique should be applied before urea and detergents are added to sample solution.

Microdialysis, based on size and shape differences, is suitable for smaller sample amounts with lower dialysis flow-rates. Unlike dialysis, microdialysis may also be performed in vivo [184,185]. Samples obtained using the method are, however, diluted and sample recovery is about 100-fold lower than achieved with solid-phase extraction [176]. As a rule, the higher mass of the component, the lower recovery through microdialysis membrane. The advantage of the technique might be a possibility of its on-line coupling to LC [186–188].

Ultrafiltration, similarly to dialysis, implicates the use of membrane, but here it plays a role of a sieving device and not a barrier between two liquid phases with different characteristics. The multi-step operation is efficient for sample concentration and purification [189], but is difficult to automate. Ultrafiltration

is considered superior in protein recovery to common precipitation techniques [190] or dialysis [191].

Gel filtration, usually carried out with the use of Sephadex [192], is also acceptable as a salt removal method with efficiency comparable to ultrafiltration and better than precipitation [190]. It was also reported that it may not achieve sufficient desalting for mass spectrometry purposes, and the procedure results in an excessive sample dilution [193].

Protein precipitation followed by resuspension in sample solution belongs to the most commonly applied procedures enabling removal of contaminants such as salts, lipids, polysaccharides, detergents, nucleic acids, etc., that may interfere with further analytical steps. There is currently no method that would allow precipitating all proteins and, consequently, only precipitated proteins can be further resolubilized. Therefore, precipitation should be avoided when screening for a complete proteome is required. Most commonly, precipitation with TCA, acetone, chloroform/methanol, ammonium sulfate or combinations of the above are being performed. TCA/acetone precipitation method, the most popular for 2-DE, is more effective than any of the reagents used alone. Precipitation with acetone (75% final concentration) is less powerful, but enables easier protein resuspension. For overviews on precipitation methods, including general procedures and limitations the references [176,181,194] are recommended.

Solid-phase extraction is a fast, versatile, easy to use, and easy to automate sample preparation technique [176]. It enables both concentration and purification of the sample. Most often, the technique bases on the reversed-phase separation mechanism employing C_{18} resin. Solid-phase filled tips may also serve as miniature chromatography columns for microscale solid-phase extraction (μSPE), and are widely applied in proteomic research to desalt, concentrate, and fractionate peptides and proteins [110,195,196]. Prior to SPE, centrifugation, filtration or precipitation are advised to be performed, in order to remove the contaminants that may block the cartridge during extraction.

Several interesting reports on comparison and evaluation of various desalting techniques have been published recently. Yuana and Desiderio [177] tested the mentioned desalting methods. Smaller proteins loss was reported while performing ultrafiltration, mainly due to the adsorption on a filter, as compared with dialysis. Column salt removal method enabled the highest protein recovery. Alternatively, commercially available microcentrifuge filtration devices (spin filters) can be applied to wash away contaminating species and to resuspend proteins in buffers compatible with digestion [197]. Lazar et al. evaluated centrifugal filters, reversed-phase high-performance liquid chromatography (HPLC), and size-exclusion HPLC. The latter, using aqueous acetonitrile as the mobile phase directly coupled to ESI-MS, provided the best performance [198].

The efficiency of four other desalting procedures (desalting column packed with Sephadex G-100, on-target washing, centrifugal filter devices and microcolumns C_{18}) was carried out by Salplachta et al. [193]. For intact proteins, the experiments showed that the best desalting procedure was the application of microcolumns C_{18}, pipette tips and centrifugal filter devices. Moreover, Joo et al. [199] developed a method for extraction of proteins from human body fluids (plasma, urine, amniotic fluid and tears) implicating the use of centrifugal filter device and the sample buffer containing CHAPS for efficient lipid and salts removal. A wide range of techniques used for salt removal including fast-protein liquid chromatography (FPLC), desalting columns, SPE, ultrafiltration or dialysis was also proposed by Visser et al. [176].

Finally, Chambers reported the automated, high throughput use of nickel and glutathione discs for protein purification [200].

2.3.2. Detergents

Most common detergent removal methods include dialysis, gel filtration chromatography, hydrophobic adsorption chromatography and protein precipitation. For detergents with high critical micelle concentration (CMC) and/or small aggregation numbers, dialysis is usually the preferred choice. Detergents can be extracted against a detergent-free buffer in about 200 excess over a period of days. For a wider spectrum of detergents present in the sample, gel filtration can be applied. This results, however in a considerable sample dilution. In turn, ion-exchange chromatography effectively excludes non-ionic and zwitterionic detergents, although Zischka et al. reported its successful application also for SDS removal [201].

Furthermore, SDS can be removed with nanoscale hydrophilic phase chromatography [202] or acetone precipitation. When carried out at −20 °C, the process is more effective than at room temperature. Finally, Dong et al. developed ceramic hydroxyapatite (HAP) chromatography for the complete removal of SDS bound to soluble or membrane proteins [203].

As to the zwittergents removal, Hannam et al. reported equal efficacy of gel filtration chromatography and a detergent affinity bead chromatography column, lightly dominating over capabilities of dialysis. Also SPE was found efficient in CHAPS removal from dilute protein solutions, offering significant advantages over standard dialysis or gel filtration methods [204,205]. Several of the above methods including nickel columns and His tags were reviewed by Seddon et al. [155] in details.

Moreover, there are commercially available kits, e.g. detergent precipitation reagents or gels effective for binding and removal milligram quantities of various detergents from protein solutions (e.g. Extracti-Gel® D Detergent Removing Gel and the SDS-Out™ SDS Precipitation Reagent and Kit, Pierce) [206]. Hydrophobic adsorption employing the use of insoluble resin (e.g. CALBIOSORB™, Calbiochem) can also be used to remove excess detergent.

2.3.3. Abundant proteins

Protein concentration in biological samples may vary even more than 10 orders of magnitude [207]. Proteomic analyses are, hence, more complicated and detection of least abundant proteins is hampered by those molecules present at higher concentration. Inter alias, high-abundant proteins prevent optimal focusing, limit loading capacity of low-abundant species and tend to mask considerable areas on the 2-DE gels. Plasma, serum and CSF, protein sources of great importance to biomedicine, diagnostics and therapeutics (see Section 1.4), contain up to

90% of highly abundant proteins such as albumin, immunoglobulins (IgG and IgA), antitrypsin, transferrin, transthyretin, α1-antitrypsin, hemopexin or haptoglobin. Removal of those proteins may increase detection of other molecules present at low concentrations, however, it may also result in a loss of other proteins, hindering identification of holistic alterations in the analyzed proteomes [208].

Various strategies have been presented for the removal of high-abundant proteins [209], most of which base on affinity chromatography employing dye-ligands, their derivatives [210,211], mimetic ligands [212,213], proteins A and G [214], and antibodies (immunoaffinity depletion) [215]. Cibacron Blue columns and their derivatives are commonly used to bind albumin whereas immunoglobins are excluded based on their interactions between with proteins G and A [216,217].

Other dye ligands include Procion Red He3B, Reactive Blue MRB, Reactive Green H4G, Reactive Green HE4BD, Reactive Yellow M8G and Reactive Brown M4R all of which can be coupled to solid supports [5]. Application of the dye-employing methods is, however, limited due to the fact that the dyes and high-abundant proteins themselves tend to bind low molecular weight proteins, lipoproteins, and enzymes present in a sample [177,191]. Hence, removal of high-abundant proteins results also in non-specific loss of other species. This effect is called "albumin sponge effect" and sometimes could be prevented by sample dilution with a buffer containing acetonitryle [218,219]. Approaches based on ultrafiltration proved to be less successful in high-abundant proteins removal [220].

Recently, Ahmed and Rice demonstrated the use of affinity dyes in conjunction with a supporting matrix, ProtoClear and Affi-Gel Blue, along in combination with Protein A (Aurum serum protein mini kit, Bio-Rad), which proved efficient in removing high-abundance proteins without a significant loss of protein profile or number of protein spots [5]. The loss of associated proteins was reported to be dependent on the treatment duration. Furthermore, the authors suggest Agilent multiple affinity removal system (Agilent Technologies) as capable of binding and retaining six highly abundant proteins and enabling enhanced detection of low-abundant molecules in a high throughput manner. ProtoClear technique was reported to be far more specific at clearing albumin and immunoglobulin G from human serum samples than Cibracon Blue Dye chromatography [219].

Furthermore, Govorukhina et al. evaluated capacity of various columns to remove albumin and/or IgG from human serum [210]. HiTrap Blue and protein G columns in combination were found more effective than Aurum columns. Another kind of affinity chromatography technology enabling high-throughput proteomic removal of abundant proteins from serum implicates the use of SwellGel protein A/G and Cibacron blue discs or resin combination [200]. For more information concerning preparation of body fluids, the readers should refer to Section 1.4.

Immunoaffinity-based protein subtraction chromatography (IASC) described by Pieper at al. was shown to effectively and reproducibly remove multiple, abundant proteins present in plasma and serum, enabling visualization of a vast number of lower abundance proteins [222]. Similarly, the use of β-casein- and bovine IgG-specific immobilized Sepharose enabled Yamada et al. to identify several low-abundant proteins of special physiological relevance [223]. Alternatively, an affinity spin tube filter technique can be applied to enrich the low-abundant biomarkers [224].

A preparative electrophoresis system, Gradiflow, is being employed for depletion of albumin under native and denatured conditions (see also Section 1.4). The technique enables separation of proteins on the basis of their molecular weight and charge. Hence, separation of the majority of plasma proteins characterized by the p*I* close to that of albumin may be carried out successfully [225]. Albumin can be also effectively removed by isoelectric trapping [226] and peptide affinity column chromatography [213].

Among the wide range of applicable precipitation methods, ammonium sulfate fractionation was reported most efficient in albumin removal [181].

Finally, solid-phase extraction constitutes another promising tool to reduce differences in proteins concentration, and thus enhancing the possibility to detect and analyze low-abundant species [177,227].

2.3.4. Lipids

Similarly to salts, lipids are widely present in biological fluids such as plasma. Numerous proteins are complexed with lipids, and this interaction reduces their solubility and might affect the p*I* and MW. Moreover, by forming complexes with detergents, lipids reduce protein enrichment/separation efficacy. Most often, if 2-DE separation is to be performed, the use of centrifugal filter device and the sample buffer including CHAPS allows for efficient lipid and salt removal, thus ensuring high percentage of proteins recovery and high-quality separation.

Joo et al. compared the use of CHAPS and subsequent centrifugation to sample boiling in SDS and dialysis [228]. Application of CHAPS allowed visualization of more proteins than achieved with the classical method, no streaking was observed on gels. Heating samples at presence of SDS cannot be performed if proteins are to be resolved by IEF [105,121]. Alternatively, precipitation in acetone or combination of TCA/acetone removes lipids efficiently. Lawless also reported a precipitation technique employing acetonitrile supplemented with 1% TFA and 1% *n*-nonyl-β-D-glucopyranoside, which was found especially helpful in dissolving membrane proteins and lipids [229]. Moreover, Watkins et al. introduced a new method for delipidation of human serum lipoproteins involving the use of a reversed-phase C_{18} solid-phase extraction cartridge. The method of delipidation produced a higher and more reproducible protein yield than the conventional liquid–liquid methanol-diethyl ether delipidation technique, and implemented a fast, sequential desalting and delipidation of the lipoproteins for subsequent mass spectrometric analysis [230].

2.3.5. Polysaccharides

Polysaccharides and nucleic acids may interact with carrier ampholytes causing streaking visible on 2D gels. Moreover, their presence in a sample buffer can result in viscous solutions, clogging the pores of the polyacrylamide gels, thus causing

either precipitation or extended focusing times, and resulting in horizontal streaking. Furthermore, some polysaccharides are negatively charged and thus may form complexes with proteins by electrostatic interactions.

In order to exclude polysaccharides from the sample, precipitation in TCA, acetone, ammonium sulfate or phenol/ammonium acetate, followed by centrifugation may be beneficial. High-speed ultracentrifugation is applied when the removal of larger polysaccharides is required. These molecules can block the matrix of chromatographic materials and the pores of membranes [231]. Furthermore, similar methods as for nucleic acids removal are advised in case of lipids and polysaccharides.

2.3.6. Nucleic acids

Nucleic acids can interfere with carrier ampholytes and proteins, and may contribute to the poor recovery of DNA-, RNA-binding protein and evoke horizontal streaks on 2D gels [114]. Silver staining used for visualization of samples separated via 2-DE detects also nucleic acids, if present in the gel, which results in background smearing. Moreover, nucleic acids increase sample viscosity, clog the pores of polyacrylamide gels and affect the accuracy of sample loading.

In order to remove DNA and RNA, digestion with protease-free DNase and RNase is often applied [115]. The treatment reduces nucleic acids to mono- and oligonucleotides. One should be aware that DNAses and RNases may appear on 2D patterns. Alternatively, protein precipitation from the solution is advised. Proteins associated with nucleic acids may be lost from the sample, unless the nucleic acid fraction is extracted with the detergent cocktail as presented by Giavalisco et al. [232]. According to Rabilloud [233], ultracentrifugation and addition of basic polyamine, e.g. spermine, is also effective in removal of large nucleic acids, as well as high MW proteins. High-ionic strength extraction and high-pH extraction appear to be potent in minimizing interactions between negatively charged nucleic acids and positively charged proteins. A convenient alternative utilizing QIAShredder (QIAgen) and subsequent centrifugation was reported by Leimgruber et al. [114].

2.3.7. Other substances

Endogenous, small ionic molecules; nucleotides metabolites, phospholipids present in cell lysates are often negatively charged, resulting in poor focusing towards anode. Other disturbances during protein separation may also be evoked by insoluble material, e.g. organelles clogging gel pores. Non-proteinaceous impurities may form complexes with proteins hampering their solubilization. Therefore, in order to remove the contaminants, TCA/acetone precipitation or other salt-excluding techniques are effectively performed. Alternatively, high-speed centrifugation [234,235] can be applied.

The presence of phenols observed in plant tissues, may modify proteins through an enzyme-catalyzed oxidative reaction. Oxidation may be prevented with the use of reductants while performing tissue extraction. Furthermore, protein precipitation with TCA, followed by extraction of phenols with ice-cold acetone or phenol adsorption to polyvinylopolypyrrolidone (PVP) [236], are advantageous.

2.4. Protein enrichment methods

Biological samples used as a source for proteomics analysis are usually very complex. As it was mentioned before, proteins concentration range in a single sample is usually beyond the dynamic range of any single analytical method, and any examined proteome could have a large and unknown complexity. Hence, prior to analysis, it is desirable to reduce the complexity of the sample by its prefractionation, or to enrich it with proteins of our interest.

The fundamental idea of prefractionation is to isolate sample into distinguishable fractions containing restricted numbers of molecules. The sample can be fractionated using a variety of approaches including precipitation, centrifugation, liquid chromatography and electrophoresis-based methods, filtration, and velocity or equilibrium sedimentation. The selection of the technique strongly depends on the nature of sample to be analyzed, and the object of the study.

The enrichment methods allow for increasing the concentration of proteins of interest. This statement is really important in the proteomics study, because usually low-abundant proteins carry valuable diagnostic information and are responsible for processes ongoing in the cells. The conventional method of sample concentration by simple evaporation results, along with the proteins, in buffer components concentration (e.g. salts and other contaminants). Therefore, universal, more convoluted methods of protein enrichment are necessary. It should be remembered that during any enrichment process, conditions must be stable to avoid protein interactions among the rest of mixture components (e.g. non-specific interactions with other proteins).

2.4.1. Precipitation

The most common methods of protein enrichment and purification rely on selective precipitation using acetone, trichloroacetic acid, ethanol, isopropanol, diethylether, chloroform/methanol, ammonium sulfate, polyethylene glycol (PEG), and a number of commercially available affinity precipitation kits [190,237,238]. Ammonium sulfate is expected to be the most widespread precipitant utilized, which causes protein destabilization. This is known as the “salting-out” effect. Addition of various organic solvents promotes an increased electrostatic attraction between particles of opposite charge in the sample solution. This effect leads to protein precipitation. Precipitation can be promoted by addition of the organic polymers such as PEG. The precipitate recovery rely on redissolving in a smaller volume, followed by centrifugation or filtration. Other type of protein enrichment routine is immunoprecipitation. The principle is based on the utilization of antibodies that are selective for one or a group of proteins with a similar epitope (e.g. phospho or glycoproteins) [239] (see also Section 2.4).

2.4.2. Centrifugation

One of the simplest methods of protein enrichment is ultracentrifugation. Separation of cell substructures can be attained by the series of runs at different centrifugal forces, or in sucrose/mannitol gradient, which allows separation of different cellular or tissue material, according to the density character-

istics of the structure [240]. This technique is useful for concentration of mitochondrial, membrane, nuclear or other locally abundant proteins.

2.4.3. Electrophoretic methods of protein enrichment

One-dimensional gel electrophoresis (1-DE) separation of proteins by their size is the traditional manner for protein enrichment and analysis (see also Section 3.1). Two-dimensional gel electrophoresis (2-DE) has the benefit that it enables simultaneous visualization of hundreds of protein spots, their post-transational modifications, and quantification of protein levels. Reproducibility of protein patterns between laboratories is more difficult, because of protocols variations, artifacts and technology. Separation based on 2-DE technique dedicated to hydrophobic and membrane proteins, as well as alkaline and low-molecular weight polypeptides, possess some limitations. Even though, membrane proteins containing up to 12 transmembrane helices, have successfully been resolved [125].

2.4.4. Membrane proteins enrichment

The membrane proteins are of great importance for proteomics, because they represent receptors, transporters, channels, and they participate in a variety of significant cellular mechanisms. Because of their function, they are considered as a major pharmaceutical target, and ability of their detection is of particular interest. Unfortunately, a vast under-representation of membrane proteins has been observed during whole cell proteome analysis.

Membrane proteins are usually enriched by ultracentrifugation in sucrose gradient, lectin affinity chromatography in combination with centrifugation, silica beads or biotinylation and interaction with immobilized streptavidin [241]. Solubilization of this fraction has to be improved by using special detergents, and the choice of them depends on the nature of experiment.

Ferro et al. used combination of chloroform and methanol to extract hydrophobic chloroplast membrane proteins [242]. The aqueous two-phase system, which employed detergents DDM, Triton X-114 or PEG, was used for membrane proteins enrichment by Sivars and Tjerneld [243] and Everberg et al. [244]. This technique requires the selective binding of one or more proteins of interest to the one of the incompatible aqueous phases. Detailed description of different detergents used during this kind of analysis, could be found in Section 2.3.

Identification of membrane proteins is not an easy task due to the lack of tryptic cleavage sites across transmembrane chain fragments. Enzymatic digestion often results in large, hydrophobic pieces, which hinder identification. To enlarge sequence coverage, a mixture of proteases and cyanogen bromide with addition of detergents could be performed [245,246].

2.4.5. Prefractionation

Preparative liquid electrophoretic methodologies are other fractionation systems for proteome profiling. The fact that protein fractions are collected here in a liquid form, promotes application of such methodologies, because sample handling is diminished, thus the risk of it loss or degradation is reduced [247,248].

One of the latest technology platforms that have been developed for proteomics includes, e.g. Rotofor, the multicompartmental instrument, capable of fractionating proteins according to their p*I* [227]. The Gradiflow is another multifunctional electrokinetic membrane device that can be used for proteins separation based on the differences in mobility, p*I* and the size of proteins. One of the major drawbacks of this strategy is that the fractions collected from above equipments contain high amount of ampholytes, which, fortunately, can be removing by microcolumns filled with C_{18} material.

It was shown by Petsev et al. that proteins can be separated on a small-scale without use of more expensive chemicals or molecules, such as antibodies or synthetic ampholytes [249]. Field gradient device separation mechanism is based on the opposition of two or more forces, from which one is constant as a function of distance along fluid channel, while the other force is changed gradually or stepwise.

Free-flow electrophoresis (FFE) is another method for purifying cells and subcellular organelles, but it is rarely used for protein enrichment because of high diffusion effect. FFE separates charged particles ranging in size from molecular to cellular dimensions, according to their electrophoretic mobility or p*I* [227,250,251].

2.4.6. Chromatographic techniques

Another most applied proteomic system for proteins enrichment is chromatographic separation, which is proficient to reduce the complexity of the sample by separating proteins according to their charge, hydrophobicity, size, or specificity. Solid-phase chromatographic techniques are capable of protein fractionation with low, as well as very high selectivity depending on the adsorbent and conditions of adsorption–elution selection. Affinity chromatography utilizes highly specific biological interactions such as that between antigen and antibody, receptor and ligand, or enzyme and its substrate, or inhibitor and it allows for very efficient protein enrichment. This technique offers many advantages over conventional chromatography, for example, specificity and selectivity to name the few.

Several technologies have been designed as important tools for biological research, including proteome- and inhibitor-based affinity chromatography, along with activity-based profiling. These techniques reduce sample complexity to access the less abundant proteins, and to help understand pathological processes [252]. During inhibitor-based procedure, the target protein is attached to the solid support, covered with inhibitor molecule. Proteome-based affinity chromatography relies on searching the protein of interest within the captured compounds. Finally, the activity-based affinity profiling is achieved by competition of target protein with standard probe (e.g. biotin or fluorophore) [253].

For investigation of post-translational modifications (PTM's), a variety of combinations of the affinity-based enrichment and extraction methods, multidimensional separation techniques and mass spectrometry are applied. More then 300 types of PTM's are currently known [254].

Glycosylation, one of the most common PTM's, plays fundamental role in a diverse set of biological processes, such as proper

folding, signaling pathways associated with the transformation of a normal cell to a cancer cell, the immune response and cellular regulation. In one study the multi-lectin column was used to enrich glycoproteins from human serum [255]. All lectins have ability to bind certain monosacharides such as mannose, glucose or fucose. It was found that the multi-lectin column was highly specific for O- and N-linked glycans present in serum. Lectin affinity chromatography may be utilized to purify a variety of proteins. Fluorescent staining methods were developed as well, in order to visualize and analyze glycoproteins and phosphoproteins [256].

The reversible phosphorylation, which occurs on serine, threonine and tyrosine residues, is a basis for regulation of fundamental cellular functions, such as DNA replication, cell division, cell cycle control, transcription, translation, protein localization, energy metabolism and signal transduction. The knowledge of the phosphorylation status of all proteins at a given time would be one of the major issues for understanding of the physiological and pathological role of phosphoproteins. Detection of phosphopeptides is possible by scanning for the neutral loss from peptide during MS/MS analysis under positive and negative ion modes [257].

Traditional metabolic labeling methods use the radioactive isotopes ^{32}P and ^{33}P [258]. The most recent approach is based on chemical modifications of phosphate groups [259], and application of antibodies against specific phosphoaminoacids [260].

Phosphopeptides, likewise glycoproteins, calcium-binding and histidine-exposed proteins can be selectively enriched by immobilized metal affinity chromatography (IMAC), which reduces the complexity of the protein mixture [227,261]. IMAC is based on formation of coordinate bonds between basic groups on protein surface, and metal ions (e.g. Fe, Co, Ni, Cu, Zn, Al) immobilized on chromatographic beads. Elution of bound proteins is undertaken by lowering the pH or using chelating agent, such as EDTA. Analysis of protein phosphorylation was introduced based on covalent modifications with biotin at the site of phosphorylation [262]. The DIGE (difference gel electrophoresis) technology allowed separation of phosphoproteins with high resolving power [263]. Phosphoproteins could be also analyzed through stable isotope labeling by amino acids in cell culture [257].

Ubiquitination is a process of biological labeling proteins which are destined for destruction. His6-tagged ubiquitin facilitate affinity isolation of ubiquitin-conjugated compounds [264].

Wide spectrum of affinity ligands are utilized for a variety of applications, but there is a requirement for a thorough knowledge of the sample content before applying affinity-based approach [265,266].

2.4.7. Solid-phase protein enrichment

A novel chromatographic carrier has been developed to perform adsorption and purification of proteins [267]. Zeolite surface interacts with proteins through chelation with Co^{2+} on zeolite nanocrystals [268]. The most important advantage of this procedure is lack of the co-concentration of salts during the enrichment process, and the peptides adsorbed on the surface of the zeolite can be directly analyzed by mass spectrometry.

Solid-phase extraction also provides sample enrichment and purification. There are plenty of solid-phase microextraction systems (SMECs), which enable simple and rapid extraction technique, including ZipTip, ZipPlate, Gelloader and MassPREP PROtarget [196,269]. Another easy sample prefractionation method was reported [270] which is based on neutral beads of Sephadex to isolate proteins according to their isoelectric points.

Antibody arrays are very useful for profiling biomarker candidates in large sets of biological samples. A variety of substrates and methods of antibody attachment have been used [271,272]. Moreover, the entire libraries of antibody microarrays are created [254,273,274].

Surface-enhanced laser desorption/ionization (SELDI) is also a useful tool for protein enrichment, since the sample preparation procedure involves surface prefractionation of protein mixtures on the derivatized target plates, in order to maximize the number of detectable peaks [227,271,275].

An additional method of sample fractionation is laser capture microdissection (see Section 1) which allows separation from neighboring cells in biopsy material, cells in cultures, etc. LCM is still an effort-demanding procedure that yields limited amounts of material and so it is not fully suitable for testing a large number of samples [271].

The above applications and technologies demonstrate the value and potential of protein enrichment in proteomics. Further improvements to the methods should broaden their exploitation and create large impact on proteomic research.

3. Samples preparation guidelines for various proteomic techniques

During sample preparation for proteomics study it has to be remembered that realistically, no single method could be applied to all possible samples, and there is always necessity to optimize the procedure for particular samples. All applied procedures should be as simple as possible and, even more important, reproducible. It is necessary to avoid proteins loss, degradation and modifications.

Before starting the experiment, it is advisable to know the fundamental problems that might appear at each step of the procedure. This is crucial, due to the fact that each next step is based on the quality of the previous one, and if the mistake is done at the beginning, there is no way to improve the results at the end.

3.1. Electrophoretic methods of protein separation

Polyacrylamide gel electrophoresis in SDS (SDS-PAGE) was described for the first time in 1949 [276]. Separation is achieved once the electric field is applied to a solution containing a protein that has a net positive or negative charge. The protein migrates at a rate that depends on its net charge, size and shape [277]. Presently, one-dimensional separation is often used as a prefractionating technique in proteomic approach, because of its insufficient resolution [278,279].

The traditional two-dimensional gel electrophoresis method was introduced in 1974 [280] and is now one of the most

commonly applied techniques in proteomics. This method is based on orthogonal separation of proteins according to different physicochemical principles. 2-DE enables separation of complex proteins mixtures in respect to their p*I*, molecular weight, solubility and relative abundance [115]. Depending on the gel size and pH gradient, it can simultaneously resolve more than 5000 proteins [281]. Moreover, 2-DE provides information on protein changes in their expression level, isoforms and post-translational modifications [282].

Detection limit depends on the dye applied for visualization of the proteins. The present detection limit achieves less than 1 ng of protein [115]. Another advantage of 2-DE is creation of protein-patterns (profiles), that can be examined using image analysis software [283]. Protein patterns may be simplified as a result of the most abundant proteins depletion methods [221,284–286].

The success of any of the protein separation and purification techniques is largely dependent on protein solubilization method [121,287,288]. A more "traditional" sample lysis procedure for 2-DE, involves cell or tissue disruption in the presence of high concentrations of urea, reducing agents and detergents. The immobilized pH gradient strip (IPG strip) is then rehydrated with sample, and proteins are separated. Unfortunately, the optimal lysis conditions for 2-DE, are not compatible with MS.

Detection of the separated proteins is usually accomplished by the use of a visible stain, whereas newer approaches apply the fluorescent dyes [289–295]. Some of the fluorescent dyes have been designed for detection of the post-translational modifications [296,297].

The major problem associated with 2-DE is the reproducibility of samples separation. It is particularly crucial in comparative proteomics, where control sample and experimental one have to be compared. To address this problem, a new method, two-dimensional difference gel electrophoresis (2-DIGE) has been developed. This technology allows for separation of two samples on the same gel simultaneously, due to labeling the proteins from different samples with different cyanine dyes prior to the first dimension [1]. This approach removes gel-to-gel variability, and is valuable in distinguishing differences in migration due to p*I* or molecular mass [263,298,299]. For statistical purposes, 2-DIGE gels also utilize pooled standards to normalize measurements of protein abundance across multiple gels in the experiment [300].

Two-dimensional Blue Native/SDS gel electrophoresis (2D BN/SDS-PAGE) merges IEF proteins in their native state with a second denaturing dimension. This method of protein separation was designed to study dynamics and interactions of membrane proteins [143,301].

Although there is a diversity of emerging proteomic techniques, there is still no appropriate method that can replace electrophoretic approach.

3.2. Capillary electrophoresis

Capillary electrophoresis is one of the liquid-phase separation techniques. Like other electrophoretic approaches (i.e. gel electrophoresis), it utilizes electrostatic forces to drive and separate components present in the sample. Because of that, this technique is best suitable for separation of charged compounds. In contradiction to gel electrophoresis, in capillary electrophoresis the separation process is conducted inside a fused-silica capillary of the internal diameter between 50 and 100 μm. Interactions between ions in the electrolyte and charged groups situated on the capillary walls are responsible for the phenomenon of electroosmothic flow (EOF), which drives electrolyte through capillary. The motion of compounds during the separation results from both electroosmothic flow and their individual electrophoretic mobilities.

It should be noted here that term capillary electrophoresis is often used instead of a more precise "Capillary Zone Electrophoresis" (CZE) which refers to a family of closely related separation techniques employing narrow capillaries. Within this text the term CE will be used as a shorthand of CZE.

Major features of capillary electrophoresis include extremely high separation efficiency, sensitivity, short analysis time and low sample consumption, as compared to capillary high-performance liquid chromatography (cHPLC). CE can be coupled to the mass spectrometer either with ESI (on-line) or MALDI (off-line or on-line) ion source, which makes it a feasible method to use in proteomics. Drawbacks include complex procedures for preparation of capillaries, their coating and preparation of necessary interfaces between CE and, e.g. mass spectrometers. Detailed description of these connections is beyond the scope of this review and a more detailed description is recommended, e.g. [302].

Here we will focus on sample preparation prior to CE analysis, as reported in literature. In a recent article Fliser at al. [303] published results on CE–MS analysis of crude urine samples. Multiple peptide signals visible on 2D electrophoregram plots prove the capillary electrophoresis is capable of removing light-, and highly mobile contaminants (like salts, present in samples at high concentration) during the run. However, high salt content causes a decrease in efficiency of separation, and desalting utilising reversed-phase [304] and/or anion-exchange solid-phase extraction [305] is beneficial for the final outcome of the analysis.

Whole-cell or tissue digests obtained in the bottom–up proteomics approach reflect problems similar to the mentioned above—complex mixtures of peptides in buffers with high salt concentration, and denaturing compounds, such as urea. Not surprisingly, the reported CE separations of cell-line [306] and body fluid digests [307] employ simple sample pretreatment protocols, limited to desalting with C_{18} solid-phase extraction and/or removal of insoluble fractions after centrifugation.

Whereas CE separation of peptides and oligopeptides in crude samples is possible, analysis of intact protein mixtures is more troublesome. In their recent study, Moini and Huang [308] applied CE for separation of *E. coli* cell lysate proteins. In the initial studies, direct injection of lysate resulted in capillary clogging caused by protein precipitation at low pH of acidic electrolyte used. To overcome this problem, the lysate was divided into basic and acidic fractions by precipitation with 0.2% acetic acid. Both fractions were analyzed separately—basic proteins at low pH conditions and acidic at high pH. Different capillary

coating was applied in both cases (APS (aminopropyltrimetoxysilane) and cellulose, respectively). Even though, the protein mixture was found to be too complex for 1D separation, thus the procedure was repeated with lysate of *E. coli* ribosomes with good results.

Capillary isoelectric focusing (CIEF) comprises another important technique in the capillary electrophoresis "family" of methodologies. Similarly to isoelectric focusing, it utilizes mixture of ampholytes to create linear pH gradient in the electric field, and thus separates compounds according to their isoelectric point. Its main advantage of the technique is its ability to preconcentrate compounds at their p*I*. However, because application of ampholytes suppresses MS ionization, direct application of CIEF in proteomics is limited due to difficulties in coupling to a mass spectrometer. Therefore, this technique is best suited as a sample preconcentration and preparation technique for further CE–MS or LC–MS analysis in the 2-DE workflow. Detailed description of complex instrumentation for linking CIEF to MS, CE–MS or LC–MS is beyond the scope of this article—more information may be obtained in the work by Simpson and Smith [302].

3.3. Sample preparation for high-performance liquid chromatography

Nowadays, high-performance liquid chromatography is an important separation technique in proteomics. It can easily be coupled to mass spectrometry, which makes it a perfect tool for separation of proteins and peptides directly prior to mass analysis. Compatibility of solvents used in the reversed-phase chromatographic separations makes this hyphenated technique most commonly used in the final stage of proteomics analysis workflow. Other liquid chromatography subtypes, including size-exclusion, ion exchange and affinity separations are commonly used during consecutive steps of sample preparation, clean-up, enrichment and prefractionation. Most chromatographic approaches are tolerant to moderate concentration of contaminants, such as weak buffers. In this part, we will summarize several examplary sample pretreatment approaches used prior to injection onto LC column.

Firstly, it should be noted that samples injected onto chromatographic column cannot contain insoluble particles or dispersed molecules that may cause column clogging and malfunction. Such contaminants are usually removed by centrifugation and/or sample filtration using spin-filters (45 μm pores). In addition, samples should not contain buffers affecting LC separation, e.g. samples injected onto column should not be dissolved in buffer with higher eluting strength than of mobile phase. High concentration of detergents should be avoided in case of RP separation whereas samples injected on the ion-exchange column should not contain high contraction of background salts and other ionic contaminants that might disturb ionic equilibrium. Volatile buffers such as ammonium acetate or ammonium bicarbonate, are recommended in this case.

Liquid chromatography may be used both in top–down and bottom–up proteomics approaches. In the first case, protein sample is separated and then individual proteins (or simple mixtures) are identified directly by means of tandem mass spectrometry. In this approach, liquid chromatography may be used for separation of proteins prior to mass spectrometry analysis. In the bottom–up approach, protein, or protein mixture is digested. Single- or multidimensional liquid chromatography coupled to mass spectrometry is then used for separation of peptide mixtures and identification of their compounds. In this chapter we will summarize exemplary sample preparation procedures that may be useful in both top-down, and bottom-up proteomics strategies.

3.3.1. Top–down proteomics

In an examplary top–down approach, Wang et al. [309] analyzed yeast cytosol proteins by reversed-phase liquid chromatography. Sample preparation included cell disruption by sonification, followed by centrifugation and desalting using 5 kDa cut-off cellulose membrane. Protein solution was subjected to denaturation and reduction in 20 mM Tris, 8 M urea and 0.1 M DTT followed by alkylation by iodoacetamide. Prior to RP–LC–MS/MS analysis, the buffer was exchanged to 10 mM Tris and 2 M urea using size-exclusion column. Desalting of proteins and buffer exchange by centrifugal ultrafiltration and anion-exchange chromatography prior to RP separation was employed by Li et al. during identification of human plasma proteins [310].

Moritz et al. [311] shown the free-flow electrophoresis-RP-HPLC (FFE IEF-RP-HPLC) approach for the analysis of human plasma proteins. In this approach, fractions obtained by FFE IEF (Free-Flow Electrophoresis IsoElectricpoit Focusing) were directly injected onto RP column. Ampholytes and buffers/salts abundant in the IEF fraction were not retained by the column, and thus easily removed from the system.

3.3.2. Bottom-up proteomics

Although LC separation of proteins is increasingly common as protein separation technique in the top–down proteomics, the 2D gel approach is still considered as basic proteomic strategy. Reversed-phase liquid chromatography coupled to a tandem mass spectrometer, is well established and commonly used procedure for identification of the in-gel digested proteins. Apart from optional drying in a vacuum centrifuge/lyophilization and solubilization in the mobile phase prior to injection onto LC column, this approach usually does not require any additional sample preparation steps. Recent results include identification of cancer cell-line proteins [312,313], or mitochondrial protein complexes [314]. However, additional step of cleaning of protein digest by, e.g. RP SPE (Reversed Phase Solid Phase Extraction) is recommended in some cases. In the work, published by Rappsilber et al. [315] the authors introduced disposable C_{18} RP extraction tips for use in conjuction with MALDI, nanoESI and RP-LC–MS approaches. The rationale for application of RP SPE prior to LC separation is removal of insoluble particles, purification from salts and sample preconcentration by the elution in small volume of organic solvents, followed by vacuum centrifugation. For similar reasons, commercially available capillary chromatography systems include trapping precolumns, where the sample is purified, desalted and preconcentrated prior to injection onto capillary column.

Strategies employing direct proteolysis of biological samples, apart from much higher complexity of peptide mixture, are very similar in contents to the gel-spot digests. In the recent work, Sun et al. [316] compared 1D-SDS-PAGE followed by proteolytic digestion and 1D-LC–MS/MS approach with 1D- and 2D-LC–MS/MS, for direct analysis of human urinary protein lysates. In all cases, sample preparation protocols were very similar. Protein content was extracted by acetone precipitation, followed by in-gel or in-solution reduction, alkylation and digestion. For both approaches, no further sample pretreatment was performed, except for lyophilization and dissolution in LC buffer directly prior to use. Similar approach in the bottom–up analysis of human serum proteins was employed by Li et al. [310]. In proteomics analysis of transcription factors bound proteins, additional desalting of the digest by RP solid-phase extraction, prior to lyophilization was performed [317], because the high salt content of the digestion buffer may affect peptide binding to the SCX (strong cation exchange) column in first dimension of 2D-LC run. The same approach was employed by Lominadze et al. [318] in analysis of human neurophil granules' proteins. Ramstrom et al. [319] analyzed human cerebrospinal fluid tryptic digest using RP-LC coupled to FT-ICR (Fourier-transform Ion Cyclotron Resonance) mass spectrometry. In this work, the digest was desalted using commercially available RP solid-phase extraction. Organic solvents after SPE were removed by vacuum centrifugation.

3.4. Mass spectrometry

For most proteomic applications, mass spectrometry is the ultimate phase of the analytical process, and is supposed to provide the reliable end-data. Their quality, however, is directly dependent on the quality of the input from all earlier sample preparation/processing steps. In general, samples entering the MS should be of the highest possible purity, not too complex, and deprived of compounds that compete with the analyte for ionization or cause signal suppression, such as inorganic salts, chaotropic agents, detergents, polymers and non-volatile components. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization techniques are most useful for proteomic studies (for a description of MS instrumentation and application in proteomics see, e.g. [320,321]. MALDI is generally more salt-tolerant than electrospray and a useful list of accepted contaminants may be found on the Internet [322]. Regardless of ionization technique, all operations must be performed with the highest cleanliness to avoid introduction of keratins or other contaminants that impair protein identification, and the reagents should be of highest possible purity to avoid high background, obscuring spectra quality. Care should be taken to avoid material loss during preparatory steps. However, depending on the type of experiment and its ultimate goal, samples may be prefractionated in order to limit the dynamic range of concentrations to unmask low-level components (see Sections 2.3 and 2.4).

Application of the proper quality of assay tubes (and plastics in general) cannot be neglected. There is a broad discussion among laboratories concerning this issue as the tubes might release remainings from the manufacturing process, thus increasing background in the mass spectrometer. Another important problem might be adsorption of a minute amount of material on the tube/pipette tip walls. It seems that the commonly accepted solution to this problem is application of the siliconized tubes of smaller volume (e.g. 250–500 μl), instead of 1.5 ml. Silicone produces several characteristic peaks in the mass spectrometer but the sample loss is lower. Moreover, sample should be processed as soon as possible after freezing the purified material. In many cases, sample loss is so significant after 4–6 days of storage that components are not detectable (R. Ekman, R. Persson, G. Karlsson, J. Silberring, personal communication).

3.4.1. MALDI-MS

MALDI mass spectrometry is one of the basic proteomic techniques and is extensively used for peptide mass fingerprinting, due to its speed, sensitivity, accuracy, satisfactory tolerance to impurities and ease of automation [323]. In order to obtain a peptide map, sample proteins are separated by 2D-PAGE and visualized by staining. Coomassie Brilliant Blue stain is robust and MALDI-compatible, although cannot be used for detection of the low-abundant proteins (ca. 100 ng detection limit). Silver staining is much more sensitive (low nanograms) but, until recently, was not suitable for MS due to the presence of glutaraldehyde [324]. This was overcome by development of MS-compatible silver staining methods [325]. Fluorescent labels, such as SYPRO Ruby can also be applied, that exhibit sensitivity similar to silver staining, have broad linear dynamic range and are fully compatible with MALDI-MS. Further information, including detailed protocols on the use of various staining methods can be found elsewhere [326–328]. After electrophoretic separation, proteins are subjected to in-gel proteolytic digestion in order to obtain peptide maps that are further analyzed by MALDI-MS. Digestion of proteins directly on MALDI target plates was also reported [329,330].

For successful MALDI analysis, the key point is the proper choice of matrix and sample deposition method, to achieve highest possible sensitivity and accuracy. Matrix purity should be of at least 99%, otherwise re-crystallization is recommended as impurities may negatively affect the formation of analyte/matrix crystals. To minimize the risk of cross-contamination, application of disposable (and prespotted with matrix) targets may be considered, instead of the multiple-use stainless-steel devices. The most popular matrices for proteomic applications include α-cyano-4-hydroxycinnamic acid (CHCA) [331], sinapinic acid (SA) [332] and 2,5-dihydrobenzoic acid (DHB) [333]. Typically, CHCA is preferred for analysis of peptide maps, SA works best for larger proteins and DHB is usually used for hydrophobic, glyco- and phosphopeptides, but these are only general guidelines. For some applications, combinations of different matrices were found useful [334,335].

A vast number of sample preparation protocols were devised that differ in the order of application of matrix and sample solution, their concentrations and solvents used. The most popular include the "dried droplet" method [336], in which the matrix solution is mixed with the sample, and the resulting mixture is deposited on the target plate. In the "thin layer" method [337],

a drop of analyte solution is deposited on a matrix-covered target surface, thereby improving accuracy and sensitivity. Useful tips on the choice of matrix and sample preparation techniques were given by Kussmann et al. [330], and a detailed study on the conditions affecting the efficiency of CHCA can be found in [338]. Depending on the protocol, the matrix may be acidified with trifluoroacetic acid to facilitate protonation. Generally, there is no universal sample preparation protocol that could be used for each and every type of sample, and in more difficult cases (low analyte and high salt concentration) careful optimization might be necessary if standard procedures fail [330].

Although it may be possible to record spectra of native biological samples, prior to analysis they might be desalted, in order to avoid signal suppression by high salt contamination. For sinapinic acid or CHCA it is possible to decrease salt concentration by on-target washing, i.e. after the sample and matrix were deposited on the target plate and co-crystallized. It is a cheap and simple method that takes advantage of the fact that, unlike inorganic ions, the crystallized matrix with incorporated peptides or proteins is water-insoluble. Here a ca. 5 μl droplet of deionized water, 0.1% TFA or 5% formic acid, is deposited on the surface of a sample dried on the target plate. The droplet is removed after several seconds and the procedure can be repeated [339]. In particular, the thin-layer method offers the possibility of effective on-target washing since the analyte and impurities are localized on top of the matrix layer. The use of DHB excludes washing as this matrix is water-soluble. The efficiency of on-target washing is dependent on the type of contaminants, their solubility and accessibility for wash solution [340]. It should be noted that some analyte molecules can also be washed away. A comparison of different protocols can be found in [341]. The addition of nitrocellulose to the matrix was found to strengthen binding to target surface and to make it more resistant against contaminants, thus allowing more effective washing [342]. Modifications of the target plate surface are also possible to facilitate on-target purification [343].

If on-target washing gives unsatisfactory results, efficient salt removal may be achieved through dialysis or the use of commercially available or home-made chromatographic microcolumns (see also Section 2.3). Desalting is often performed together with concentration, as is the case with reverse-phase microcolumns. In popular Millipore ZipTips, the stationary phase is placed in the outlet of a pipette tip, and elution occurs in the microliter volumes [344]. Their convenience stems from the ease of operation, as washing and elution are performed by simply plunging and releasing the pipette plunger. Therefore, they are compatible with many robotic platforms for proteomics. Chromatographic microcolumns can also be prepared in-house, using gel loading pipette tips, fused silica capillaries or small columns equipped with frits and filled with beads of different stationary phases [330,345–347]. Independent on the shape, microcolumns offer the possibility of stepwise elution of bound components, which lowers sample complexity. A useful comparison of experimental conditions for the use of RP microcolumns (bed volume, solvents, etc.) can be found in [348]. In general, best results are obtained if the sample is eluted in the lowest possible volume to ensure maximum concentration. Therefore, in order to maximize their final concentration on the target plate, analytes may be eluted with a matrix-containing solvent [340,349]. Although each transfer of low-concentrated samples between tubes and columns may lead to significant material loss [348], the benefits of thorough purification might well outweigh the risks. The use of chromatographic beads immersed in the analyzed solution was reported as well. The beads were then transferred to the MALDI plate, where the bound material was eluted and analyzed [350]. A sophisticated and efficient means of sample purification and separation is offered by the LC–MALDI technology that combines the advantages of capillary liquid chromatography (LC) with the sensitivity and accuracy of MALDI-MS.

In high-throughput screening (HTS) studies, automated machines are used for sample preparation and deposition on target plates that offer unparalleled precision in low-volume operations, speed and elimination of human errors [351–357]. These devices make possible the efficient application of LC–MALDI for confident protein identification and, most importantly, are able to operate on picoliter sample volumes. Some of the robotic platforms enable automatic completion of other stages of the experiment as well, such as protein digestion [358–360], or chromatographic separation [357,361].

Apart from typical stainless-steel MALDI target plates, prestructured targets are available that include hydrophilic anchors distributed over a hydrophobic surface in order to concentrate the analyte on the spot centre, thus increasing sensitivity. For prestructured targets, dedicated protocols were developed [362–364]. Recently, disposable matrix-precoated AnchorChip™ (Bruker Daltonik, Germany) targets with calibration spots were introduced [365]. Their major advantages include the ease of automated sample spotting, elimination of cross-contamination risk, and possibility of several months' long storage. Various in-house methods of modifications of the target surface were also reported [343,366–369] and reviewed recently [370].

Furthermore, the atmospheric pressure MALDI (AP-MALDI) was introduced in 2000 [371]. Although its applications in complex proteomic studies are still limited, it should be noted here that for AP-MALDI typical matrices may be used, such as CHCA or SA. However, the use of liquid matrices, including water as matrix was also reported in combination with an infrared laser [372,373]. This gives the possibility of the on-line combination of liquid chromatography and AP-MALDI [374,375].

3.4.2. SELDI

As a conceptual modification of MALDI-TOF measurements, surface-enhanced laser desorption/ionization technique is marketed by Ciphergen that combines chromatographic separation and mass spectral measurement for proteomic profiling and biomarker discovery (see also Section 1.4). The SELDI chip contains chromatographic coating of selected type (i.e. hydrophobic, ion-exchange, metal-binding, etc.), on which sample components of a given type are captured. Unbound compounds are washed off, thus contaminants are removed and sample complexity is markedly reduced. After application of

a proper energy-absorbing matrix, such as CHCA or SA, proteins bound to stationary phase are analyzed for MS profiling. The company also released chips with covalently bound matrices. Thus, background in the low *m/z* range is eliminated and small molecules can be successfully analyzed by this technique.

The great advantage of SELDI lies in its ability to remove salts and other impurities prior to MS analysis, thanks to which crude sample can be analyzed, such as urine [376,377], cerebrospinal fluid [378,379], serum [380,381], etc. However, sample preparation steps such as denaturation or depletion of high-abundance proteins may be considered [382] (compare Section 2.3). For reviews on the application of SELDI in proteomic profiling, see [383].

3.4.3. Electrospray ionization

Electrospray (ESI) is a "soft" method of ionization, which rarely promotes spontaneous fragmentation of analytes. However, electrospray is often coupled with ion trap or quadrupole analysers as this combination enables efficient peptide sequencing by induced fragmentation (MS/MS). This makes ESI well suited for peptide identification in complex mixtures, such as protein digests. In an ESI ion source the ionization process occurs under atmospheric pressure and the sample is introduced as liquid, therefore electrospray may be coupled directly to liquid chromatography systems. These features make ESI a useful tool in proteomics.

As already mentioned, the ESI ion source is sensitive to inorganic salts that cause signal suppression and adduct formation, lowering sensitivity and signal-to-noise ratio. Additionally, some inorganic salts, such as phosphates, could precipitate in the heated capillary of the ESI source leading to its permanent damage. Certainly, any solid particles (also from the bleeding chromatographic columns!) in the analyzed samples must be strictly avoided. In proteomic applications, the positive ion mode is mostly used, in which ionization is based on protonation of the analyte molecules, therefore the sample is often acidified, e.g. with formic or trifluoroacetic (TFA) acids, to facilitate ion formation.

Desalting is the main stage of sample preparation for ESI measurement (a list of accepted salt concentrations can be found in [322]). For direct sample infusion, in which sample is pumped into the ion source through a syringe pump, typical desalting strategies may be used, as described in Section 2.3. Nevertheless, such high-volume approach is rarely used in proteomics where only minute amounts of sample are typically available.

In many, if not most, ESI-MS-based proteomic studies, the spectrometer is coupled on-line to a chromatographic system, where contaminants are efficiently removed. Chromatographic separation also lowers sample complexity, which makes the analysis more sensitive for low-level components. In such case, however, final eluents must be chosen carefully so that they do not interfere with ionization. Usually sample components are introduced into the ion source after they are eluted from the reversed-phase (RP) column, in which water/organic solvent systems are used that are easily accepted by the ESI source. Typical organic solvents used in RP separations include methanol or acetonitrile with an ion-pairing additive to strengthen the analyte-stationary phase interaction. A list of various ion-pairing agents and their extensive characteristics, including pros and cons of the use of most popular TFA, can be found in [384]. Based on a sound literature overview, the author concluded that, despite its drawbacks, TFA is probably the best ion-pairing agent available. On the other hand, TFA may cause significant signal suppression in the mass spectrometer, thus decreasing sensitivity of measurements.

Direct coupling of ESI-MS with other types of chromatography (i.e. ion exchange, size-exclusion, affinity, etc.) is less frequently used [384], since they require non-volatile inorganic buffers. Nevertheless, these types of chromatography are often used in biochemical purifications (using volatile buffers that overcome such problems) as they preserve the function of proteins, therefore, in order to facilitate ESI-MS analysis of eluents, on-line desalting methods were developed based on microdialysis [385], or other principles [186,386].

For the efficient analysis of highly complex proteomic samples, non-ESI-compatible chromatographic techniques may be combined with RP separation and electrospray detection. In particular, strong cation exchange (SCX) chromatography is usually used as a part of two-dimensional systems, where sample components are first separated on an SCX column and then transferred to the RP column. In this setup, although increasing salt concentrations are used to elute fractions from the SCX column, the analytes are then trapped on the RP stationary phase and eluted in an MS-friendly water/organic eluent system directly to the ion source. If complex protein digests applied to the first dimension already contain concentrated buffers that could interfere with binding to the SCX column, the use of an additional first-in-line RP precolumn might be considered for effective desalting. On-line hyphenation of 2D-LC system with ESI-MS, although still technically challenging, is the basis of the multidimensional protein identification technology (MudPIT) [387] and is more and more routinely applied to complex proteomic projects (see [388,389] for methodological reviews and [390–393] for recent applications). More detailed guidelines for sample preparation prior to LC separation can be found in Section 3.2.

Very recently, a new ionization technique was devised termed desorption electrospray ionization (DESI). Here the charged droplets of solvent are sprayed onto the analyzed object, so that molecules present on its surface are ionized [394]. DESI can be applied to solids, liquids (including complex biological samples) and adsorbed gases. In proteomics, proteins and protein complexes can be detected, and peptide maps can be analyzed including MS/MS fragmentation. Importantly, DESI apparently does not require sophisticated sample pretreatment and tissue sections could be analyzed directly. For liquids, such as urine or plasma, several microliters are deposited on an appropriate surface and left to dry before analysis. The applications of DESI cover, among others, clinical diagnostics, especially in dermatology, microorganism characterization and MS imaging. Up-to-date reviews of this promising technique can be found in [395,396].

3.5. Quantitative proteomics

Based on ESI ionization, quantitative proteomic analyses are possible to measure relative abundances of peptides or proteins in samples taken from biological fluids, cells or organisms in different states, e.g. controls versus diseased. Typically, components of one sample are labeled with the light reagent of standard isotopic composition, while components of the other sample are modified with the heavy reagent, enriched in stable heavy isotopes of a selected element, e.g. containing ^{2}H instead of ^{1}H atoms. Labeled samples are pooled, digested, subjected to additional prefractionation if needed (the order of operations may differ between methods), and analyzed by LC–MS. In the spectra pairs of peaks are observed whose *m/z* values differ proportionally to the molecular weights of the labels, and whose intensities correspond to relative abundances of the peptides/proteins. For in-depth description of quantitative proteomics and its methods, see [397–400].

From the sample preparation point of view, the labels may be introduced to the sample in vivo during cell growth (metabolic labeling, SILAC—see below) or after proteins have been isolated from the biological material (enzymatic labeling, ICAT, iTRAQ). Potential errors in quantification may result from differences in preparation of samples to be compared, e.g. during protein isolation from crude biological material, their concentration or fractionation. These errors can be minimized if the number of operations is limited, or if as many operations as possible are conducted on pooled samples.

In one approach of in vivo labeling, compared cell lines are grown on media containing only either ^{14}N or ^{15}N isotopes [401]. The function of the isotope labels may also be served by modified amino acids (stable isotope labeling by amino acids in cell culture, SILAC). In the latter and more popular approach, one population of cells is grown on a standard medium, containing all necessary amino acids in their typical isotopic compositions, while the other population is grown on a medium containing a selected amino acid labeled with heavy atoms, e.g. Leu-d_3 instead of Leu-d_0 [402] but other amino acids can also be used [403,404]. Simultaneous analysis of three distinct states by differentially labeled arginine derivatives was also performed [405], and the heavy metal SILAC approach was used for identification and quantitation of methylation sites [406]. In both these in vivo methods protein isolation, proteolytic cleavage and chromatographic separations are performed on pooled samples, which helps minimize errors.

Among in vitro labeling methods, probably the most straightforward and universal is enzymatic labeling, where one of the samples to be compared is proteolyzed in the isotopically enriched $H_2{}^{18}O$ instead of usual water [407]. This approach does not require any special sample preparation compared to routine proteomic strategies but has some limitations as the mass of peptide increases. Lower resolution at higher *m/z* does not allow for a satisfactory separation of the peak envelope.

On the other hand, popular isotope-coded affinity tag (ICAT) technique is a multistage process, in which the heavy reagent contains eight deuterium atoms instead of eight ^{1}H atoms and labeling occurs via a thiol-reactive group [408]. Labeled samples are pooled and proteolyzed. Derivatized tryptic peptides are then isolated from by affinity chromatography thanks to the presence of biotin moiety in the ICAT reagent molecule, and analyzed by LC–MS. However, biotin might obscure the analysis by shifting peptide masses to a higher mass range and by generation of additional fragments in MS/MS spectra. In an updated version of the ICAT experiment, the labeling reagent was immobilized on solid support by a photocleavable linker so that modified peptides could be more easily separated by filtration and bond cleavage [409]. A drawback of all proton/deuterium containing labels stems from differences in their behavior in RP chromatography, as deuterated species elute a bit earlier than corresponding ones, containing only ^{1}H atoms [410]. These problems were addressed by the introduction of $^{12}C/^{13}C$ labeling system in which, additionally, the biotin tag may be easily removed from the labeled peptides in acidic environment after they have been isolated by affinity chromatography (cleavable ICAT), in order to lower the overall size of the tag prior to MS analysis and improve fragmentation efficiency. This approach proved effective in the analysis of complex proteomes [411]. What is more, the synthesis of further types of ICAT reagents was reported [412,413].

Recently, the isobaric tags for relative an absolute quantitation (iTRAQ) were developed by which four samples can be analyzed simultaneously [414]. This allows for analyzing sample composition, e.g. in different time points or in duplicates. Proteolytic peptides are labeled with amine-specific isobaric tags which, upon induced fragmentation in the mass spectrometer, yield different reporter ions in 114–117 *m/z* range. Reporter ions have different isotopic compositions and hence molecular weights but thanks to balance groups the tags are isobaric and no shift in mass spectra is observed for different ions, which simplifies the analysis, including the MS/MS measurement. Although this technique is relatively new, it was applied in several interesting projects [415–418]. An informative recent overview can be found in [419].

An absolute quantification (AQUA) strategy was also developed, in which a synthetic standard peptide is introduced into the cell lysates at known concentration [420]. The peptide contains stable heavy isotopes and mimics a tryptic peptide present in the protein of interest. The selected reaction monitoring (SRM) measurement during MS analysis allows detection and quantitative assessment of the native peptide, compared to the isotopically enriched standard.

3.6. Imaging mass spectrometry

Imaging mass spectrometry is a new tool for revealing the spatial distribution and relative concentration of compounds in biological samples such as tissue sections. It seems to be a valuable method in comparative studies, where profiles and images of tissue sections in different stages could be matched. It enables to find the differences in protein/peptide expression between those stages, without the necessity of knowing which proteins have changed. Because of that, this technique seems to be very useful for biomarker discovery [421].

Development of MS imaging was possible only due to advantages of mass spectrometers with MALDI ion source [422,423]

or TOF SIMS (Secondary Ion Mass Spectrometry) [424] for peptides and smaller molecules. It allows for soft ionization of the components of the sample, which means that the molecules which possessed relatively high masses, like proteins (but also peptides), could be observed. Moreover, the ionization is usually not multicharge, as ESI source, so relatively simple spectra of complex mixtures can be obtained. Another advantage of this technique is its great sensitivity, which means that even proteins present in the femtomole range could be detected.

In MS imaging the thin section of tissue, or the membrane with direct tissue blotting, is placed on a MALDI target plate, then the matrix is applied on the surface. Inside the spectrometer, laser beam causes the ionization of proteins, which are present exactly in this particular section, and in the place where the laser beam is focused. For every such point a spectrum could be obtained. The mass to charge ratio can range from 1000 to more than 100,000. Using this method, a profile of a given spatial point on the surface of the tissue section could be generated and then, from the intensity of a given *m*/*z* value monitored in each spectrum, a density map or image could be constructed. Virtually, all signals from the section could serve to generate a specific density map for this section. Proteins of interest can be then identified based on its peptide map, after isolation and trypsin digestion.

All those steps lead to create specific molecular image of the tissue, which provides useful information about local proteomic/peptidomic composition, relative abundance and spatial distribution of the components in the tissue. A more detailed description of the method can be found elsewhere [425–427].

Sample preparation is a crucial step in MS imaging. Especially, it is important to maintain the integrity of the spatial arrangements of all compounds. Any mistake done here may cause delocalization and degradation of the analytes. At the beginning, it is essential to surgically remove the tissue samples very carefully to retain its native shape. Fresh tissue samples must be frozen immediately after dissection. Usually liquid nitrogen is used here. The sample, loosely wrapped in aluminum foil should be gently lowered into the liquid gas over a period of 30–60 s. The foil helps to stabilize more fragile fragments of tissue and to protect it against the adhesion to the container walls. Sample prepared in this way may be stored at −80 °C until analysis.

As it was mentioned before, in the MS imaging experiment, tissue sections may be examined directly or a protein blot may be analyzed. Material obtained from the laser capture microdissection experiment could also be used [12]. The blotting procedure is a quick and easy way to generate global protein profiles from a given tissue and the obtained protein profiles seem to be reproducible between animals of the same strain [428]. Proteins can be transferred from the fresh tissue by contact blotting on an active surface, such as C_{18} [429], or a carbon-filled polyethylene membrane [430].

During this procedure, the membrane is wetted in methanol for 30 s and then attached to the target plate by a conductive tape. Fresh cut tissue section is placed on the membrane and contact blotting is performed for 5 min. During this time, the tissue should be covered by a glass slide to avoid drying. Before matrix deposition, the blotted area should be rinsed with water, to remove tissue fragments, cell debris, blood, and salts. Caution must be taken here, because more hydrophobic proteins and peptides that are weakly bound to the membrane could be lost [430].

Apart from blotting, MS imaging could be performed directly on thin tissue section, which eliminates the problems associated with blotting procedures. Thin tissue sections are obtained from the snap-frozen tissue samples using a cryostat. The thickness of the slices is not critical and can be adjusted to assure easy handling. Typically, for mammalian tissue, the thickness should be about 10–20 μm, i.e. in the order of mammalian cell diameter, so the majority of the cells are cut open, exposing intracellular contents. The temperature of the sample during cutting should be kept between −5 and −25 °C. The exact value depends on the tissue type (for example, fatty tissues require lower temperature to obtain high-quality sections). Usually, slicing is performed at −15 °C and slices are 12 μm thick.

In traditional tissue sectioning before cutting, the tissue is embedded in the optimum cutting temperature polymer (OCT) or agar, which stabilizes the tissue and provides its smooth surface. It is important to avoid cutting the OTC with the cryostat blade, as this could leave a thin film of the polymer on the top of the section, which may cause poor MALDI-MS analysis.

Tissue slices are then thaw-mounted on the target plate. The preferred way to do it is to put the tissue section on the cold target plate and then quickly warm them together. With this method there is no loss of water-soluble proteins. It is important to transfer the sample very carefully to the target plate to preserve its native shape. Slices are then allowed to dry in a vacuum desiccator for 1 h [421,430,431].

Cells obtained by LCM may also be the source of sample for imaging MS analysis. Following microdissection, polymer with adhered cells may be placed directly on a target MALDI plate [12].

As already mentioned, to obtain the spectra, matrix must co-crystallize with the sample, so it has to be deposited on the surface of the tissue section, or blotted area. Three main conditions have to be fulfilled to obtain the high-quality images. First, the process of covering the surface of the sample with the matrix cannot change the native localization of proteins. Second, matrix solution has to wet the tissue surface in order to form crystals with the proteins. Last, the size of crystals must be smaller than the image resolution [421].

There are two ways in which matrix can be placed on the sample surface: “drop deposition” method, and covering the surface with using a glass spray nebulizer. The simplest way is to deposit a drop of matrix using an automatic pipette, at a given coordinate of the section. Typically, about 100–200 nl of matrix is used.

In the glass spray nebulizer, about 500 μl of matrix solution should be sprayed on the section at the distance of about 15–30 cm. Then the surface is allowed to dry at room temperature. This spray cycle should be repeated up to 10 times with air-drying in between, to obtain homogenous crystal field. It is important to remember that excessive wetting of the sample should be avoided in order to protect proteins from delocalization.

Both methods give spectra of comparable quality but when high-resolution image is demanded, homogenous coating obtained by glass spray nebulizer is preferred.

Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, SA) (saturated solution in a solution of 50/50/0.1 acetonitrile, water and trifluoroacetic acid, v/v/v) seems to be the best matrix for higher molecular weight proteins. Replacement the acetonitrile with ethanol also gives spectra of high quality [432].

Samples are usually analyzed in the linear mode and mass spectra are usually at an average of 250–1000 laser shots aimed randomly at different positions across the spot. Usually, internal calibration is performed by the addition of proteins of known molecular weight.

Signals correspond to proteins and peptides found in given tissue. Mass to charge ratio is in a range from 2 to 100 kDa and signals represent a wide range of intensities within three orders of magnitude. To generate a map of signals, the section surface is covered by grid and each spectrum is taken at each grid coordinate with image resolution limited to the laser spot size, which, for commercially available instruments, is about 50 μm in diameter.

As examples of application of this new proteomics technique, we could mention imaging of the rat brain [421] and searching for tumor-specific biomarkers in colon and prostate cancers [433]. This kind of study might have a great importance for clinicians because it could permit molecular assessment of tumor biopsies, with the potential to identify subpopulations of cells that are not based on the cellular phenotype determined microscopically. In the future, assessment of surgical margins could be possible due to molecular assessment using MS imaging [434].

4. Conclusions and future prospects

The results published during recent years suggest that proteomics is still in its infancy. There is no standardized strategy for samples preparation, proteins purification and separation. Moreover, various mass spectrometers are used for proteins identification, and each instrument and laboratory produces its own set of more or less specific data. Despite the methodological problems, bioinformatics is still the Achilles tendon of the global analysis of proteins. In particular, a mixture of peptide maps that belongs to several proteins cannot be identified using presently existing tools.

Future prospects will keep in focus systems biology where the entire networks are identified for faster and more efficient diagnostics dedicated for individual patients (personalized medicine).

The best strategy for the future would be NO SAMPLE PREPARATION at all, but today instrumentation (mass spectrometers) do not allow for direct identification/quantitation of hundreds of proteins present in complex biological mixtures. Until then, a high-throughput strategy must be involved, with an increasing number of microchip technologies (lab-on-the-chip), simultaneously and automatically preseparating a large number of components. Such process will certainly be more focused on isolation of organelles, dedicated proteomics/peptidomics such as phosphopeptides, glycated proteins and other post-translational modifications, as they play vital role in signal transduction. The goal is no longer proteins identification but a better recognition and understanding of global processes, occurring within cells. Preparation of the samples dedicated for such purpose should also be mild and suitable for extraction of non-covalent complexes between proteins, peptides, nucleic acids and metabolic products. Application of activated surfaces, e.g. SELDI concept, might also be a future direction for proteins prepurification directly on a MALDI plate. Two-dimensional gel electrophoresis might soon be replaced or at least limited, by a combination of other methods such as isoelectrofocusing in solution, followed by, e.g. one-dimensional PAGE and capillary LC–MS/MS. This approach might gain better reproducibility and much better dynamic range of proteins to be identified. In general, a number of optimized procedures are necessary to compare data between laboratories, and to gain good reproducibility.

Another question is availability of the highly advanced instrumentation and expertise, often not available in the university laboratories. Therefore, it might be advisable to establish well-equipped core facilities while samples preparation and preseparation procedures will be performed by the end-users. As the direction of proteomics switches from simple identification of proteins to differential proteomics and functional studies of possible disease markers, there is no need for the end-user to keep equipment and staff to identify protein profiles just for one particular project.

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