Chapter 4

Application of Some Proteome Analysis Techniques in Animal Reproduction

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Additional information is available at the end of the chapter

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Abstract

This chapter focuses on the application of proteome analysis techniques to animal reproduction and provides general information on one-dimensional (1D) and two-dimensional (2D) electrophoresis, chromatographic methods, and mass spectrometer (MS), widely used in proteomics studies. Proteome consists of an entire complement of proteins expressed by a cell, tissue, or organism. Proteomics reveals functions of proteins encoded in the genome. These functions include posttranslational modifications (PTMs) and alterations in the protein synthesis. Animal reproduction takes a key role in livestock, and increasing the reproduction rate in flocks plays an important role in livestock management. Studying the proteins related to reproduction could guide on how to increase fertility. Recent studies addressed the proteome constitution of both male and female reproductive system. Follicular fluid, endometrium, and ovary proteins were analyzed in females by proteomics study, while in males, sperm proteomics was more focused. Information obtained on this issue is also beneficial for the development of reproductive technologies such as *in vitro* fertilization and embryo transfer. Strategies to increase fertility in animals can be revealed by proteomic studies, and a more profound knowledge on proteomics may become helpful to develop and enhance the efficiency of reproductive technologies.

Keywords: animal reproduction, protein screening, chromatography, electrophoresis, mass spectrometer, proteomics

1. Introduction

Reproduction is crucial for the existence and survival of a species. Also, being successful in animal farming depends on the reproductive efficiency of the individuals. There are three

main aims to the livestock reproductive efficiency: maintaining of herd size, providing the productivity and effectiveness in selection or breeding, and culling. Fertility is influenced mainly by genotype and environment. Besides, anatomical fitness and hormones have an important impact on fertility. In livestock, various reproductive technologies have been developed to escape infertility. Artificial insemination, embryo transfer, *in vitro* fertilization, and cloning are some of them. The development and enhancement of the techniques have benefit from the understanding of reproduction system in a proteomic basis.

Proteomics was first described by Wilkins and Williams in the mid-1990s [1]. Very simply, proteomics is a branch of biotechnology that involves gene-level protein expression studies. Two definitions of proteomics have occurred; first is restricting the large-scale analysis of gene products to studies involving only proteins, and second is combining protein studies with analyses that have a genetic readout, such as mRNA analysis and genomics [2]. However, the goal of proteomics is to obtain a more global and integrated view of biology by studying protein and genome. Furthermore, proteomics can provide exciting new opportunities to dramatically advance knowledge in a system perspective view of biology. Moreover, it also enables identifying new drug targets, leads to the development of superior diagnostics and therapeutics, and correlates biological pathways and molecular mechanisms in the disease [1]. Proteomics approaches can be used for protein profiling, comparative expression analysis of two or more proteins, the localization and identification of posttranslational modifications, and the determination of protein-protein interactions [3].

Crops not consumed in human diets are utilized by farm animals and turn to good quality products such as meat, milk, and others. Hence, livestock provides the quality source of protein for the human consumption, contributing to a balanced diet for the majority of the populations.

However, proteomics has been partially omitted from farm animal research [4] until recently. Proteins in animals were first studied in 1975 on guinea pigs and mice [5, 6]. In twentieth century, the studies were conducted with laboratory animals, mainly for the understanding of human protein metabolism [7]. The usage of proteomics in farm animals is more related with meat and milk industry. However, proteomics takes part of the clinical diagnostics of mastitis, intestinal and respiratory diseases, or parasites and helps to understand their pathogenesis [8]. Proteomics can get different names whereby according to usage in research areas: nutriproteomics, immunoproteomics, histoproteomics, meat proteomics, serum proteomics, etc. Taking from the names, it seems that proteomics has a wide variety of subject areas or matrices in animal science.

1.1. Proteomics in the reproduction field

At a molecular level, reproductive processes are complex phenomena that occur by interactions of many proteins. Although reproduction is understood at the molecular level, it is far from being understood in what way the reproductive phenotype and behavior relate to its

molecular foundations. It has been conjectured that female and male reproductive proteins can be identified by proteomics to ease the understanding of this mechanism. Different works have been developed using a proteomic approach in the reproduction field.

For example, the litter size is important parameter of sow productivity. It is emphasized that placental efficiency and litter size were highly correlated. It has further been shown that the placental proteins profiling as biomarkers could be used to estimate porcine litter size. Six proteins were identified in pigs with small size, contrasting to the 13 produced by pigs with large litter size [9]. Aminopeptidase (PSA, 70 kDa) and retinol-binding protein 4 (RBP4, 23 kDa) were the dominant proteins in large litter size group, as confirmed by western blot. In addition, PSA was determined as two different molecular weights (70 and 100 kDa) in both groups. PSA with 100 kDa was more expressed in the small litter size group [9].

Another example could relate to the understanding of hyperprolificacy in sheep. Using a proteomic approach, it was determined that a low level of ribosome-related proteins in the follicular fluid with isobaric tag for relative and absolute quantification-based proteomics was associated to high ovulation rate in Han sheep [10]. In addition, the follicular fluid provides a microenvironment for nuclear and cytoplasmic maturation of the oocytes. For *in vitro* fertilization, it is important to collect as many oocytes as possible. The oocyte selection in an *in vitro* fertilization program relies mainly on morphological criteria [11]. Precisely at this point, the proteome of the ovarian follicular fluid may provide information on the potential fertility in all animal species. The amount of serotransferrin, zinc-alpha-2-glycoprotein-like, complement factor B, and complement protein C3 changes in the follicular fluid according to the oocyte size [12]. The knowledge on the protein constitution of the follicular fluid could be useful to optimize the success of *in vitro* embryo production [12].

Similarly, changes in follicular fluid proteomics have been revealed in less-fertile dairy cows [13]. As known, the low fertility is common in high-yielding dairy cows, leading to important economic losses mainly on regards to the number of the inseminations, the increased maintenance costs, even treatment costs, and a lower number of calves. A total of 219 proteins were identified in the follicular fluid of preovulatory follicles in dairy cows, 26 of them uncharacterized [13]. Alpha-1-antiproteinase, metalloproteinase inhibitor 2, inter-alpha-trypsin inhibitor heavy chain H1, basement membrane-specific heparin sulfate proteoglycan core protein, complement component C8 alpha chain, collagen alpha-2(I) chain, prothrombin, alpha-S1-casein, interleukin-1 receptor accessory protein, and one uncharacterized protein (its gene named LOC781004) were differentially expressed in low-fertile dairy cows compared to controls [13]. Other studies identified 143 proteins in Holstein cows' follicular fluid during follicle development [14] and 363 proteins in buffalos follicular fluid [15], while other studies identified 38 and 463 protein spots in the female dog [16] and the mare [17].

Success in pregnancy depends on a good implantation and the uterus fitness. Although pigs have high fertility ratio, early embryonic losses are relatively common in this species. In this context, identifying the endometrium proteins can be significant. Several proteins were identified from sow endometrium, which play a role in the preparation of the endometrium for implantation [18]. In addition, some changes reported in the protein constitution have been

associated to the action of both maternal and embryonic hormones in the endometrium, during the embryo-maternal interaction, a crucial event for fertility [19].

Overall, in humans, 50% of the infertility causes are attributed to men. Male infertility is primarily caused by sperm dysfunction [20]. Biochemical fitness, DNA fragmentation, sperm abnormalities, and immunological problems are at the origin of this problem. Many studies, conducted on ejaculated semen, showed that the conventional methods have limited capacity to collect information on spermatogenesis and epididymal maturation. Many proteins were characterized during sperm epididymal maturation [21, 22]. Knowledge on the changes in the content and the location of these proteins during the sperm maturation process is critical for understanding spermatogenesis as well as fertility. As it is known, the spermatozoa leave the seminiferous tubules for posttesticular maturation with scarce content in cytosolic organelles and a complete loss of transcriptional ability [20]. In human, it has been shown that 35% of sperm-located proteins are of testicular origin and 48% of epididymis, while 17% are common to both organs [23]. Besides, sperm proteomic may also be useful in the field of *in vitro* fertilization. Different protein content was identified between male patients who experienced a failure in an *in vitro* fertilization procedure and healthy males [24]. Moreover, sperm proteomic also allows identifying the cryopreservation ability of semen within species. For instance, in boars, sperm membrane proteins can be used as markers for semen cryopreservation [25].

There are some limitations as well as advantages in the proteomics analysis. Limitations in proteomic generally depend on the characteristics of the tissue, fluid, or body system to be studied. Low-abundance, hydrophobic, and basic proteins show certain limitations in proteomics [26]. A good knowledge of quantities and properties of reproductive system proteins can increase the success of proteomic analysis and in determining the right instruments to be used in the analysis.

2. 1D and 2D electrophoresis

The studies on the separation of proteins are based on a history of about 250 years. The 1960s and 1970s are the most popular years for protein and enzyme studies and for the development of separation techniques. Since 1989, the interest in protein purification has increased considerably due to efforts to search for genes. It is often stated that the twenty-second century will be important for the properties of genes and corresponding proteins that are discovered and for understanding the functions of the organism [27, 28].

Proteins are macromolecules that combine a large number of amino acids. An amino acid is composed of a group of positively charged amino (NH_3) and negatively charged carboxyl (COOH); the charged components in the formation act on the net charge of a protein. According to the media, protein net charge can sometimes be positive and sometimes negative (amphoteric). Due to this basic feature of proteins, they can be separated in electrophoretic environments and their biological characterization can be facilitated. However, there is a certain pH value at which the net charge of each protein is "zero," which is called the isoelectric point (pI). Proteins are positively charged when their pI values are higher than

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their pH values (pI > pH), and they are negatively charged when pH > pI. Proteins can also be separated according to their molecular weights. In this process, proteins are degraded into peptides and then charged minus with a detergent called sodium dodecyl sulfate (SDS). The peptides are then separated using a polyacrylamide gel. This method was first named as SDS-PAGE by Laemmli in his scientific article in 1970 [29]. Thus, SDS-PAGE can be considered as the ancestor of the novel gel-based separation techniques. In this method, two different polyacrylamide gels (stacking and separating) can be prepared and combined. This gel system is defined as discontinuous. With the aid of a comb that is placed on the "stacking gel" before it is gelatinized, the wells where the protein samples can be loaded into are formed—hence, it is also called "comb gel." This gel intents to sift and organize the high kilodaltoned proteins before they pass into the "separating gel." Many proteins can be examined in a separating gel with an acrylamide concentration of 10–12.5%. **Figure 1** shows an image of 1D electrophoresis according to Laemmli while the system is working. **Figure 2** shows the image of a 10% gel obtained from the quail serum after the 1D electrophoresis process was performed.

In 2D electrophoresis, proteins are first separated according to their PI and subsequently separated according to their molecular weights. With this method, up to 1000–2000 proteins can be separated at one time [30]. In the 1D stage, immobilized pH gradient (IPG) strips are used. One side of the IPG strips is covered with a polyacrylamide gel at different pH values (available in varying pH range and length), and the other side is covered by a plastic strip.

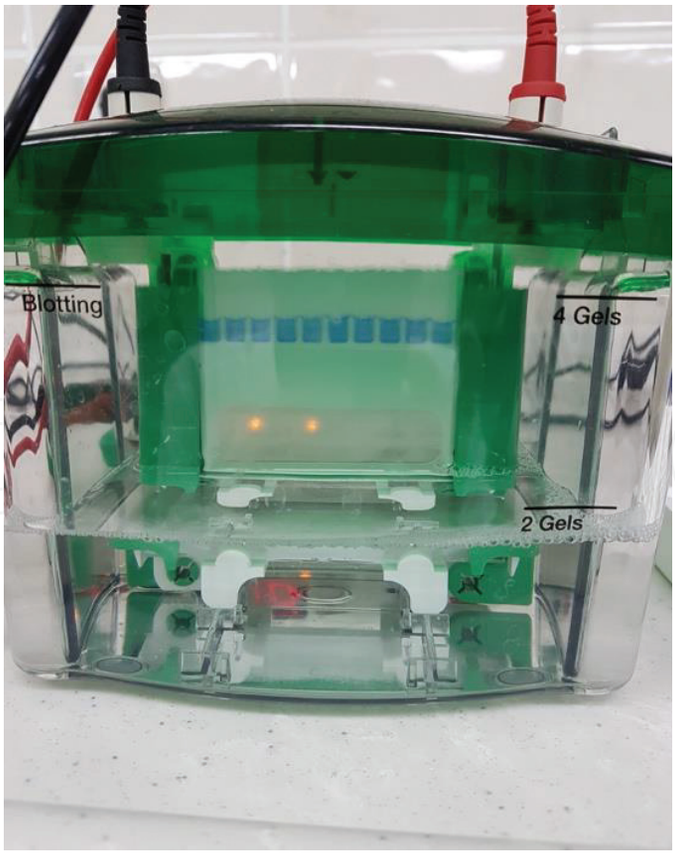

Figure 1. 1D electrophoresis according to Laemmli.

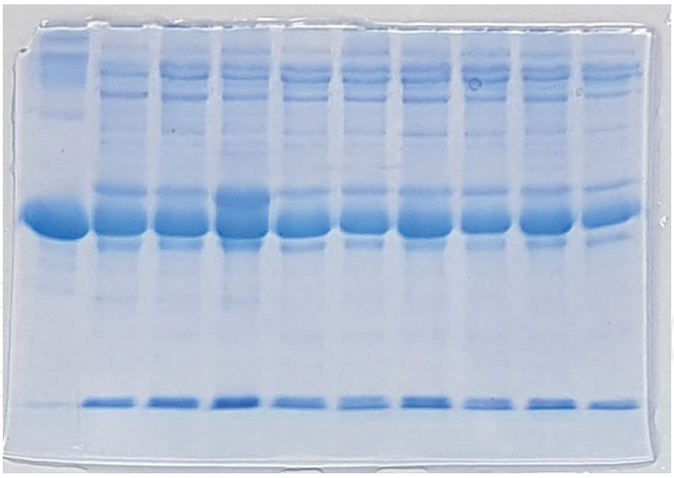

Figure 2. 10% gel image of quail serum after destaining in 1D electrophoresis.

IPG strips are placed over the protein samples (in a strip holder) prepared for analysis. The strip holder is then held in an electrical field (in 12,000 V for 1 h) until the net charge of the proteins is zero. After this step, strips are immersed in an equilibration solution, which is intended to the proper denaturation of proteins for an effective separation in 2D stage. The equilibration solution, usually prepared in a volume of 20 mL, is then divided into two equal portions: 10 mg dithiothreitol (DTT) is added to the first portion and 25 mg iodoacetamide (IAA) to the second. DTT is used for breaking disulfide bonds in the proteins, while IAA prevents the reformation of these broken disulfide bonds.

The 2D stage is performed in a vertical electrophoresis system. These systems generally consist of plates, casting stands, electrode buffer tanks, lids, and a power supply. The plates are assembled at regular spacing (0.75 or 1 mm) with the aid of spacers. In these plates, gel takes shape until gelatinization is complete. A casting stand is used to hold the plates in an upright position. Electric current is conducted in a buffer. The most commonly used electrode buffer is Tris-glycine (pH = 8.3). The electrical stream direction is from the cathode to the anode because proteins are negatively charged with SDS. The gel cassette placed in the buffer tank is filled with the electrode buffer and the system becomes ready by closing the lid.

IPG strips are placed horizontally on top of the prepared SDS-PAGE gel. A molecular weight marker is loaded on the first well of the gel to distinguish the proteins by their weight. However, unlike in 1D electrophoresis, the agarose gel pours over the stacking gel. The agarose gel prevents the development of an air gap between IPG strips and the separating gel. After separation, proteins are visualized by staining. Coomassie brilliant blue, silver stain, and fluorescent dyes are widely used. If fluorescent dyes are used, fluorescent imaging systems are required. Specialized software programs are used to measure the studied proteins. **Figure 3** shows a 2D gel image from the analysis of sheep caruncular endometrium. Mass spectrometry (MS) systems (MALDI-MS, ESI-MS, and LC-MS/MS) may also be used after the 2D stage for more detailed analysis and characterization of proteins. If MS will be used after 2D stage, the gel, SDS, and other residues such as bromophenol blue have to be removed. Trypsin, a serine protease enzyme, is needed for MS analysis. This enzyme separates proteins into appropriate lengths at which the MS device can measure [31]. 2D gel/MALDI-TOF MS may be a good choice for proteomic analysis as a starting point [26].

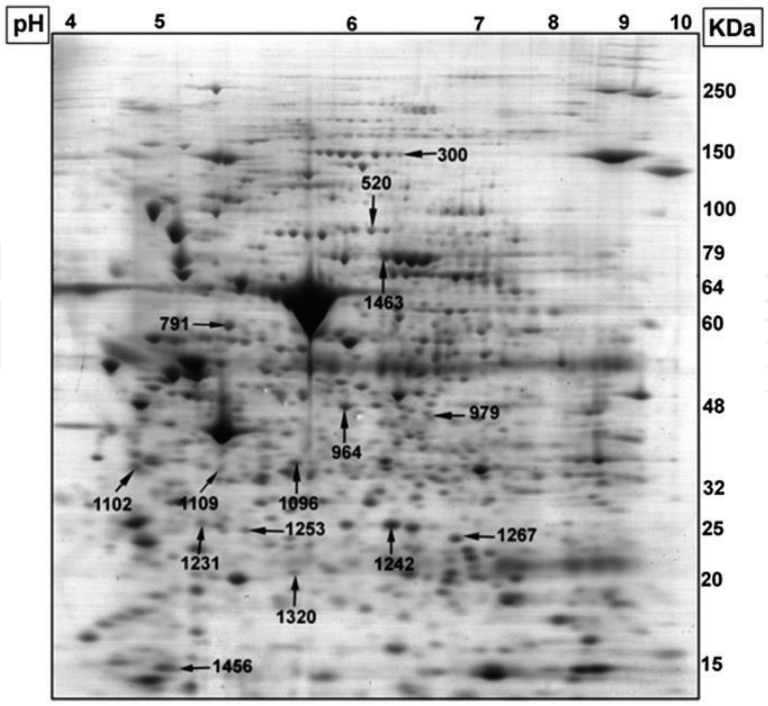

Figure 3. 2D gel image of sheep caruncular endometrium. Increasing pH from left to right and molecular weights of proteins from bottom to top [32].

3. Chromatography and MS

Chromatography is a widely used technique for separating the molecules. It is based on the principle that the molecules are separated when passing through a mobile phase from a stationary phase. While the mobile phase may be liquid or gas, the stationary phase is only solid or liquid. This technique is named according to the character of the mobile phase used. If the mobile phase is liquid, it is called "liquid chromatography (LC)" and if mobile phase is gas, it is called "gas chromatography (GC)." Apart from these, it is also classified according to the type of bedding (column or paper chromatography) and separation mechanisms (ion-exchange, partition, surface adsorption, and size exclusion chromatography) [33–35].

Proteins can be purified based on their size and shape, total charge, hydrophobic groups, and binding capacity with the stationary phase. These characteristics of the proteins allow the use of chromatographic methods, and column chromatography is the most frequent method for protein separation. There are many stationary/mobile phase combinations that can be employed. The chromatographic equipment needed depends upon the usage purpose. In chromatography systems, there must be established the conjunction with columns for the move through of stationary and mobile phase in the columns. Other accessories such as valves, fittings, fraction collectors, and autosamplers may be used to enhance the efficiency of output. For the data storage and evaluation, a computer console is usually combined with the system. **Figure 4** shows the scheme for an LC system.

Molecules are not normally charged, and mass spectrometers (MS) convert ions into charged ionic molecules. If the proteins get a polarity, the detailed analysis can be performed on them. The basic principle of mass spectrometry is based on a deflection of atoms toward different

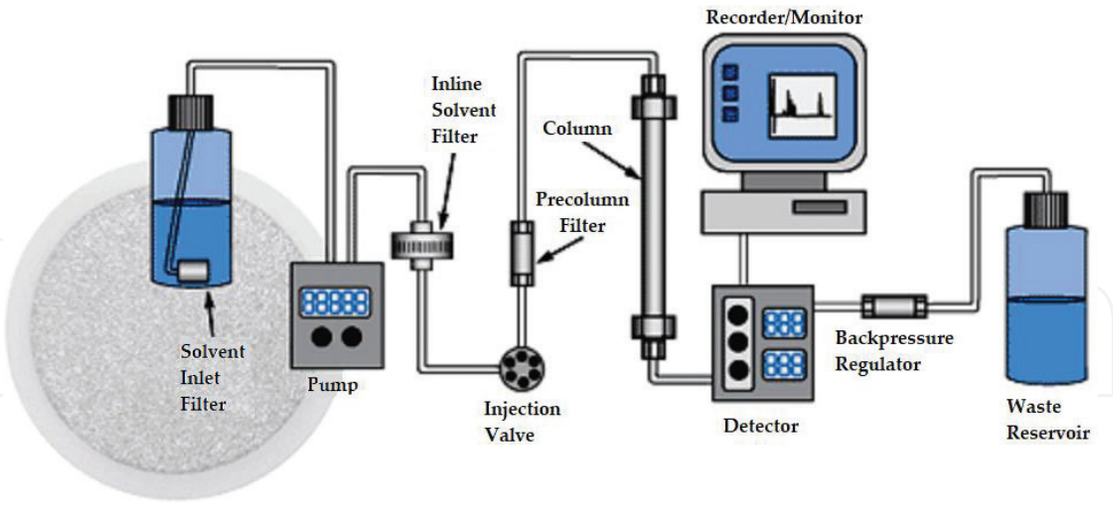

Figure 4. The schematic LC system [36].

orbits with the acceleration process in a magnetic electric field depending on their weights. Molecules separated in the LC method are more specific and quantitatively assessed with MS and the quality of LC's chromatographic property is increased. In MS, however, the particles separate not only according to their weight but also according to their charge. In the first stage of MS, the "ionization," particles are usually given a positive charge, and electrons are lost. The deflection velocity in the magnetic field will also change depending on the electrons lost by the particle. The particles that loss more electrons or are more positively charged will deflect more. It is important to take the air through vacuum at this stage, because the ionized particles may collide with air molecules impairing the obtention of a smooth flow. The particles are then accelerated to have the same kinetic energy (acceleration stage). Particles flowing at a certain velocity start to deflect according to their weight and charge (deflection stage). Finally, the particles are determined by the mass/charge ratio (m/z) to obtain data. For example, if a mass of a particle is 12, charge ratio is +1, m/z = 12, the mass of another particle is 24, but the charge ratio is +2, m/z = 12 again. **Figure 5** presents the general workflow scheme of MS.

Another available technique for protein identification and measurement is called tandem mass spectrometry or MS/MS. In this process, certain particles separated by m/z in MS1 are stimulated by the neutral gas (usually argon) contained in the collision chamber, and the particles are redispersed and transferred to MS2 and remeasured in m/z to m/z (**Figure 6**). Multiple MS cycles (MS/MS/MS or MS^n) can be developed for more precise studies [37].

However, particles with similar charge during the acceleration stage may show similar flow under the same kinetic energy, and therefore, particles are separated according to their flight times in the spectrometer. This technique is called time-of-flight mass spectrometry (TOFMS), and it is used for particle separation in many laboratories [40].

The quantitative analysis of biomolecules (proteins, peptides, nucleic acids, etc.) susceptible to breakage can be difficult in these techniques. At the same time, these techniques are labor and time-consuming, as well as costly. Matrix-assisted laser desorption/ionization (MALDI) technique has been developed to avoid measurement losses in the analysis of biomolecules susceptible to breakage (protein, peptide, nucleic acids, etc.). In this

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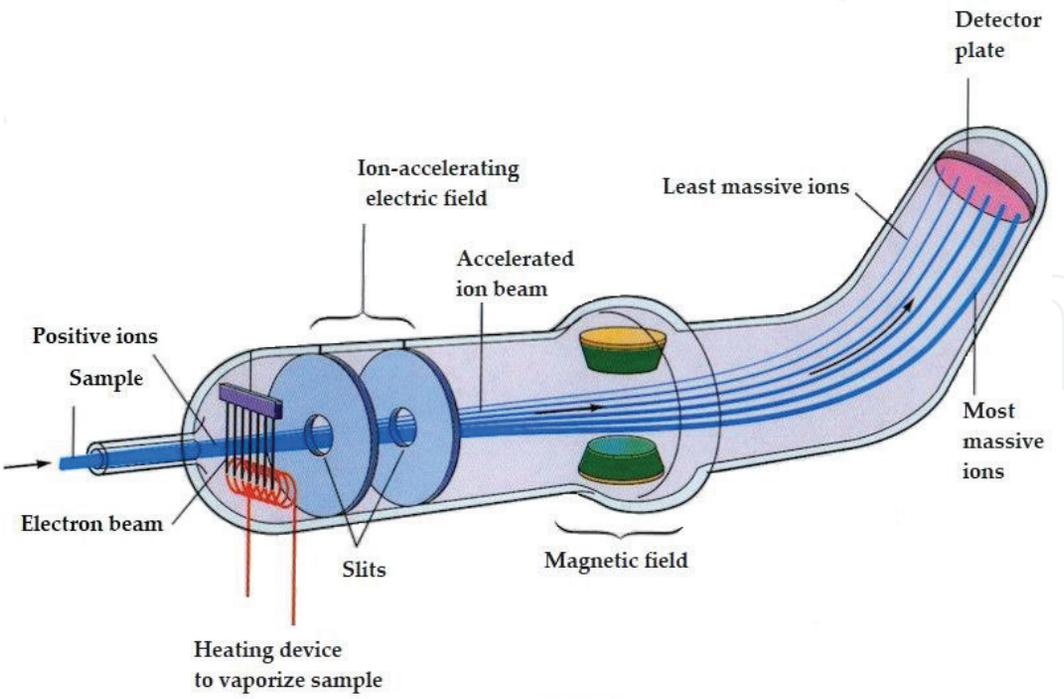

Figure 5. General workflow scheme of MS [38].

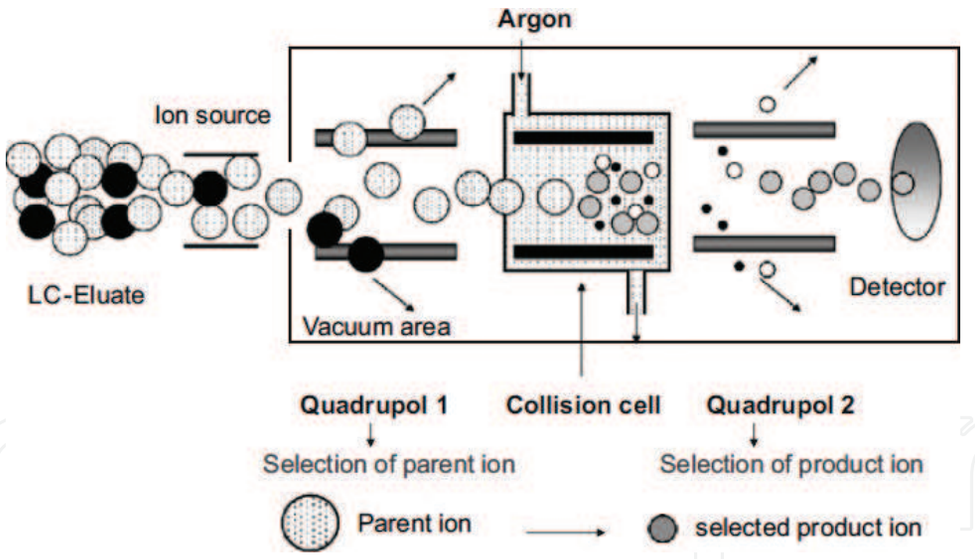

Figure 6. Principle of tandem mass spectrometry (MS/MS) [39].

technique, the first stage of MS, "ionization," is applied gently to prevent breakage. Briefly, in MALDI-MS, the sample is mixed with an ultraviolet light absorbing solution (the matrix, which is a compound of crystallized molecules). The liquid fraction in the matrix is then evaporated, and the matrix-sample mixture is crystallized. The sample is ionized via the matrix by sending the laser light to this crystallizing compound. The ions produced by the MALDI technique can also be measured with TOF (MALDI-TOF) to provide a high sensitivity and quantitative measurement. In a study that comparing MALDI-TOF MS with other automated methods provided by various companies [41], the error rates of the

Matrices	Peptides	Proteins	Nucleotides	Application
α-Cyano-4-hydroxycinnamic acid (CHCA)	✓			Smaller than 5000 Da
2,5-Dihydroxybenzoic acid (gentianic acid)	✓	✓		Low-weighted molecules
3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid)		✓		Larger than 5000 Da
3-Hydroxypicolinic acid (HPA)			✓	Oligonucleotide detection

Table 1. Matrix types available for some biomolecules [44, 45].

Specifications	Quadrupole	Ion trap	TOF
Range (m/z)	3000	2000	∞
Resolution	Typical	Medium	Low
Adaptability	Poor adaptive for MALDI Well suited for ESI	Well suited for MS/MS	Suitable for MALDI

Table 2. Some specifications of quadrupole, ion trap, and TOF [46].

techniques (MALDI-TOF MS *vs.* VITEK GN+® in VITEK® 2, bioMérieux) were determined as 9.4 and 0.39%, respectively. At the same time, 6-h recovery and eightfold lower cost were achieved by MALDI-TOF compared to conventional methods (VITEK GN+® in VITEK® 2, bioMérieux) [41]. However, there are some factors that limit MALDI-TOF. It is difficult to combine this technique with other chromatograms, and it can work unstable with ions having a low m/z. In **Table 1**, matrix types for various biomolecules and some of their specifications are presented [41].

The electrospray ionization (ESI) is another "soft ionization" technique for break-sensitive biomolecules. This technique was first reported by Yamashita and Fenn in 1984 [42]. In this technique, there is a transition of ions aid by electrical energy into a gas chamber. In this system, movement of liquid occurs only by electrical power. The electrospray injection method is one of the most suitable techniques for providing uniform flow and particle size distribution in a low flow rate [43]. Some features of quadrupole, ion trap, and TOF, and their usability with other techniques are shown in **Table 2**.

4. Conclusions

Proteomics may become a helpful tool, allowing to better understand the mechanism underlying the biology of reproduction and fertility in both animals and men. The potential contributions of proteomics to the study of fertility can be considered as two directional: first, in the diagnosis of reproductive diseases and the second, in the identification of breeds and/or individuals with high fertility. Proteomics is also quite useful in the development of reproductive technologies and in supporting assisted reproduction. Proteomics has been slightly neglected in livestock research due to its high cost and labor. In the future, proteomic studies

in animal science will have an important place owing to the understanding of the benefits of proteomics in terms of animal health and production and the allocation of research funds by governments or funding organs.

Conflict of interest

The authors have no affiliations with or are not involved in any organization or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this chapter.

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