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Proteomics in nutrition research: principles, technologies and applications

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The global profiling of the whole protein complement of the genome expressed in a particular cell or organ, or in plasma or serum, makes it possible to identify biomarkers that respond to alterations in diet or to treatment, and that may have predictive value for the modelling of biological processes. Proteomics has not yet been applied on a large scale in nutritional studies, yet it has advantages over transcriptome profiling techniques in that it directly assesses the entities that carry out the biological functions. The present review summarizes the different approaches in proteomics research, with special emphasis on the current technical ‘workhorses’: two-dimensional (2D)-PAGE with immobilized pH gradients and protein identification by MS. Using a work-flow approach, we provide information and advice on sample handling and preparation, protein solubilization and pre-fractionation, protein separation by 2D-PAGE, detection and quantification via computer-assisted analysis of gels, and protein identification and characterization techniques by means of MS. Examples from nutritional studies employing proteomics are provided to demonstrate not only the advantages but also the limitations of current proteome analysis platforms.

Proteomics: Two-dimensional gel electrophoresis: Mass spectrometry: Principles: Examples

Every nutritional process relies on the interplay of a very large number of proteins that are expressed at the level of the cell, the organ or whole organism. Alterations of mRNA levels, and in turn of the corresponding protein levels (although these aspects of gene expression do not necessarily change in parallel), are critical parameters in controlling the flux of a nutrient or metabolite through a biochemical pathway. The overall composition of the diet, the levels of specific nutrients and non-nutrient components of foods, and indeed many other aspects of life-style can affect essentially every step in the flow of genetic information from gene expression to protein synthesis and protein degradation, and thereby alter metabolic functions in the most complex ways. The proteome is the whole protein complement of the genome expressed in a particular cell or an organ. Proteome analysis (‘proteomics’) allows both changes in protein expression patterns, and the identity of the proteins themselves, to be displayed and determined simultaneously. Moreover, for individual proteins, post-translational modifications that may be crucial for biological activity and even amino acid substitutions (polymorphisms) can be detected. The potential value of proteomics for the nutritional sciences has been recognized for some years (Trayhurn, 2000). But, in contrast to the techniques for large-scale transcriptome analysis that are already used by the nutrition research community and which have led to numerous

published studies, only a very few reports have described the use of proteome analysis as a tool in nutrition research. With the present article we hope to introduce the general concepts, techniques and potential applications of proteomics to a broader section of the nutrition research community.

The extraction, display and analysis of the individual proteins of tissues and cells together comprise a complex multistage process, involving a variety of biochemical and biophysical principles. This is a rapidly developing field, and new and improved techniques continue to emerge. However, two-dimensional (2D) gel electrophoresis, coupled with spot analysis by MS, is still the most widely used technical approach. In the present review we consider each of the main steps in proteomic analysis, from sample preparation through the various aspects of electrophoresis to protein identification. We then briefly review some recent publications to illustrate the various ways in which proteomic approaches can be applied in nutrition research.

Sample preparation

The protein preparation is a critical step in the analysis of the proteome, since both the quality and the quantity of the protein isolate determine the reliability and reproducibility of the

Abbreviations: 2D, two-dimensional; ESI, electrospray ionization; IEF, isoelectric focusing; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; M_r , molecular weight; PMF, peptide mass fingerprint; SELDI, surface-enhanced laser desorption/ionization; TOF, time-of-flight.

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findings. Both the source of the biological material available, such as body fluids, tissue samples or cells, and the principal goal of the proteome analysis itself determine which protein isolation technique is most suitable (Dunn & Gorg, 2001; Shaw & Riederer, 2003). Different techniques for protein isolation and separation are available (Shaw & Riederer, 2003), depending on whether the goal is to separate as many proteins as possible from a given sample or to isolate a certain subset of proteins (i.e. from a distinct cellular compartment or a subclass such as phosphoproteins). The analysis of proteins from liquid biological samples like serum, plasma, urine and cerebrospinal fluid, or from aqueous extracts of cells and tissues, requires generally fewer preparative steps than are needed for solid tissue samples. A special problem is encountered in plasma/serum containing proteins of high abundance, such as albumins and Ig, which make analysis of minor protein components extremely difficult. However, for the enrichment of low-abundance proteins and the removal of high-abundance proteins prior to 2D-PAGE, new affinity columns are commercially available (Pieper *et al.* 2003; Thongboonkerd *et al.* 2004). High amounts of salts, as found for example in urine (Thongboonkerd *et al.* 2004), or very low protein concentrations, as in a sample such as tears (Joo *et al.* 2003), can cause problems as well. A desalting of the protein lysate by dialysis or protein precipitations, for example using TCA or acetone (Plymoth *et al.* 2003), may help to solve these problems.

Protein preparation from tissues and cells

The first step in preparing a protein extract from a tissue sample is mechanical disruption (Thongboonkerd *et al.* 2004). This is extremely important and therefore the tissue is usually processed with a liquid N₂-cooled mortar (Thongboonkerd *et al.* 2004), by sonication (Gorg *et al.* 2000) or using a glass/Teflon homogenizer (Carboni *et al.* 2002). The main problem with the analysis of tissue samples is the heterogeneity of the sample caused by the different cell types present in various quantities in a tissue (Rekhter & Chen, 2001). There are methods available for the enrichment of individual cell types by immuno-affinity separation techniques (Pieper *et al.* 2003). The isolated cells can then be used directly for 2D-PAGE. Another possible approach to obtaining a homogeneous cell population from heterogeneous tissues is provided by microdissection techniques. Laser-capture microdissection in particular is used widely to isolate a distinct cell population (Craven *et al.* 2002). By focusing a laser beam on the target area of the tissue, under direct microscopic visualization, the desired cells are caused to adhere to a polymer film via activation with laser pulses. The polymer film on the tissue is then lifted, so that the cells are liberated from the surrounding tissue. The technique makes it possible to conserve the native morphology of the cells, and the macromolecules can be extracted after microdissection for subsequent molecular analysis (Rekhter & Chen, 2001; Fuller *et al.* 2003).

Circulating cells such as lymphocytes (Vuadens *et al.* 2002) or cells from suspension cultures (Thongboonkerd *et al.* 2004) can be easily obtained by centrifugation, washed with PBS or an isotonic sucrose solution and diluted in a lysis buffer (Gorg *et al.* 2000). For adherent cells the medium has first to be removed before the cell layer is washed with PBS or sucrose solution, and the cells are harvested by scraping them off the support before dilution in lysis buffer (Fuchs *et al.* 2005a).

One of the major aims in proteome analysis is to conserve the native physico-chemical features of the proteins isolated without

changes in molecular mass by hydrolysis. However, often proteins need solubilization, disaggregation, denaturation and reduction (Shaw & Riederer, 2003; Thongboonkerd *et al.* 2004). Other technical influences that can alter the quality of separation based on isoelectric focusing (IEF) are associated with the necessity to remove nucleic acids, lipids, salts, polysaccharides, ionic detergents or proteases (Thongboonkerd *et al.* 2004). Nucleic acids in particular can cause problems due to an increase in sample viscosity that causes background smears (Garrels, 1979). Nucleic acids bind to proteins through electrostatic interactions and thereby impair focusing, whereas high-molecular-weight nucleic acids may occlude gel pores (Westermeier, 2001). Nucleic acids can be removed, for example with a mixture of protease-free RNases and DNases, or via precipitation of the proteins by TCA or acetone (Gorg *et al.* 2003). Membrane proteins are particularly difficult to separate by standard separation techniques since they form complexes with lipids which results in reduced solubility and changes in the charge and molecular weight (M_r). Moreover, the binding of lipids to proteins lowers the solubilizing efficacy of detergents. This problem can partially be overcome by a high-speed centrifugation step and newly developed but fairly expensive membrane protein enrichment kits (Gorg *et al.* 2003). However, these kits have so far not been proved to solubilize integral membrane proteins efficiently. Polysaccharides included in samples can occlude the gel pores, leading to precipitation or extended focusing times with horizontal streaking. Polysaccharides with negative charges bind to proteins by electrostatic interactions (Westermeier, 2001). A precipitation of the proteins with TCA or acetone not only eliminates the lipids but also removes the polysaccharides and nucleic acids (Gorg *et al.* 2003). SDS should not be included in the protein preparation process since, by its negative charge, the resulting protein complexes do not separate and focus during IEF (Westermeier, 2001). Finally, proteolytic activity in the sample can result in artificial protein spots; to minimize hydrolysis, samples should be kept cold at all times with minimal handling (Gorg *et al.* 2003). In addition, proteases can be inactivated either by precipitation of the sample with TCA/acetone (Gorg *et al.* 1997) or by the use of protease inhibitor cocktails (Thongboonkerd *et al.* 2004; Fuchs *et al.* 2005a).

Sample fractionation

The high dynamic range and diversity of proteins expressed in a sample may require a reduction in the complexity of the protein sample or a special enrichment of low-abundance proteins or of alkaline proteins that are difficult to separate (Gorg *et al.* 2000). Therefore, a pre-fractionation step of the raw sample usually provides a higher yield of low-abundance proteins (Huber *et al.* 2003). The available pre-fractionation methods are based on either the physico-chemical features of the proteins within a sample or a fractionation into different subcellular compartments. Sub-fractionation may be achieved by high-speed centrifugation, by sequential extraction procedures with buffers of increasing solubilizing power (Weiss *et al.* 1992, 1993; Herbert, 1999) or by precipitation (TCA, acetone; Gorg *et al.* 1997). It is also possible to use other separation methods such as free-flow electrophoresis (Corthals *et al.* 1997), or any kind of chromatography and/or affinity purification of protein complexes. Isolation of cell compartments makes it possible to focus on certain protein entities (Gorg *et al.* 2000). A further increase in resolution may be obtained by combining various fractionation procedures.

Two-dimensional gel electrophoresis

2D-PAGE separates proteins according to their two prominent physico-chemical properties: the net charge at a given pH and the M_r . In the first dimension, the proteins as isolated from a biological sample are separated by IEF according to the isoelectric points of the individual proteins. The second dimension, separating the proteins according to their M_r , is performed using classical SDS-PAGE.

Two-dimensional PAGE – first dimension

The introduction of immobilized pH gradients has caused a significant increase in the reproducibility of separations by IEF, and allows the use of narrow pH intervals (Bjellqvist *et al.* 1982; Gelfi *et al.* 1986; Gorg *et al.* 1988). The cup loading technique with a separated strip rehydration step is a widely used sample application procedure (Barry *et al.* 2003). Samples can also be applied by in-gel rehydration (Rabilloud *et al.* 1994), which offers the advantage that sufficient quantity of protein can be loaded from samples with low protein concentrations (Rabilloud *et al.* 1994). Various protocols for IEF-based separations have been described by Gorg *et al.* (1998, 2000).

Use of standard, wide-range (pH 3–10), immobilized pH gradient strips allows 1000–3000 protein spots to be resolved per gel (Hoving *et al.* 2002). For an increase in the resolution of protein clusters, or for the visualization of low-abundance proteins, narrow pH-range gels, called zoom gels, are frequently used (Wildgruber *et al.* 2000; Hoving *et al.* 2002).

Two-dimensional PAGE – second dimension

After an equilibration step of the immobilized pH gradient strip, which enhances the protein transfer from the first to the second dimension (Gorg *et al.* 1988), SDS-PAGE is run in a horizontal or vertical system with the standard buffer system of Laemmli (1970). For the horizontal second-dimension system, gels are fixed to a plastic support that prevents alterations in gel size during the staining process (Gorg *et al.* 1980). The vertical SDS-PAGE systems available are more suitable for high-throughput applications in comparative proteome analysis studies, as they allow multiple gels to be run in parallel, with a corresponding increase in reproducibility (Beranova-Giorgianni, 2003). In addition, they provide the possibility of working with homogeneous or gradient-resolving gels (Zhan & Desiderio, 2003). The use of an acrylamide gradient in the second dimension can increase the resolution of protein separation in a certain M_r range whereas the total range is essentially the same as with linear gels (Scheele, 1975). Protocols for SDS-PAGE are provided by Gorg *et al.* (1988, 2000).

Protein detection techniques

Organic dyes

Staining of proteins with colloidal Coomassie brilliant blue is still very popular (Chrambach *et al.* 1967; Patton, 2002) as it allows almost a background-free detection of proteins, a good quantitative linearity and compatibility with MS. Moreover, it is an easy-to-use and low-cost staining (Westermeyer, 2001; Patton, 2002). Coomassie brilliant blue staining occurs in an acid environment

that improves the ionic interactions between the dye and the basic amino acid moieties of the protein, and increases secondary dye–protein interactions based on hydrogen bonding, Van der Waals' attraction and hydrophobic interactions (Wirth & Romano, 1995).

Silver staining

Due to its high sensitivity, silver staining is used to visualize trace quantities of proteins of as small as 1 ng (Patton, 2002; Wirth & Romano, 1995). Three basic silver staining procedures exist: (i) diamine or ammoniacal silver stains; (ii) chemically developed non-diamine type; and (iii) photoreduction silver stains (Wirth & Romano, 1995). The AgNO_3 procedure can be modified in order to make it compatible with subsequent protease digestions by removing glutaraldehyde, which otherwise cross-links the proteins and thereby inhibits the required digestion needed for mass analysis (Shevchenko *et al.* 1996; Lopez *et al.* 2000). The disadvantages of the silver stains are the low specificity (Steinberg *et al.* 1996), the restriction to a tenfold linear dynamic range and the complexity of the methods, all of which reduce reproducibility. For example, staining has to be finished at an arbitrary time point in order to avoid over-development of the gels, and this often results in an unsatisfactory gel-to-gel reproducibility, with variations of up to 20% in spot intensities (Quadroni & James, 1999; Patton, 2002).

Fluorescence staining

Although the fluorescent stains, such as Nile Red or SYPRO Red and Orange, allow a high sensitivity, with a detection limit of about 5 ng of protein (Alba *et al.* 1996; Patton, 2002), their use is not as widespread as that of Coomassie brilliant blue staining. This arises because they are more difficult to handle, more expensive (Wirth & Romano, 1995) and require a fluorescent scanner or a charge-coupled device camera for detection (Patton, 2002). Fluorescent stains like SYPRO Red and Orange, however, offer a better specificity than silver stains, which also detect nucleic acids, lipids, lipopolysaccharides and glycolipids (Steinberg *et al.* 1996). In comparison with the silver staining methods, the fluorescent dyes possess a higher reproducibility, in particular in the quantification of low-intensity protein spots in a 2D gel profile. Moreover, they provide a better sequence coverage in subsequent peptide mass profiling (Patton, 2002).

Fluorescence-based difference gel electrophoresis

The difference gel electrophoresis technique offers currently the highest reproducibility, since up to three different protein samples, individually stained, can be run together in a single gel (Tonge *et al.* 2001). Prior to separation of the proteins by IEF, individual samples are labelled with different dyes, such as Bimane or CyDyes, that differ in excitation and emission spectra. By using a scanner that can excite the fluorescent dyes and measure their emission, proteins from different samples can be quantified within the same gel. The technique abolishes problems associated with shifts of the isoelectric points or molecular masses between independent gels (Unlu *et al.* 1997). However, fluorescence labelling is less sensitive than silver staining and requires

a costly fluorescence scanner or charge-coupled device camera system (Alba *et al.* 1996).

Image analysis

For computerized quantitative image analysis, the gels have to be transformed into a digital format. Therefore, gels are scanned by the use of modified document scanners, laser densitometers, charge-coupled device cameras, or fluorescent and phosphor imagers (Dowsey *et al.* 2003). To analyse the complex protein profiles of 2D gels it is necessary to use software that automatically detects the spots, corrects the background staining and quantifies the spot volumes. The software must precisely allocate identical spots in different gels, and determine the quantitative differences in all spots between various gels (Westermeier, 2001; Patton, 2002). Various software solutions and packages are available, providing quite different features and grades of automation in the analysis of gels. It is strongly recommended to seek expert advice from colleagues with experience before investing in the generally very expensive software.

As a minimal requirement for analysis we suggest that six gels be run from at least three independent protein preparations (two gels per sample). It is generally advisable to increase the number of samples, and not the number of gels per sample (less than or equal to three), for identification of homogeneous regulation of protein spots. The number of regulated spots between

samples or groups should be identified based on the significant level of differences, which is often calculated automatically by modern image-analysis software. If protein preparation and 2D-PAGE are well standardized, the images of gels and the numbers of protein spots identified from individual samples separated under identical conditions are highly reproducible. This is shown in Fig. 1 for protein extracts prepared from peripheral blood mononuclear cells of six different human volunteers separated by 2D-PAGE with a pH gradient from 3 to 10 in the first dimension and a mass range of 20 to 200 kDa in the second dimension. The software (ProteomWeaver; Definiens AG, Munich, Germany) identified between 420 and 485 proteins above background in the individual samples, demonstrating the low coefficient of variation between samples (D Fuchs, R Piller, J Linseisen, H Daniel and U Wenzel, unpublished results).

It should be kept in mind that the number of proteins identified in a gel depends on many variables such as the pH gradient for the first-dimension separation, the second-dimension mass range, the loaded protein quantity and the staining type. For example, in an analysis of the rat liver proteome, we identified on average 709 (SD 13) protein spots in a gel with pH 3 to 10 in the first dimension and a 20–200 kDa mass range in the second dimension (tom Dieck *et al.* 2005). Using the same samples, but with a required higher protein load and separated in a narrower pH range of 6 to 11, about 750 proteins were detected. When separated in a pH range of 4 to 7, on average more than 900 proteins could

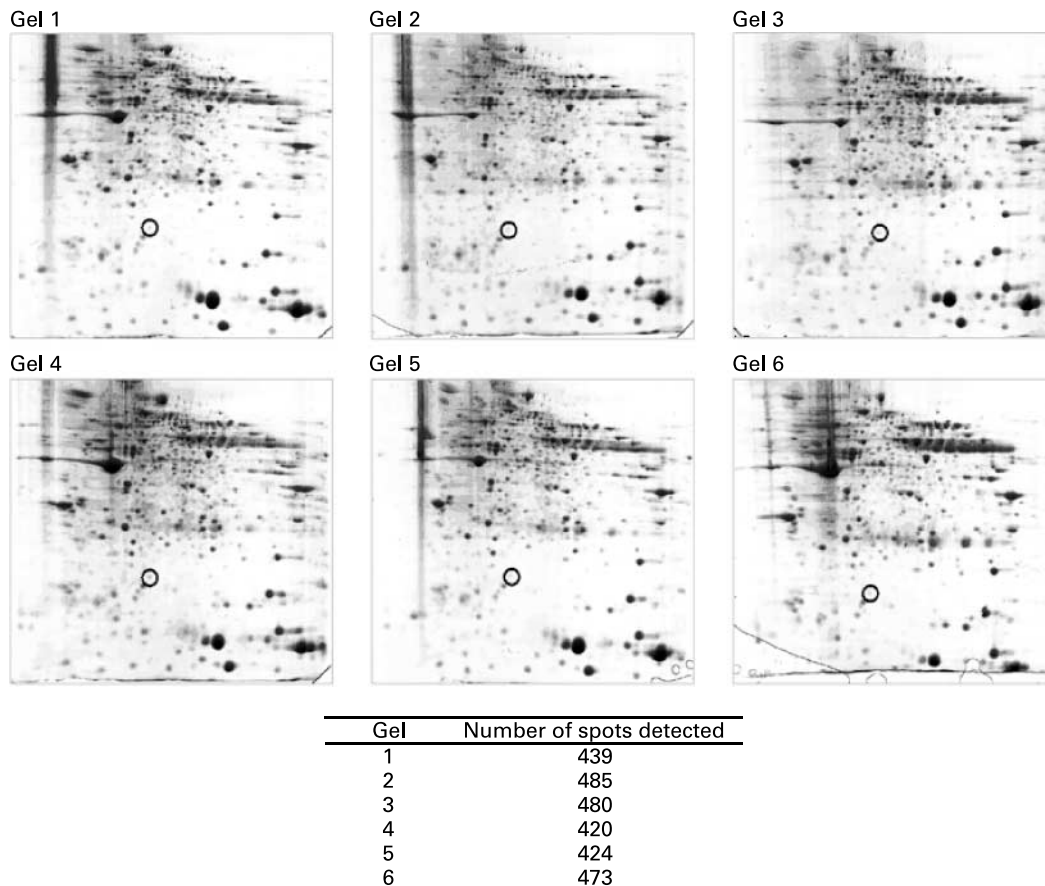


Fig. 1. Scans of two-dimensional gels of protein extracts prepared from human peripheral mononuclear cells of six individual volunteers and separated under identical conditions.

be detected in the same samples (D Fuchs, R Piller, J Linseisen, H Daniel and U Wenzel, unpublished results). Defining the proper separation conditions with highest resolution and the best sensitivity depends on the amount of protein available, and is essentially a trial-and-error optimization procedure.

Protein identification via MS: principles and components

Basic principles

MS was first successfully introduced in the 1940s for the purification and accumulation of nuclear isotopes, and for the analysis of volatile and low-molecular-weight substances, i.e. from petroleum (Polednak & Frome, 1981). In 1946, the concept of the time-of-flight (TOF) technique was introduced (Stephens, 1946) and the world's first commercially produced mass spectrometer became available in 1948. It was based on the electronic ionization technique and had a limited resolution with a maximum mass range of 300 Da. In the 1950s, quadrupole techniques and the coupling of GC with MS (GC-MS) were developed (Gohlke, 1959). At this time it was possible to analyse substances with a maximum mass of 1–2 kDa. In the late 1980s, Karas & Hillenkamp (1988) and Tanaka *et al.* (1988) were able to identify and define the masses and structures of the first larger biomolecules, by inventing a method that combined matrices with the laser desorption/ionization technique and with TOF mass analysers, leading to the matrix-assisted laser desorption/ionization (MALDI)-TOF technique.

The most commonly used tools for proteome analysis are 2D-PAGE followed by MS via MALDI-TOF for protein identification via peptide mass fingerprint (PMF). Protein degradation by residue-specific cleavage into peptides is performed mainly enzymatically. The peptide masses can be acquired and afterwards matched against theoretical peptide libraries generated from the proteins as deposited in protein sequence databases. Due to the increasing demand for high-throughput proteome analysis, automated mass analysis and protein identification tools are already available (Gorg *et al.* 2000; Chamrad *et al.* 2003). Proteome analysis chips that utilize surface coatings to enrich certain protein species from a sample are now available. When used in combination with MS (surface-enhanced laser desorption/ionization (SELDI)-TOF) these appear to be an alternative to classical 2D-PAGE, but the chips currently lack reproducibility and are far less well validated than DNA chips, for example (Phelan & Nock, 2003; Jain, 2004).

MS in general utilizes the differences in the mass-to-charge ratio (m/z) of ionized compounds or molecules for their separation in a high vacuum. The ionization of the analyte is obtained through absorption or release of an electron. The ability to identify an unknown compound, determine its structure, and define its chemical characteristics in quantities as low as 1 pg, makes MS a universal tool. For the analysis of heterogeneous mixtures, the mass spectrometer can be combined as a detector with GC (GC/MS) or liquid chromatography (LC/MS).

The main components of a mass spectrometer are an ion source, which generates the ions, a mass analyser where these ions are separated, and a detector delivering the mass spectrum. After application of the various existing ionization and desorption methods used upon the molecules or peptides (ions, atoms or photons), generated ions will be dissociated in the vacuum of the mass analyser based on their m/z value.

Table 1 lists the key and most frequently used components of mass spectrometers.

Ion sources

The ion sources predominantly used in bioanalysis, especially in peptide and protein analysis, are electrospray ionization (ESI) and MALDI.

Electrospray ionisation. ESI is a soft ionization method but the analytes have to be soluble in a low-boiling matrix (e.g. acetonitrile, methanol). A solution of the analyte–matrix mixture is sprayed under atmospheric pressure through an LC capillary into a strongly charged field. The high voltage between the tip of the capillary and the obverse electrode is responsible for the fine nebulization and ionization of the analyte molecules. These molecules are loaded with one or more protons (or Na^+ , K^+) coming from the solvent, while the charging depends on the surface of the analyte and therefore represents its secondary or tertiary structure. In this case there are also quasi molecular ions produced, and fragmentation is suppressed. ESI is an applicable method for analysing polar and large biomolecules, polymeric structures and metal complexes that are difficult to vaporize and to ionize. ESI also makes it possible to monitor the folding or unfolding of proteins (Hamdan *et al.* 2001). Because solvents can be used, ESI can be easily combined with HPLC or capillary electrophoresis (Johnson *et al.* 2001) for separation prior to mass analysis.

Matrix-assisted laser desorption/ionisation. The MALDI method is a soft, pulsed laser-ionization method that can be used for analysis of intact biomolecules, like proteins, after one-dimensional or 2D gel electrophoresis separation, and for gathering their sequence or molecular weight information. Methods developed by Tanaka, Hillenkamp and co-workers allow the embedding of the analyte into an appropriate organic matrix and a laser pulse (usually an N_2 laser) with a duration of 1–15 ns ablates material from the surface of a matrix–analyte mixture spotted on a metallic target. The laser energy is absorbed by the small organic molecules of the matrix that are dashed out of the spotted surface, carrying along the analyte molecules. After the subsequent desorption into the vacuum, the photoionized radical matrix molecules are able to ionize the analyte molecules through proton transfer. The protonated analyte molecules are accelerated in the electrostatic field towards the mass analyser. Because of its high sensitivity and a minimal fragmentation of the analyte, MALDI is the method of choice for large

Table 1. General components of mass spectrometers for proteome analysis

Ion source	Mass-selective analyser	Ion detector
Electron impact ionization (EI)	Magnetic sector	Faraday cup
Chemical ionization (CI)	Electric sector	Micro channel plate
Fast atom bombardment (FAB)	Quadrupole	Secondary electron multiplier (SEM)
Electrospray ionization (ESI)	Electric ion trap	
Matrix-assisted laser desorption ionization (MALDI)	Ion cyclotron Time-of-flight (TOF)	

biomolecules (proteins, oligonucleotides, DNA, RNA), big synthetic polymers up to 500 kDa (or even 1000 kDa) and polar components (Rehm, 2002; Marvin *et al.* 2003).

Mass-selective analysers

Magnetic sector. Generated ions enter a curved flight tube located between the poles of a magnet and are accelerated to a high velocity. By varying the strength of the magnetic field over the measuring time, ions with a specific m/z ratio passing through this sector, and the whole flight tube, reach the detector while the others are deflected. The detector records the prevailing strength of the magnetic field during the impact of an ion. There are single-focusing analysers available where beam paths with 60, 90 or 180° are used, or double-focusing analysers where an electrostatic analyser is added for separation of ions with different kinetic energies. Double-focusing systems offer several advantages including high resolution, accurate mass determination and a large mass range, but they are very large and costly. The resolving power and exact mass determination, however, are superior to other mass analysers. The dynamic range is good and the potential for MS/MS analysis is excellent when used as the first stage of a hybrid or four-sector instrument. Magnetic sectors are not well suited for pulsed ionization techniques such as MALDI, but can be applied together with ESI (Loo & Muenster, 1999).

Electric sector. An electric sector analyser focuses ions according to their kinetic energy. A capacitor is generated out of two conducting sheets and creates a radial electrostatic field. By changing this electrostatic field, ion beams with different energies are detected while others are deflected. In combination with the magnetic sector a double-focusing system is achieved.

Quadrupole. In this type of mass analyser only electric fields are responsible for the separation of the ions based on their m/z values. The quadrupole is composed of four parallel, symmetrically located, hyperbolic-shaped rods, where the ions pass through their centre for separation. The poles (with different charges next to each other) are working with fixed direct currents and alternating radio-frequency voltages. Only ions with a special m/z value can be focused on the detector when a particular produced electric field is maintained, while the other ions will be diffracted into the rods. A mass spectrum consisting of diverse ions is obtained by altering the strength and frequency of the electric fields. Quadrupole analysers are often combined with ESI, and the obtained maximum mass range amounts to 4 kDa.

Electric ion trap. In the ion trap, the electric fields are generated between three electrodes in order to trap the ions into a small volume. This technology is of compact size and allows an enhancement in the signal-to-noise ratio of a measurement via accumulation of the ions. The trap consists of a ring electrode, which separates two hemispherical electrodes. By changing the electrode voltages and thus ejecting the ions from the trap, a mass spectrum can be acquired. This technique is characterized as 'dynamic' trap.

Ion cyclotron. Contrary to the electric ion trap, ion cyclotron resonance mass spectrometers are seen as 'static' traps. In this system, ions are forced through a homogeneous magnetic field in a circular path with a mass-dependent rotational frequency. The ions have to be brought into phase with an excitation pulse, and by measuring the cyclotron frequency their mass can be determined. Ions with different masses but the same m/z value would show the

same cyclotron frequency. By applying the Fourier-transformation technique, these effects can be eliminated.

Time-of-flight. In a TOF mass spectrometer the generated ions are extracted and accelerated from the source into a field-free drift zone within the instrument, by an electric potential that is applied across the source. Within this zone the analyte ions are separated according to their m/z values. The span of time (time-of-flight) between the acceleration process of the ions and their impact on the detector is measured very exactly. From the acceleration voltage and the length of the drift region, the m/z value, and therefore the mass, can be calculated. The TOF technology can be operated in two different modes, linear or reflector mode. The latter is able to compensate for the possibility that ions with the same mass can start with different kinetic energies and therefore with a different velocity; thus the mass resolution is increased by using the reflector mode. Almost all MALDI systems are using the TOF or TOF/TOF technique, which is well suited for pulsed ionization methods, and obtains the highest practical mass range within all mass analysers.

Ion detectors

Faraday cup. Because the construction of the Faraday cup detector is relatively simple, it is generally more robust and reliable than the secondary electron multiplier (see below). It consists of a metallic cup placed in the path of the ion beam, where the electric potential is held constant. The incident ions strike the dynode surface and the associated (high) resistance emits electrons for neutralization; the resulting current is amplified and recorded. The attached electrometer provides an absolute measurement of the electron-beam current. Its sensitivity is constant throughout the measuring time, and not mass-dependent. This detector is well suited for isotope analysis but its detection sensitivity is lower than that of the secondary electron multiplier.

Microchannel plate. This detector is composed of glass capillaries coated in the interior with an electron-emissive substance. These capillaries (inner diameter: 10–25 μm) are held under high voltage, and an incident ion striking the surface releases a cascade of secondary electrons, which produce a measurable current. Through this effect the increase of the primary impact can be 10^3 - to 10^4 -fold.

Secondary electron multiplier. In this device, ions hitting the surface (composed of half conductors) of the initial amplification dynode cause an emission of secondary electrons which are accelerated towards other surfaces, thus creating an electron cascade by generating more secondary electrons in a repeating process. The fundamental principle of the Faraday cup is extended here because there is not just one, but a series of dynodes with increasing potentials in use, resulting in drastic signal amplification of the order of 10^6 .

Analysis of proteins or peptides by matrix-assisted laser desorption/ionization–time-of-flight MS

There are different techniques available for the purification of complex protein mixtures prior to MS. Amongst the most commonly used are LC and GC. A quite effective and fast method of sample purification is achieved via a reversed-phase nanocolumn. Here the analyte molecules are eluted with a matrix solution directly onto the MALDI target after sample loading on

the column, with subsequent washing steps (Gobom *et al.* 1999). During SELDI, complex mixtures of proteins are separated chromatographically on protein biochips. The chip-bound proteins are coated with the matrix for co-crystallization and, by a method similar in principle to MALDI, are then separated on the basis of their m/z value. There are many different chromatographic methods available for the enrichment of samples on chips, including reversed-phase or hydrophobic interaction, and ion exchanger matrices. Even immobilized metal affinity chromatography can be performed. Moreover, the surface can be coated with receptor ligands, DNA (to enrich DNA-binding proteins) or antibodies. With a protein chip system, antibody-antigen binding studies and peptide mapping can be accomplished. SELDI is appropriate for low-molecular-weight compounds (below 20 kDa) and can be seen as an addendum to 2D-PAGE. Samples gathered from serum, urine or tissue lysates are often processed by SELDI because here salt and detergents do not hamper analysis. SELDI chips are commercially available for example from Ciphergen (Fels *et al.* 2003). The main advantage of SELDI appears to be that it easily allows pattern analysis of a large set of samples in order to identify a characteristic protein pattern associated with, for example, a pathological situation.

MALDI is most frequently combined with TOF mass analysers, or the more recently developed and more sensitive tandem MS (TOF/TOF or MS/MS); the latter is also frequently combined with ESI. The MS/MS technique, where two mass analysers are connected in series, provides a tool for a detailed analysis of the chemical nature and/or structure of the biomolecule of interest. MALDI-TOF analysers also provide, by means of post-source decay, the possibility of obtaining further information about the analyte through ongoing fragmentation. This is applied most frequently for studying post-transcriptional modifications, such as phosphorylation of proteins or peptides, but also for sequence analysis of peptide fragments (fragmentation analysis and structural TOF). Post-source decay has become a

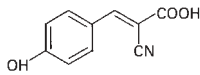
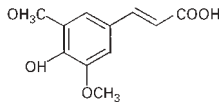
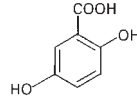
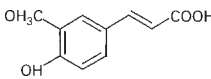
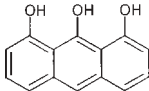
well established method in MS-based bioanalytics (Chaurand *et al.* 1999).

Applied aspects of matrix-assisted laser desorption/ionization-time-of-flight analysis in proteomics

The reliability and reproducibility of MALDI-TOF analysis depend strongly on quality assurance measures. As the detection methods are very sensitive, any contamination causes serious problems in analysis. Contamination of samples with dust, skin, hair or breath needs to be completely prevented. It is advisable to wear powder-free latex gloves, and it is essential to work in a clean environment.

Sample preparation and matrix. Protein spots identified by 2D-PAGE that are of interest for further analysis have to be excised from the gel, washed and digested enzymatically (usually with trypsin) or chemically (e.g. cyanogen bromide; Conrads *et al.* 2000). The excision may be done by hand with a skin-picker, or automatically with a robotic system as provided by various suppliers. When using Coomassie brilliant blue-stained gels, the excised spots have to be washed to remove coloration from the sample and to avoid any extra peaks during mass analysis. Washing is usually done with NH_4HCO_3 and acetonitrile before the spots are shrunk with acetonitrile and dried in a vacuum centrifuge. The dried spots can be stored at -20°C for some months or may be processed directly. When using tryptic digestion methods, one has to be aware that there are autolytically derived peptides produced from trypsin, with m/z values of 842.50, 1045.56 and 2211.10, which have to be masked out from the mass spectrum. After an in-gel digestion of the excised and dried protein spot, the released peptides need to be extracted, which is done using an acidic solution, mostly 1% trifluoroacetic acid. As in the case of the spot-picker, there is automated equipment available for washing and digesting the spots, for example the Ettan™ Digester from Amersham Biosciences. For the subsequent MALDI-TOF analysis, the sample containing the peptides is then spotted onto a metallic target by using a special matrix

Table 2. Selected matrices frequently applied for matrix-assisted laser desorption/ionization MS analysis

Matrix	Structure	Molecules suitable for
Cinnamic acid (4-hydroxy- α -cyanocinnamic acid, HCCA)		Peptides Proteins Oligosaccharides
Sinapinic acid (SA; 3,5-dimethoxy-4-hydroxycinnamic acid)		Proteins Polymers Glycoproteins
Gentisic acid (2,5-dihydroxy-benzoic acid, DHB)		Peptides, proteins Lipids Nucleic acid Oligosaccharides
Ferulic acid		Peptides Proteins
Dithranol		Synthetic polymers Lipids

from an existing assortment. Choosing the right matrix is crucial for MALDI analysis because the analyte is embedded into the crystals of the matrix molecules, absorbing energy at the wavelength of the specific laser. Selected matrices frequently applied for MALDI-MS analysis are given in Table 2.

The different matrices can be dissolved in diluted acetone or acetonitrile containing 0.1% trifluoroacetic acid. Benzoic and cinnamic acid, for example, seem to be a good choice for matrices when analysing proteins or oligosaccharides (Jespersen *et al.* 1998; Sigma-Aldrich, 2005).

The matrix in which the analyte molecules are embedded assists in the volatilization and ionization of the analyte molecules by absorbing the laser energy. The small organic molecules of the matrix, which are used in 100- to 10 000-fold excess of the analyte molecules, have therefore to be active at the specific laser energy wavelength. When using an N₂ laser this is at 337 nm, and with a Ne-YAG laser (yttrium-aluminium-garnet crystals doped with neodymium) this will be at 335 nm (tripled frequency) or 266 nm (four-fold frequency; Bruker Daltonik GmbH, 2002). Moreover, in order to serve as Brönstedt base or acid, the matrix should have similar resolution characteristics to the analyte (especially when the dried droplet method is applied), but a minimal affinity to protons. To prevent artifacts, there should be no chemical reactions between matrix and analyte, or between the analyte molecules themselves. Thus the analyte molecules are also protected against decomposition. When

the solvent is evaporated, a co-crystallization between matrix and analyte has occurred.

There are different methods used for spotting the samples on a special target. The most common one, preferably used for MALDI-TOF MS, is represented by the dried droplet method, which tolerates the presence of salts and buffers very well. In this case either a premixed matrix/protein solution is used (final protein concentration of 1–10 mM) or the two components are spotted one after another, starting with the saturated matrix solution. A droplet is placed on the target, dried at room temperature and the deposit is washed with 0.1% trifluoroacetic acid solution. Further methods are the fast evaporation method, multi-step perfusion chromatography and single-step perfusion chromatography (Gobom *et al.* 2001). The latter is a quite new modification of the multi-step perfusion chromatography, but faster and easier in handling and therefore more applicable for high-throughput applications (Choi *et al.* 2003).

Peptide mass fingerprinting. It is reasonable to perform internal or external calibration with a test series of protein fragments having known masses, such as bombesin, substance P or the adrenocorticotrophic hormone 18–39 clip. There are also internal calibration methods where the autolytic peptides of the enzyme used for digestion are selected, for example the trypsin fragments at *m/z* value 842.50, 1045.56 and 2211.10, which can yield a mass accuracy up to 20 ppm (Friedman *et al.* 2004). In

Matched peptides shown in grey and underlined

MEALPLLAAT TPDHGRHRL LLLPLLLFLL PAGAVQGWET EERPRTREE CHFVAGGOVY PGEASRVVA
 DHSLHLSKAK ISKPAPYWEG TAVIDGEFKE LKLDYRGKY LVFFYPYPLDF TFVCPTEIIA FGDRLEEFRS
INTEVVACSV DSQFTHLAWI NTPRQGGGLG PIRIPLLSDL THQISKDYGV YLEDSGHTLR GLFIIDDKGI
LRQITLNDLP VGRSVDETLR LVQAFQYTDK HGEVCPAGWK PGSETIIPDP AGKLKYFDKL N

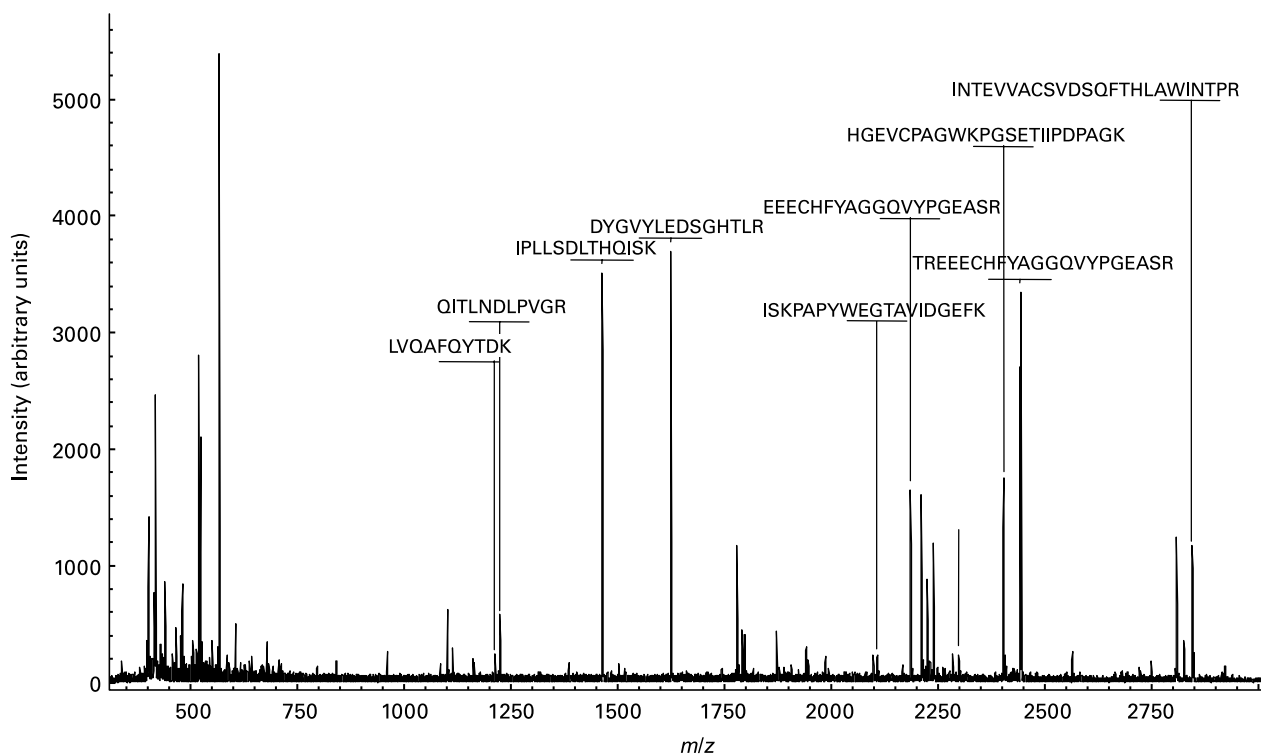


Fig. 2. Peptide mass fingerprint of a protein from human peripheral blood mononuclear cells. Sequence coverage and allocation of detected peptide fragments in the amino acid sequence identified the protein in the database as a putative thiol-specific reductase. *m/z*, mass-to-charge ratio.

Fig. 2, a mass spectrum obtained from a tryptic digest of a protein identified in human peripheral blood mononuclear cells by MALDI-TOF MS is shown.

A database search with the PMF of the analyte can be performed against different databases like MSDB, NCBIInr (<http://www.ncbi.nlm.nih.gov/>), PIR (<http://pir.georgetown.edu/>) or SwissProt (<http://us.expasy.org/sprot/>) using different search engines like Mascot (www.matrixscience.com), Prospector or ProFound (prowl.rockefeller.edu). With these different search engines, very convenient automation steps are possible. The validation procedures of the gained PMF differ between search engines depending on the internal algorithms used. The parameter settings should be based on the taxonomy of the sample species, the enzyme used for hydrolysis and the mass accuracy. It is also not advisable to allow more than one missed cleavage. It is recommended that each PMF analysis should be based on at least two separate digests from the same protein picked from two different gels. The most concise criterion for final protein identification in the database search is its score, with the level of significance for the match. Depending on the database and the taxonomy, these parameters may differ. As a second line, the match between the predicted molecular mass and *pI* value of the suggested protein, and the corresponding known mass and *pI* from its in-gel location, may be taken into consideration. Finally, the total number of peptides that matched the sequence, the percentage of sequence coverage, and the mass accuracy error should guide the decision as to whether the protein with the highest probability has been correctly identified. Fig. 3 shows two examples as screen shots for the identification of proteins based on PMF with different qualities in detection.

After analysis and identification of the protein via PMF, confirmation may be obtained by peptide sequence analysis by means of post-source decay or by sequencing of the peptides via classical Edman degradation. Both methods, however, require much higher protein amounts than PMF in order to obtain reliable results. A Western blot of a second 2D gel with an available antibody of good selectivity for the identified protein may also be performed. Blotted gels may also be exposed to commercially available antibody cocktails with different labelling and detection procedures. Customized libraries of antibodies, or sets of antibodies for specific pathways or processes such as cancer- or apoptosis-relevant proteins, can be purchased.

Recently, *in vivo* stable isotope labelling of proteins has been combined with one-dimensional and 2D gel electrophoretic separation followed by MALDI-TOF MS peptide fingerprinting. This approach not only reveals the identity of individual proteins, but also, from the relative rate of incorporation of the label, information about the metabolic turnover rate can be abstracted from the spectrum at the same time. Applied to mature 3T3-L1 adipocytes, a high rate of remodelling of extracellular matrix proteins and in particular of the type-I collagen fibres was demonstrated (Bouwman *et al.* 2004).

Proteomics applied in nutrition research

Nutritionists face formidable problems as they seek to understand the impact of nutrients at the molecular level, but it is a challenge that must be accepted if nutrition science is to match the advances of other branches of biology in the post-genomic era. Many of the techniques described herein have been under development for decades, but it is only since the mid-1990s that they have become fully integrated with the advancing fields of

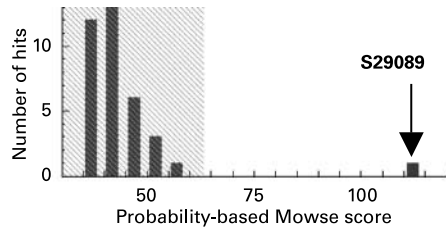
bioinformatics and computing to create the science of proteomics (Wilkins *et al.* 1996). This powerful new approach to understanding patterns of gene/protein expression has proved particularly valuable for drug discovery (Petricoin *et al.* 2002), for medical microbiology (Washburn & Yates, 2000) and for various aspects of pathology including the characterization of tumours (Mariani, 2003; Rai & Chan, 2004). Surprisingly, there have been relatively few studies in nutritional proteomics published so far, and most of these have involved the use of rodent models (tom Dieck *et al.* 2005) or human cells in culture (Herzog *et al.* 2004*a,b*; Wenzel *et al.* 2004; Fuchs *et al.* 2005*a,b,c*). However, a few recent examples will serve to illustrate the potential of the technique.

By combining microarray and proteome analysis techniques to assess the hepatic responses to experimental Zn deficiency in a rat model, a unique pattern of genes/proteins was identified that indicated a down-regulation of all pathways involved in glucose utilization as well as fatty acid oxidation, but increased mRNA and protein levels of enzymes and transporters required for *de novo* fatty acid and triacylglycerol synthesis (tom Dieck *et al.* 2005). These findings correlated well with an increased liver fat content (tom Dieck *et al.* 2005) and impaired fatty acid metabolism frequently observed in Zn deficiency. Proteome analysis was also applied to identify the target proteins of flavonoid action in colon cancer cells and led to the identification of a new mechanism by which flavone selectively induces apoptosis in transformed cells (Herzog *et al.* 2004*a,b*; Wenzel *et al.* 2004). Putative anti-atherosclerotic activities of genistein in endothelial cells stressed with oxidized LDL or homocysteine have also been explored by use of proteome analysis (Fuchs *et al.* 2005*a,b,c*).

As mentioned earlier, samples of body fluids are relatively easy to process for proteomic analysis because, unlike complex solid tissues, the separation of cellular fractions is usually not necessary. Gianazza *et al.* (2003) used 2D gel electrophoresis to investigate the effect of cobalamin deficiency on the proteome of cerebrospinal fluid in two rat models. In totally gastrectomized animals there was an increase in total protein in cerebrospinal fluid, which reached a peak 4 months after surgery. In rats maintained in a cobalamin-deficient state by dietary depletion, a similar peak occurred 6 months after introduction of the diet. The 4-month peak in protein concentration was associated with a specific increase in α_1 -antitrypsin, and *de novo* appearance of thiostatin and haptoglobin- β , but all these changes were corrected by supplementation with cobalamin.

More recently, Linke *et al.* (2004) described the application of proteomics to plasma samples from rats with differing retinol status. Plasma samples were analysed directly using SELDI-TOF MS. Pre-fractionation of the samples by anion-exchange chromatography, using 96-well filter plates, was shown to markedly increase the total number of peptides and proteins detectable by this method. Three proteins with molecular mass between 10 000 and 20 000 were shown to be present at reduced concentration in the plasma of retinol-deficient rats, and the authors concluded that their approach provided a promising means of detecting changes in nutritional status at the whole-body level.

Park *et al.* (2004) recently described the use of proteomic techniques to explore the impact of atherogenic diets on hepatic protein expression in C57BL/6J mice (B6, atherosclerosis-susceptible strain) and C3H/HeJ mice (C3H, atherosclerosis-resistant strain). Both strains were fed atherogenic diets and each showed a different pattern of both plasma lipids and intracellular lipid droplets. Proteomic analysis of liver from the two strains revealed a

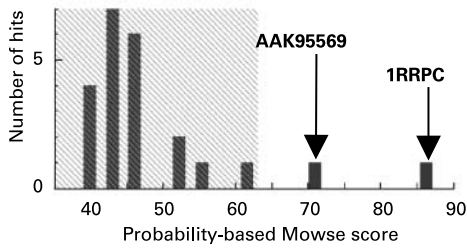


Concise protein summary report

Format As Help
 Significance threshold $p < 0.05$ Max number of hits

Re-Search All Search Unmatched

1. **S29089** Mass : 42701 Score : 112 Expect : 7.7e-07 Queries matched : 12
 alpha-centractin – human



Concise protein summary report

Format As Help
 Significance threshold $p < 0.05$ Max number of hits

Re-Search All Search Unmatched

1. **1RRPC** Mass : 20728 Score : 86 Expect : 0.00029 Queries matched : 8
 ran, chain C – human
2. **AAK95569** Mass : 26246 Score : 71 Expect : 0.0091 Queries matched : 7
 AY050633 NID :- Home sapiens

1RRPC: ran, chain C

Measured M_r/pI 21 kDa/6-8

Theoretical M_r/pI 21 kDa/9-6

AAK95569: ribose-5-phosphate isomerase

Measured M_r/pI 28 kDa/6-8

Theoretical M_r/pI 26 kDa/6-9

Fig. 3. Probability-based scores from two different matrix-assisted laser desorption/ionization–time-of-flight mass spectra as gained in reflector mode with a mass accuracy of 50 ppm. The upper part shows a well-defined search result with one hit that identified human α -centractin with twelve matched mass values, a sequence coverage of 36% and a mass error of 22 ppm. The lower part shows, for two different proteins, much lower but significant scores. In this case ribose-5-phosphate isomerase and ran chain C were predicted. Despite the lower scores, ribose-5-phosphate isomerase was accepted here (but not ran chain C) since the predicted mass and pI value matched well with the corresponding values identified in the two-dimensional gels. Sequencing of two peptide fragments from the predicted ribose-5-phosphate isomerase by post-source decay mass analysis finally confirmed the nature of this protein.

complex response. Overall, a total of thirty hepatic proteins were significantly changed by exposure to the diets, and, of these, fourteen including carbonic anhydrase III, senescence marker protein 30 and Se-binding protein 2 were differentially changed only in B6 mice. The remaining sixteen proteins, including glutathione S-transferase, apo E and chaperonin proteins, were changed in

both strains. Another twenty-eight proteins were differentially expressed in the livers of both B6 and C3H mice, regardless of diet feeding condition. The authors concluded that the proteomic approach had revealed clear differences in expression of both oxidative stress proteins and lipid metabolism-related proteins between the two strains in response to the atherogenic diets,

and that these differences could account for their differing susceptibility to atherogenesis.

Conclusion

Proteome analysis techniques allow the changes in steady-state levels of numerous proteins in a biological sample to be determined simultaneously. As we have seen, the classical approach to proteome analysis employs 2D-PAGE for protein separation in combination with PMF of the separated proteins after digestion with proteases for determining the protein identity. PMF is most conveniently accomplished by MALDI-TOF MS and more sophisticated mass analysis techniques allow peptide sequencing and characterization of post-translational modifications. Depending on the nature and quantity of the starting material and the general goal of the analysis, certain pre-fractionation steps or particular protein isolation procedures may be necessary before protein separation is performed.

Although the application of proteomics requires access to specialized equipment and a significant investment in skills and expertise, there can be no doubt that proteomics is a central technology in post-genomic nutrition research for assessing the effects of diet composition, specific nutrients and non-nutrient components on the genome and on mammalian metabolism. With the European Nutrigenomics Organisation, proteomics expertise becomes more widely available for the nutrition research community by training programmes and through collaborative research efforts. We may hope and reasonably expect that nutritionists will find new ways of answering the challenge posed at the start of the new millennium (Trayhurn, 2000) to apply this new approach to solving major scientific problems in human nutrition.

As helpful guides to proteome analysis using 2D-PAGE and MS, including hands-on and trouble-shooting advice, we recommend the following references: Westermeier (2001), Pennington & Dunn (2001) and Gorg *et al.* (2003).

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