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Review

Role of proteomics in taxonomy: the *Mytilus* complex as a model of study

J.L. López*

Departamento de Genética, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

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Dedicated to A.K. Hugo.

Abstract

Several strategies carried out for the application of proteomic methodologies to species and populations are summarized. Species of the genus *Mytilus* are used as a model organism to explain these strategies. The proteomics needed to differentiate populations, species, following some different approaches are provided. Moreover, there is an explanation of when it is most critical to carry out a rigorous analysis of this type. Sample preparation, selection of the most appropriate tissue of this organism for a comparative analysis, two-dimensional gels, computer analysis, mass spectrometry (MS) combined with two-dimensional electrophoresis (2-DE) are described. Then, the inconveniences of working with species like those of the genus *Mytilus* that are poorly represented in databases will be presented along with a description of how to approach this problem. Likewise, the review will include the strategy to follow when dealing with species, like mussels, that present a high degree of genetic polymorphism. The different protein-expression-based strategies used to approach the problem of differentiating *Mytilus* species will also be shown. Examples are presented to illustrate the use of 2-DE and MS to differenciate populations, species in taxonomic analysis.

Keywords: *Mytilus*; Proteomics; Taxonomy; 2-DE

Contents

* Tel.: +34 981 563100x13238; fax: +34 981 596904.
E-mail address: jllopez@usc.es.

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1. Introduction

Proteomics is defined as the study of proteins expressed by a genome or tissue. Two main tools used in proteomics, especially when referring to protein expression, are two-dimensional electrophoresis (2-DE) and mass spectrometry (MS). Two-dimensional electrophoresis, first developed by O'Farrell [1], is a powerful and sensitive technique for analysing complex protein mixtures which involves combining isoelectric focusing gel electrophoresis (IEF) with sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). If then the resulting protein patterns are studied using computer-assisted programs, the technique becomes a unique method for the qualitative and quantitative analysis of protein expression. Furthermore, in recent years the identification of 2-DE separated proteins has been improved with the development of highly sensitive protein sequencing and mass spectrometric methods [2,3]. The applications of two-dimensional electrophoresis are numerous, particularly, in the context of proteomic studies for detecting and quantifying modifications in genome expression during development, under different stresses or in response to different environmental conditions [4,5]. Two-dimensional electrophoresis is a unique method for large-scale protein characterization and combined with mass spectrometry allows to identify the protein repertoire of specific tissues [6].

The goal of proteomics is to study the proteome. The proteome is defined as the complete protein complement of a genome [7,8], One can say that there is one particular genome per given organism or cell, but there are an infinite number of proteomes, when referring to the protein expression at a particular moment and under specific conditions. The "expression proteome" is like an instant photograph of the protein expression at that moment and under particular conditions. A comprehensive description of the proteome of an organism not only provides a catalogue of all proteins encoded by the genome but also data on protein expression under defined conditions. Proteomics allows to obtain a quantitative description of protein expression and its changes under the influence of biological perturbations, the occurrence of post-translational modifications and the distribution of specific proteins within the cell [4]. Thus, the complexity of a biological system can be approached in its entirety due to that proteomics allows a multiplicity of proteins to be studied simultaneously [9]. The applications of the proteomic approach are numerous in many areas of biology, biochemistry and biomedicine [4,10–13].

Proteomics, known as a discipline that is complementary to genomics, provides additional and basic information for a global understanding of gene expression and regulation. Furthermore, the analysis of transcription alone provides a limited view of gene expression and it does not take into account regulatory steps at the level of mRNA translation [13]. Protein function and the phenotypic traits of a particular genotype depend, not only on the possible post-translational modifications, but also on the protein expression. Therefore, proteomics implies a higher level of analysis in the understanding of gene function in particular and of biology in general.

Two-dimensional electrophoresis analysis of proteins is a source of monogenic and codominant markers for population genetics analysis and variability studies [14]. However, in the marine world this technology has scarcely been applied; it has been used particularly for the identification of species and genetic variability [15–18]. There are many studies in which genetic markers obtained by 2-DE are used to study genetic variability in diverse species, such as man [19–29], cheetah [30], *Drosophila* [31], *Pinus pinaster* [32], etc. They have also been used in phylogenetic studies [33–35] and in gene mapping [36].

Other authors have also referred to 2-DE as an important source of genetic markers [31,36]. These can be quite useful to study population genetics, since, as mentioned before, the protein spots found on two-dimensional gels are useful monogenic and codominant markers that are probably not affected strongly by natural selection [36]. Moreover, 2-DE is a high-resolution technique, able to separate thousands of genetic products on a single gel and detect changes in the order of 0.1 pH units in the first dimension and of 1 kDa in the second [27]. Hence, it allows obtaining numerous markers randomly distributed throughout the genome. The possibilities offered by this proteomic tool for taxonomic studies are notable.

The genus *Mytilus* exists in the European coasts as three taxonomic forms, two of these, *Mytilus edulis* and *Mytilus galloprovincialis*, being predominant. The other species, *Mytilus trossulus* is restricted to the Baltic Sea. All forms can be recognised by analysis of morphological traits and genetic markers. In *M. galloprovincialis* different genetic markers have been described, including of allozyme-type [37–41], of nuclear DNA [42] and of mitochondrial DNA [43–46], however, only one 2-DE study was performed for the analysis of genetic variability [47]. The modern biogeographical distribution of *M. edulis* and *M. galloprovincialis* appears related to water temperature. Thus, *M. edulis* occupies temperate cold-water areas of Europe and North America [48] while *M. galloprovincialis* is a warm-water form that occurs in the Mediterranean and extends northward to the coast of France and the United Kingdom [38,49]. In accordance with this, physiological differences between these two taxa are observed when mussels are exposed to different thermal environments [50]. However, the taxonomic status of these three mussel forms continues to be controversial [48,51,52]. As indicated later on, proteomic methodologies can be used as different strategies to resolve taxonomic problems. Specifically, different results have helped explain, at least in the *Mytilus* complex, its taxonomy, as well as the biogeographical distribution of these species.

Moreover, it should also be considered that the identification of marine species is gaining notoriety in industry because of commercial regulations imposed by many countries all over the world. As in the case of other organisms not being marine, quality control measures and criteria of origin are becoming more strict and rigorous [53]. The search for

new markers allowing precise and rapid species identification makes this technique interesting not only for the prevention of possible commercial fraud but also to differentiate forms of ambiguous taxonomic status. This is the case in the marine mussel species belonging to the genus *Mytilus*.

Although proteomics allow the rapid and detailed characterisation of proteins, it has not been applied routinely to the analysis of species poorly characterised in databases, where interpretation of data generated from mass spectrometry cannot be automated as of yet. For this reason, development of methods for the application of these techniques to the analysis of proteins from species not present in databases is of considerable interest in fields such as biotechnology and food industry.

In the present review, the strategies carried out for the application of proteomic methodologies to species and populations will be summarised. Species of the genus *Mytilus* present in European coasts will be used as a model organism to explain these strategies. The proteomics needed to differentiate populations, species, following some different approaches will be provided. Moreover, there will be an explanation of when it is most critical to carry out a rigorous analysis of this type. Sample preparation, selection of the most appropriate tissue of this organism for a comparative analysis, the analysis of two-dimensional gels, etc., will also be described. Then, the advantages and inconveniences of working with species like those of the genus *Mytilus* that are poorly represented in databases will be presented along with a description of how to approach this problem. Likewise, the review will include the strategy to follow when dealing with species, like mussels, that present a high degree of genetic polymorphism. The different protein-expression-based strategies used to approach the problem of differentiating *Mytilus* species will also be shown.

2. Experimental approach

Although "proteome" refers to the total protein complement able to be encoded by a given genome [7], it is unlikely that: (i) the totality of this protein expression exist at any one given instant, and (ii) the detection threshold of 2-DE for low copy number molecules enable all translated proteins to be visualised [2]. In higher organisms, like mussels, much of the coding potential of a genome will be devoted to different stages. Thus, effective proteome analysis must depend upon experimental design and the need to examine several physiological states. Furthermore, DNA sequence information is unable to predict if gene-products are translated and the phenotype of multigenic phenomena, including drug administration, cell-cycle, ontogeny, aging, stress and disease. It is here that proteome research has much to offer [2]. The application of the proteome approach must be broadened to include those organisms like *Mytilus* which are poorly defined at the molecular level. The three taxonomical forms of the genus *Mytilus*, also known as *Mytilus* complex, can be recognised by expert marine biologists according to their external anatomical and morphological traits and by means of genetic markers. As mentioned above, although substantial genetic divergence has clearly occurred among them for allozymes and mitochondrial DNA [43,48,52,53], reliable markers for total characterisation should be defined. The identification of specific protein or peptide markers would be useful for the efficient diagnosis of these species.

Two-dimensional gel electrophoresis is the core technology for analysis of the proteome [7]. 2-DE can refer to any electrophoretic method capable of resolving protein mixtures on the basis of two independent separation criteria. The basic principle and methodology of 2-DE were published over 30 years ago [1,54], but technical developments in the last few years have supported a renaissance in the application of 2-DE [55–57]. A general description of those methods relevant to the analysis of mussels proteins is presented below. Assessment of genetic polymorphisms using 2-DE has not been widely reported. Studies of genetic variability in natural populations of animal species by means of 2-DE has been relatively scarce, because 2-DE is technically more difficult and time consuming than 1-DE and furthermore, because the first results revealed substantially less genetic variation than had been estimated by 1-DE [58–60]. Moreover, most studies have been focused on a few species (particularly man and *Drosophila*) so that the available information is to a large extent redundant and biased as a means of getting an appropriate view of the levels of genetic variability detected by 2-DE. Two-dimensional electrophoresis can be considered nowadays as a source of numerous monogenic and codominant markers, distributed randomly throughout the genome, making it a useful technique to study the genetic structure of populations and for genetic mapping. Examples of genetic polymorphisms detected by 2-DE are shown in Fig. 1. For each *locus* the possible genotypes are shown for two alleles Fig. 1A and for three alleles Fig. 1B. From left to right, acid homozygote, heterozygote and basic homozygote. The arrows indicate the position of the allelic variants, present and absent.

There have been constant developments in technology in two principal areas that benefit from the analysis of proteome expression. Firstly, the refinement of 2-DE, permitting reproducible analyses of complex protein mixtures between laboratories, and secondly, the development of sensitive micro-sequencing techniques capable of analysing the small amounts of protein recovered after 2-DE. Mass spectrometry as a tool for the identification of 2-DE separated proteins overcomes the limitations of protein amount and is not constrained by blockage of the N-terminus. More recently, sequencing via nanoelectrospray MS [61,62] has become by far the most sensitive technique for the identification of gel-separated proteins.

The amount of information obtained from these studies is so abundant that specific computer image analysis systems and programs are needed to handle all these data. The results obtained are stored in protein databases [63–65], which in

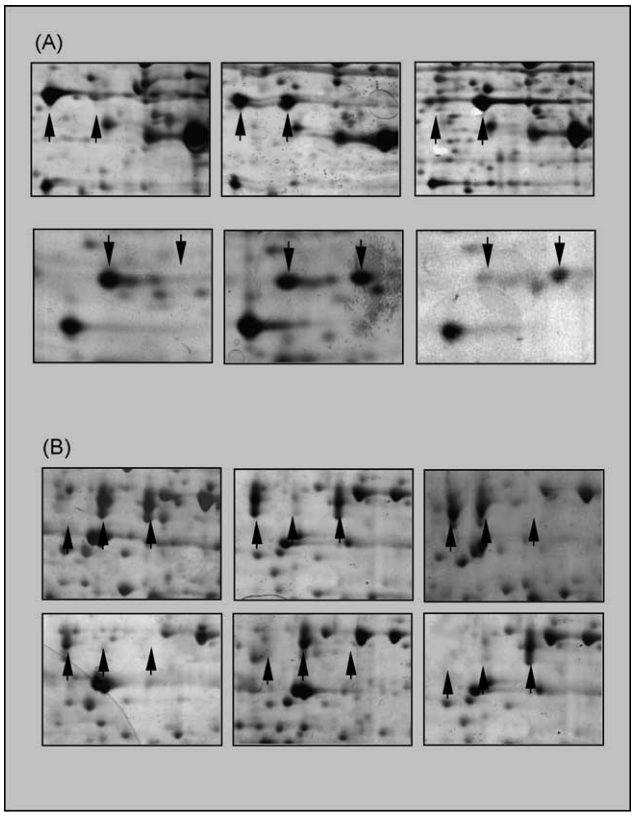

Fig. 1. Examples of genetic polymorphisms detected by 2-DE. For each *locus* the possible genotypes are shown for two alleles (A) and for three alleles (B). From left to right, acid homozygote, heterozygote and basic homozygote. The arrows indicate the position of the allelic variants, present and absent.

recent years have gained notable scientific interest due to the possibility of connecting this information with that of DNA databases [66,67].

For any type of analysis or study, whether comparative or taxonomic, it is vital to check each of the steps and stages of the proteomic tools, especially the 2-DE. In adjusting the high-resolution two-dimensional electrophoresis technique it is of high priority to make the correct selection of sample or tissue to study. The reproducibility of the protein patterns obtained and the computer treatment of the two-dimensional images using different programs for two-dimensional gel analysis must be verified and be as rigorous as possible.

2.1. Sample preparation

Appropriate sample preparation is absolutely essential for good 2-D results. The method of sample preparation depends on the aim of the research and is key to the success of the experiment [68]. Due to the great diversity of protein sample types and origins, only general guidelines for sample preparation are provided in this review.

Different treatments and conditions are required to solubilise different types of protein samples: some proteins are naturally found in complexes with membranes, nucleic acids, or other proteins; some proteins form various non-specific aggregates, and some proteins precipitate when removed from their normal environment. The effectiveness of solubilisation depends on the choice of the cell disruption method, protein concentration and solubilisation method, choice of detergents, and composition of the sample solution. The protein extraction from the raw material is not total. Hydrophobic proteins, such as membrane proteins, are the most affected. The process of solubilisation continues to be, even today, a critical step in 2-DE [69,70]. The protocols have been improved, incorporating the use of new reagents in the solubilisation and/or carrying out sequential protein extractions [71]. The absence in the gel of some proteins present in the sample can also be due to losses during the processing and to limits in sensitivity.

Proteolysis greatly complicates analysis of the 2-DE result, thus the protein sample should be protected from proteolysis during cell disruption and subsequent preparation. Proteases can be inhibited by disrupting the sample directly in strong denaturants such urea, TCA, or SDS [72–74]. Individual protease inhibitors are active only against specific classes of proteases, so it is usually advisable to use a combination of protease inhibitors. Broad-range protease inhibitor "cocktails" are available from a number of commercial sources. For more-comprehensive discussions of proteases inhibition, see [75–78].

Protocols on sample preparation can be obtained from papers describing specific 2-DE applications. There are excellent reviews which present detailed protocols for gel preparation and electrophoresis conditions and prospective users of 2-DE [79–81].

2.1.1. Sample preparation in mussels

The solubilisation method commonly used for different mussel tissues is a modification of that originally described by O'Farrell [1]. The sample must be treated to break protein bonds and eliminate non-protein substances that can interfere in the electrophoresis, leaving behind single polypeptides, and avoiding, as much as possible, any type of chemical modification of the proteins [82]. The system is sensitive to small changes in charge, and certain modifications can make that a single protein appear as two or more spots [1]. There is not a universal solubilisation protocol; each type of sample has its own specific characteristics. The solubilisation of the sample under denaturing conditions allows to obtain higher yields in the protein extraction and better reproducibility [83]. For samples of diverse origin, like for example certain types of biopsies, and specifically for most mussel tissues, good solubilisation is achieved by first lyophilising the samples and then pulverising them. Introducing changes at this stage should be avoided and special care should be taken in preparing the samples to avoid producing chemical changes in the proteins that could lead to changes in charges and doubling of spots. Temperatures above 37 °C for the lysis buffer are not recommended, given urea breaks down and the resulting isocyanate would carbamylate the proteins, causing

significant changes in their charges [1,84]. Temperatures below 18–16 °C should not be reached either, given the crystallisation of urea would result.

2.1.2. Tissue selection for comparative analysis

Choosing the appropriate tissue for comparative studies between different populations and species is crucial. Not only is the tissue giving the higher resolution and best patterns to be considered but also other factors. First, the different tissues should be tested to assess which yield the best patterns.

The mantle, for example, presents good resolution patterns. However, this tissue can be problematic, given it contains most of the gonad and therefore can undergo gametogenic cycle associated with the annual reproductive cycle [85]. Hence, it would be difficult to determine in comparative taxonomic or population studies, if the changes detected are due to interpopulational or interspecific differences, or to actual differences in the gametogenic cycle. If then, on the other hand, a tissue is selected "a priori" given its attractive nature, rich in proteins for example, as is the digestive gland, another variable would be introduced. In this case, possible exogenous protein contaminants derived from the diet could alter the two-dimensional patterns, thereby giving false results. A similar problem would occur with the branchia. This tissue is likely to accumulate residual proteins from the physiological process of filtration. Both the digestive gland and the branchia can be adequate for the detection of contaminants or pollutants in general. An interesting study on the detection of changes in protein expression profiles in bivalve mollucs exposed to environmental pollutants by 2-DE was performed [86].

The foot of *Mytilus* is an ideal example of what tissue to study, given it does not present the problems indicated above. The mussel foot is a structure of great mobility containing several glands that secrete a series of substances that will form the bisus, used by the mussel to adhere to the substrate [87]. The protein components of the bisus, for example adhesion proteins, have been studied by several authors [42,87–89].

2.2. Protein quantification

Qualitative as well as quantitative studies of the separated proteins in the two-dimensional maps can be, on occasions, problematic if similar amounts of proteins are not used loading the gel in the first dimension [1,90]. This effect is particularly important when using a sensitive detection method like silver staining that allows the detection of amounts up to 0.05–0.1 ng protein/mm^2 of gel [10]. Knowing the concentration of the sample is fundamental to control the amount of protein to be loaded onto the gel of the first dimension. The concentration of protein dissolved in lysis buffer cannot be determined directly by the Bradford method, nor by any other protein determination method (Biuret or Lowry), the reasoning being that the components of the lysis buffer (urea, detergents, ampholytes, DTT) alter the spectrophotometric reading. To avoid this problem, Ramagli and Rodriguez [90] have developed an assay (modified Bradford) consisting in acidifying the lysis buffer, before dilution of the sample proteins. Thus, reliable recordings of the protein concentration can be taken in the range 0.5–50 μg.

The principal problems of two-dimensional gels are the streaking of certain proteins and the background staining. Both could be due to the individual properties of the proteins, to the properties of the sample or to the 2-DE technique used. In most cases, the quality of the patterns is conditioned by the nature of the sample. However, the fact that the spots with streaks are surrounded by clear or well-defined spots indicates that this problem is due to the intrinsic characteristics of particular proteins [91]. These streaks are produced mainly during the first dimension and could be due to a high-protein concentration, or that these proteins tend to be insoluble before reaching their isoelectric point [1,91]. The gels run with muscle samples present a high number streaking-associated proteins, given muscle proteins are difficult to extract and solubilise (data not shown).

It is known that different tissues or sample of diverse origin have a high-salt concentration, an "enemy" of 2-DE [10,92]. The use of concentrators or dialysis gives good resolution patterns. However, the method involving protein precipitation with acetone and/or TCA, and the later resuspension in the same lysis buffer gave the best patterns (data not shown).

2.3. Two-dimensional electrophoresis

The original method for first-dimension IEF depended on carrier ampholyte-generated pH gradient in polyacrylamide tube gels [1,54]. Carrier are amphoteric molecules with a high-buffering capacity near their p*I*. Commercial carrier ampholyte mixtures are comprised of hundreds of individual polymeric species with pIs spanning a specific pH range. Although this basic method has been used in hundreds of 2-DE electrophoresis studies, it has several limitations that have prevented its more widespread application. Thus, carrier ampholytes are mixed polymers and suffer from batch-to-batch manufacturing variations. These variations reduce the reproducibility of the first dimension separation. Ampholyte pH gradients are unstable and have tendency to drift toward the cathode. Gradient drift affect reproducibility by introducing a time variability, rendering the 2-DE technique less useful at basic pH. Carrier ampholytes have other drawbacks including an inability to load the large amounts of protein required for micro-sequencing minor proteins study of minor abundant proteins, theoretically more variable. The gel rods may stretch or break, affecting reproducibility. Results are often dependent on skill of the operator. This will be one the greatest sources of variation in the two-dimensional patterns, since not all are synthesised in the same way.

The principal modifications in 2-DE, from the description of O'Farrell and Klose simultaneously in 1975 [1,54], have been in the first dimension. The use ampholytes and the introduction of commercial IPG strips offer greater

reproducibility and allow to establish comparisons among laboratories [93,94].

The use of immobilised pH gradients (IPG) for the charge separation of 2-DE overcomes many of the problems associated with carrier ampholytes [95]. IPGs are prepared using immobilines made by covalently linking the buffering compounds to the acrylamide monomers. Immobilised pH gradients are stable and capable of simultaneously focussing both, acidic and basic proteins on a single gel prepared with a broad pH gradient. Another important advantage of these strips is the possibility of loading greater amounts of sample, making the running of preparative gels possible for later characterisation analyses.

An important feature of IPGs is their high reproducibility between laboratories [93,94], a characteristic which makes them ideal for developing 2-DE protein databases. The studies of populational and interspecific variability are clearly limited in the "classical" method since these studies are not reproducible, for the reasons mentioned, in other laboratories. However, numerous, interesting studies of genetic variability in different species have been described by the classic 2-DE in man [22,24–29,96–99], cheetah [30], mouse [100], *Drosophila* [31,101] and mussel [47].

The second dimension of 2-DE separates proteins on the basis of their apparent molecular weights in polyacrylamide gels in the presence of SDS. The gels, in the form of slab gels, are prepared as either single-concentration or gradient-polyacrylamide gels which can be optimised to separate proteins over a specific M_r range.

One objective of 2-DE for proteome analysis is to maximise the number of proteins amenable to analysis. This requires the optimisation of both, the gel resolution and protein detection. The dimensions of the gel clearly influences protein resolution. Typically, high-resolution 2-DE systems use 1–1.5 mm thick slab gels with dimensions on the order of 20 cm × 20 cm capable of resolving over 1800 proteins. Large gels provide a three- to fourfold increase in the number of proteins detected. However, increasing the gel dimension leads to enhanced complexity in profile analysis, and practical problems arise in handling these larger gels [91]. At the opposite end scale, 2-DE can be carried out on smaller gel system where relatively simple 2-DE protein profiles are obtained [95]. An alternative approach is the production of 2-DE "contigs" in which a series of several gels are prepared, each of which resolves proteins within a limited range of pI and Mr. The protein profiles produced by these gels are then combined by computer analysis to form a single large "virtual" gel covering broad p*I* and M_r ranges.

On the *y*-axis of the gel, the distribution of spots depends on the length, density and pH of the second dimension gel [1,91]. An appropriate acrylamide concentration is 12.5%. Lower percentages worsen the resolution of two-dimensional maps and higher percentages (15%) make the extraction of proteins from the gel more difficult for later studies. The two-dimensional protein maps that allow the visualisation of more spots are called "analytical gels". These gels present the best patterns for a comparative analysis. To increase the concentration of several specific proteins for posterior characterisation, either by mass spectrometry or by any other analytical method, a greater sample load is applied in the first dimension, thereby obtaining protein maps containing greater amounts of protein. These gels are referred to as "preparative gels".

The absence of overlapping and the separation between spots make the analysis and comparison of gels easier, along with the isolation of proteins from the two-dimensional gels for later analysis. Also, the free spaces on the gel between spots can be covered by new spots corresponding to small amounts of sample proteins. These spots could be detected by increasing the load or the sensitivity of the staining method. The distribution of the proteins throughout the surface of the gel is not usually problematic [91]. The focusing of proteins during the isoelectric focusing depends on the length of the strip of the first dimension, the voltage applied and the temperature used.

2.3.1. Two-dimensional electrophoresis in mussels

A representative 2-DE gel of foot proteins from *M. galloprovincialis* is shown (Fig. 2). These pictures compare 2-DE protein profiles of foot for mussels analysed using ampholines versus immovilines. The patterns are equally clear. The difference is in the number of proteins resolved. Using the classical method an average of 800 spots are resolved, whereas with the IPG strips the number of resolved spots rises to an average of 1400. Apart from the obvious advantages of having more spots, since many more *loci* are available to potentially analyse to search for qualitative and/or quantitative differences among conditions or species, the use of IPG strips also allows (as commented before) to overcome the inability to load the large amounts of protein required for micro-sequencing minor proteins when gel rods are used.

2.4. Staining

Once the electrophoresis is finished, the proteins are detected using a variety of staining methods of different sensitivities. Coomassie Brilliant Blue (CBB) detects approximately 0.1 μg of protein. Highly sensitive silver stains can be used to increase the number of proteins detected in a sample. Silver staining improves protein detection up to fivefold compared to CBB staining. Densitometric analysis of the 2-DE protein patterns is frequently used to locate quantitative protein changes. CBB and the silver staining procedure show linear responses over a range of protein concentrations of 0.5–20 μg whereas silver staining is linear at protein concentrations of 0.02–0.8 ng/mm^2 [102]. A limitation to quantitative analysis using either CBB or silver staining is that the response slopes differ between proteins [103,104]. Radiolabelling proteins with radioactive amino acid precursors can be used as a high-sensitivity detection method for studies in which prokaryotic and eukaryotic cells can metabolise in vitro to incorporate amino acid tracers during protein

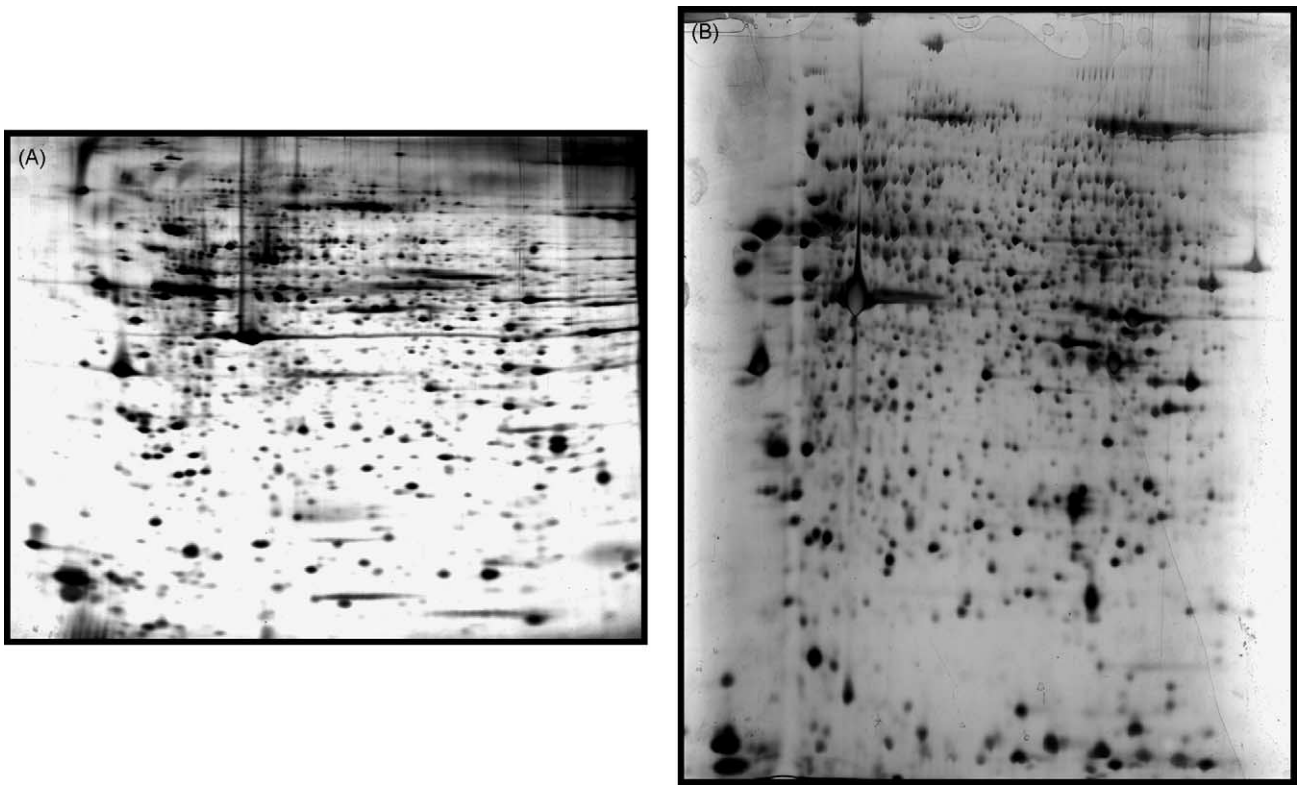

Fig. 2. Two representative 2-DE gel of foot proteins from *M. galloprovincialis*. (A) 2-DE protein profiles of foot for mussels analysed using ampholines and (B) protein profiles of foot for mussels analysed using immovilines.

synthesis [20,24,105]. Obviously, for quantitative studies, radiolabelling is limited by the incorporation of the amino acid tracer.

On numerous occasions and when working with mollusks, obviously it is difficult to radiolabel proteins. Hence, one must resort to using other staining methods. Silver staining is useful when searching for qualitative variations (presence/absence of spots). Therefore, silver-stained gels give good resolution (analytical gels). It is necessary to decide what is preferred among number of spots, good definition, and relative isolation of these. However, when the detection of quantitative variations is wanted the staining with coomassie was more reliable [106]. Fig. 3 compares two gels having the same protein load, one stained with coomassie and the other with silver.

Coomassie-stained gels gave good resolution for comparisons [17,106]. Incrementing the protein load in first dimension gels (IPGs), staining the gels with CBB, destaining following the standard protocols and finally leaving the gels immersed in abundant distilled water gave practically the same number of spots as did a silver-stained analytical gel [17].

The use of CBB with the assistance of computer programs all carrying out reliable quantitative comparisons [106].

2.5. Resolution and reproducibility

In the analysis with two-dimensional gels, it is necessary to fine-tune and optimise the resolution of the protein maps as a function of the nature and characteristics of the samples studied. Testing in the first dimension with strips "Immobiline Dry Strips" of different pH ranges and lengths is recommended. Then, if most of the proteins focus at intermediate pH ranges IPG strips in the range 4–7 should be used. This allows for a uniform distribution of the protein "spots" on the gel. To analyse other types of samples in which basic proteins are abundant strips of this pH range could not be used. Many proteins would be unresolved and concentrated at the basic end of the gel. In this case, broader pH ranges (i.e. 3–10) should be used.

A good two-dimensional protein map should show spots that are not overlapping, well distributed throughout the gel, dark, having well defined borders, free of streaks and background staining. In general, all these characteristics should be present when comparing gels. The absence of overlapping and the separation between spots is particularly important for the detection of polymorphic variations of specific proteins. Moreover, this makes the analysis by densitometric imaging and the isolation of proteins for two-dimensional gels for posterior structural analyses easier.

Figs. 4A and B show gels loaded with 255 and 8 μg of sample, respectively. As observed, gel A (255 μg) shows a greater number of spots and these are larger due to the greater amount of protein; the streaks and the overlapping are also more intense in gel A than in gel B (8 μg). To the most part, the overlapping can be reduced by preparing larger gels (data not shown).

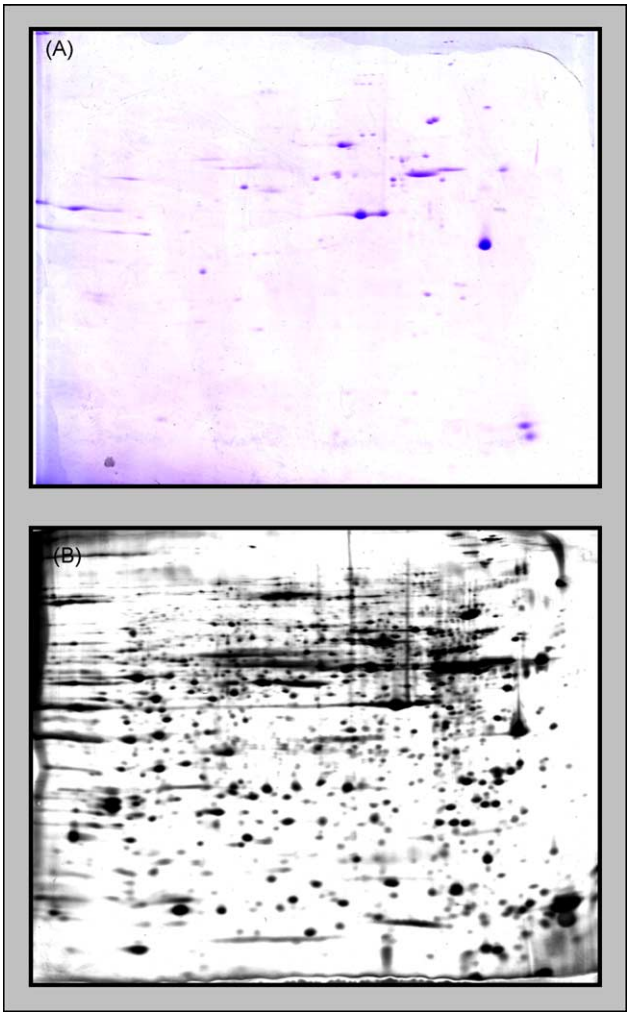

Fig. 3. Comparative figures of two gels having the same protein load, one stained with coomassie and the other with silver satining.

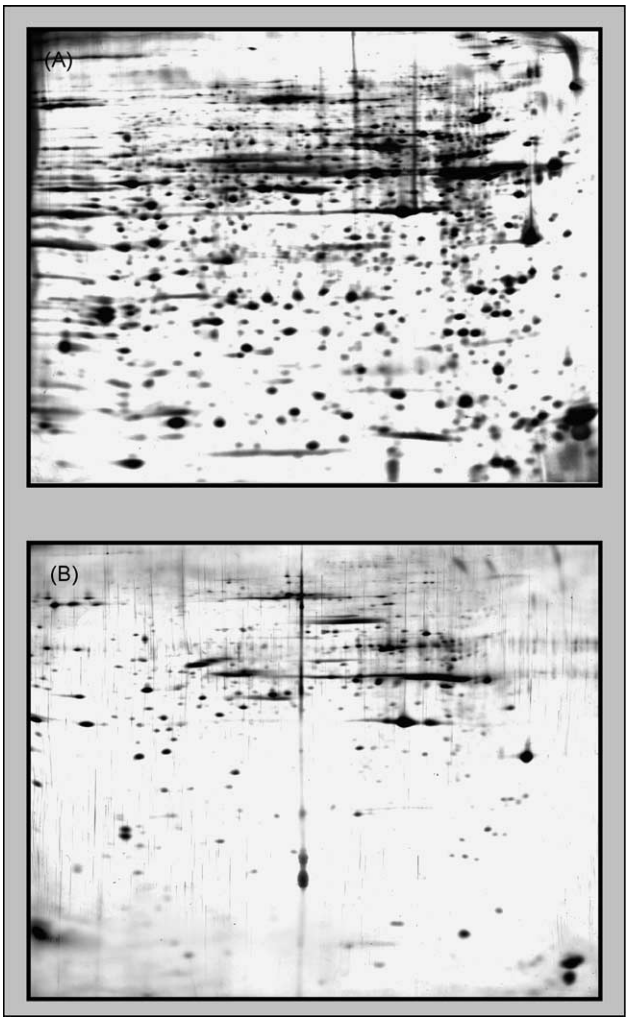

Fig. 4. Reproducibility analysis. (A) and (B) show gels loaded with 255 and 8 μg of sample, respectively. Gel A (255 μg) and gel B (8 μg).

For good reproducibility, samples between the same individual as well as among different individuals should be run. The detection of differences due to variations among individuals is indicative of inter-individual polymorphism. These data will allow future variability studies.

The reproducibility of the patterns should even reflect characteristics such as the size or intensity of the spots in gels run with similar amounts of protein. This, along with the wide distribution of relative amounts of proteins of the sample, allows to recognise the spots even when complex patterns are obtained [1]. Fig. 5 illustrates 2-DE protein profiles for foot of *M. galloprovincialis* analysed by the classical (O'Farrell) method and shows patterns of the same individual at different protein concentrations.

2.6. Computer analysis of 2-DE patterns

For small numbers of gels, and especially when the gels present simple patterns, the 2-DE protein profiles can be compared simply by overlaying the gels and manually inspecting the profiles for proteins with any electrophoretic mobilities. As the number of proteins being screened increases, the use of computer programs for profile matching becomes necessary. In addition, the detection of quantitative changes in protein expression between different populations growing in different conditions, different species or any other condition, has an absolute requirement for computer-assisted programs. Specialised computer programs have been developed for the analysis of 2-DE protein profiles [108–112]. However, for the detection of qualitative changes visual analysis is essential.

To study quantitative changes, intensity levels of the spots must be normalised by expressing the intensity of each protein spot in a gel as a proportion of the total protein intensity detected for the entire gel (relative volume, %Vol) [17,106,113–115].

2.7. Identification of the proteins resolved by 2-DE

Once the proteins have been separated by 2-DE, the next step is to identify the proteins. These methods have a wide

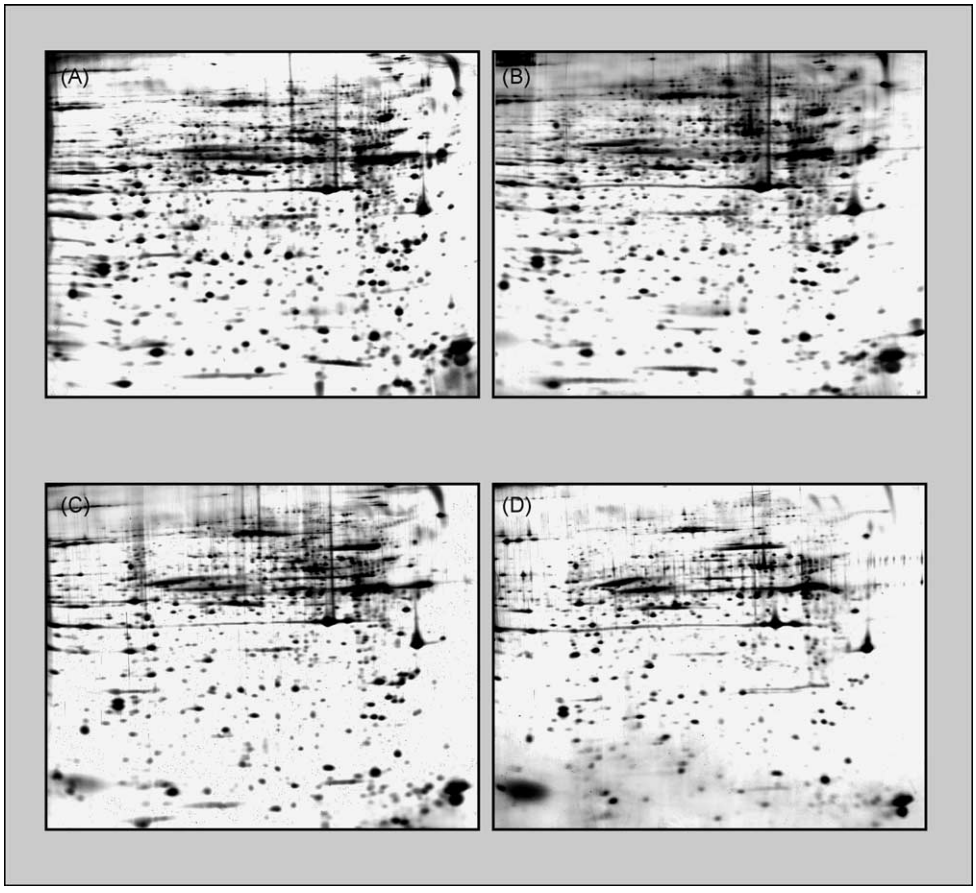

Fig. 5. Reproducibility analysis. Two-dimensional protein profiles for foot of *M. galloprovincialis* analysed by the classical (O'Farrell) method and shows patterns of the same individual at different protein concentrations.

application to protein characterisation and are based on the determination of either the amino acid composition or the partial amino-acid sequence or a peptide mass fingerprint. Data derived from a combination of these methods improves the reliability for protein identification. Although others propose a hierarchical approach to protein identification in which rapid low-cost methods (e.g. amino-acid composition analysis) are used first, before turning to more expensive and time-consuming methods, culminating in micro-sequencing mass spectrometry [8], when working with molluscs or other species that are poorly represented in DNA and protein databases, the strategy, as will be mentioned later, must be substantially different.

Although these are sensitive analytical methods, it may be necessary either to use an enrichment step prior to electrophoresis or to pool and extract the protein of interest from replicate gels [66] in order to characterise minor proteins present in a cell extract. When IPGs are used for the first-dimension separation, it is possible to apply large amounts of sample to the gel to recover sufficient amounts of the minor proteins from a single gel.

Peptide mass fingerprinting using matrix-assisted laser desorption ionisation (MALDI) MS has developed into a widely used method to identify proteins resolved by 2-DE [116–118]. Proteins are digested using specific proteases and the masses of the peptides produced are accurately determined using MALDI MS. These masses provide a "fingerprint" for the protein spot that is matched against computer determined mass fingerprints using dedicated software [118]. An extension of peptide mass fingerprint is the use of tandem mass spectrometry to derive a peptide sequence tag (comprising three or four amino acids) from the peptides. The derived peptide tag can be used in combination with the data from peptide mass fingerprint for protein identification [119]. For each of the identification methods described above a list of possible matches to the unknown protein, ranked according to similarity, is derived. The search may be refined by including experimentally determined estimates of the pin and M_r of the intact protein.

3. Taxonomic applications

The simultaneous use of several allozymic markers to allow identify in a non-ambiguous way the different species of the *Mytilus complex*. However, this approach forces the characterisation of species from a populational point of view. The analysis of numerous individuals and several allozymic

markers is required to infer statistically the differences in the allelic frequencies of these markers [37–40,44].

A proteomic approach at first seems quite is promising. The search for protein markers would not be novel. However, it would novel in the analysis of global proteins, from a proteomic perspective. Both abundant and non-abundant proteins will be studied. Could expression proteomes provide clues about the populational and inter-specific differentiation? The answer is yes.

In view of these findings, it is not difficult to imagine the possibilities of a proteomic approach and in particular of 2-DE in detecting qualitative and quantitative differences to differentiate species, subspecies and populations. The data that could be extracted can complement others previously obtained by genomic techniques and contribute a new perspective to an analysis.

One approach, that could be referred to as classical, could consist in the generation of reference maps and subsequently to detect, quantify, and compare the global protein expression between two populations or related species of marine mussels (or any other of related organisms) growing in their own geographical habitats [17,106,115]. This technique samples a large portion of the total genome and it is ideal for detecting new specific molecular markers at species, stock or individual level. This approach could represent a starting point in the search for uncommon and/or unknown proteins, and for the identification of proteins differently expressed and associated with particular processes. With certain species, as those of the genus *Mytilus*, there are difficulties that force changing the strategy for the analysis. The elevated degree of genetic polymorphism, as mentioned before, hinders knowing if the qualitative differences found in the analysis of the two-dimensional patterns are due to inter-individual or inter-specific differences. This implies a change in the strategy, defining the comparative analysis of the patterns of protein expression as exclusively quantitative differences in those proteins (spots) that are common to all the individuals studied. The most important hindrance is the problem and limitation inherent of the cross-species identification, as the mussel is poorly characterised at the genome and proteome level.

The technique of peptide mass fingerprinting using MALDI–TOF and/or nanoelectrospray MS/MS enabled the unambiguous identification of several few expressed proteins. For the identification of most proteins in many cases it is necessary to resort to homologies with other species. It is expected that once there is software available to automatically identify proteins not present in databases this type of analysis will be applied to a greater scale, comparable to other works with species that are already well represented.

The identification and characterisation of proteins can expand our understanding of the molecular differentiation of these organisms and serve as a useful base for future ecological, physiological and genetic studies. These studies provide new clues or points of light for the interpretation of physiological differences, genetic divergence, taxonomic status and a better understanding of the ecological environments these species of marine mussels occupy. Hence, the quantitative differences observed between *M. edulis* and *M. galloprovincialis* are not only due to their distinct genetic composition but also to the environmental variations they find themselves in, given they originate from different geographical locations. The results found contribute valuable phenotypic information. They provide evidence that the northern species, *M. edulis*, could be more thermally sensitive than the southern species, *M. galloprovincialis*. These results are compatible with the findings of Hilbish et al. [50] who demonstrated that these two mollusc species were physiologically differentiated with respect to temperature, and indicate that this approach constitutes a useful key for future analysis of protein expression, and for the characterisation of different species at genetic, ecological and physiological levels. [17,106]. Fig. 6 summarises the steps followed in this type of approximation of classical proteomics.

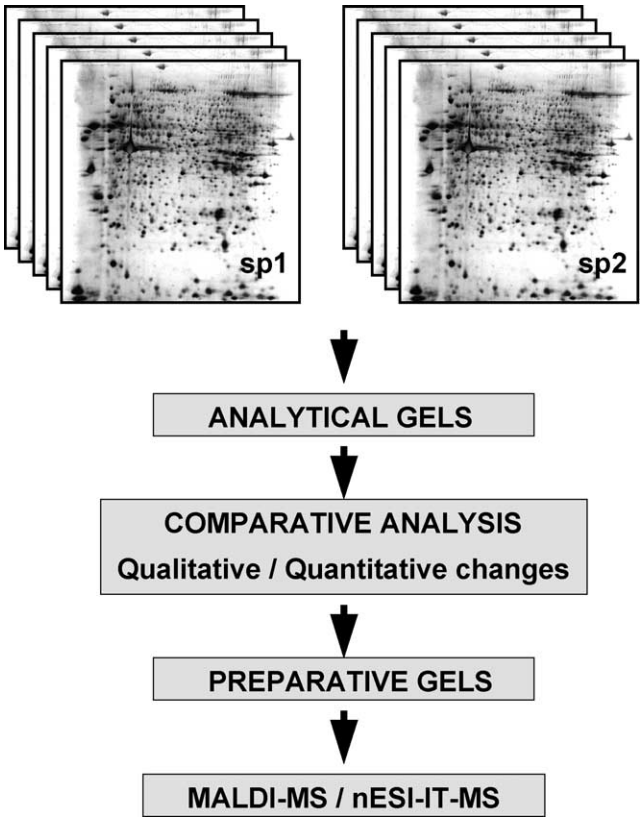

Fig. 6. A schematic overview of classical expression proteomic analysis.

The search for new markers allowing precise and rapid species identification, is interesting not only to differentiate forms of ambiguous taxonomic status but also for the prevention of possible commercial fraud. This is the case in the marine mussel species belonging to the genus *Mytilus*. For this reason, development of methods for the application of these techniques to the analysis of proteins from species not present in databases is of considerable interest in fields such as biotechnology and food industry.

With the aim of developing new strategies for the identification and characterization of marine species, powerful techniques based on biochemical methods are arising [120]. Species identification based on protein analysis by isoelectric focusing, polyacrylamide gel electrophoresis, capillary electrophoresis, 2-DE, and HPLC has been carried out [121–126]. Two-dimensional electrophoresis allowed the identification of protein spots which showed interspecific variations in molecular weight and/or isoelectric point. These variations were indicated changes in protein sequence and MALDI–TOF peptide mass maps were used for specific identification of species. The combination of high-resolution 2-DE and MS, proved to be useful, reproducible and sensitive strategy for the molecular characterisation of peptides in fish products for both identification and characterisation purposes [16].

Taking advantage of the speed and throughput of MS, species-specific peptides may also be identified from prominent protein spots "not" showing apparent alterations in 2-DE behaviour. It was demonstrated that this approach may be useful even in organisms difficult to diagnose, such as those of the genus *Mytilus*, which present a high degree of intraspecific polymorphism at the genetic and protein level, and are poorly characterised at the genome and proteome levels in databases. This method was applied to identify peptides and was useful to differentiate individuals belonging to the one species, among individuals belonging to the other species of *Mytilus*, and may potentially be applied to any problem where a fast and rigorous identification of species is needed.

The utility of this approach by characterising the sequence of a peptide which allows a fast and highly-specific identification of individuals belonging to the *M. trossulus* taxonomic form, when compared to those belonging to *M. edulis* and *M. galloprovincialis*.

Differences in mass maps, however, were not sufficiently robust to allow for a systematic characterisation of mussel species, since not all the differential peaks were predominant, and we had to expand considerably the mass maps in order to detect some of these peaks. In addition, sample preparation for MALDI–TOF analysis is well known to be an inherently heterogeneous process, and some variations in peptide intensities are expected to occur in different analyses even in the same sample. A different MS approach was explored. The results also showed how the identification of

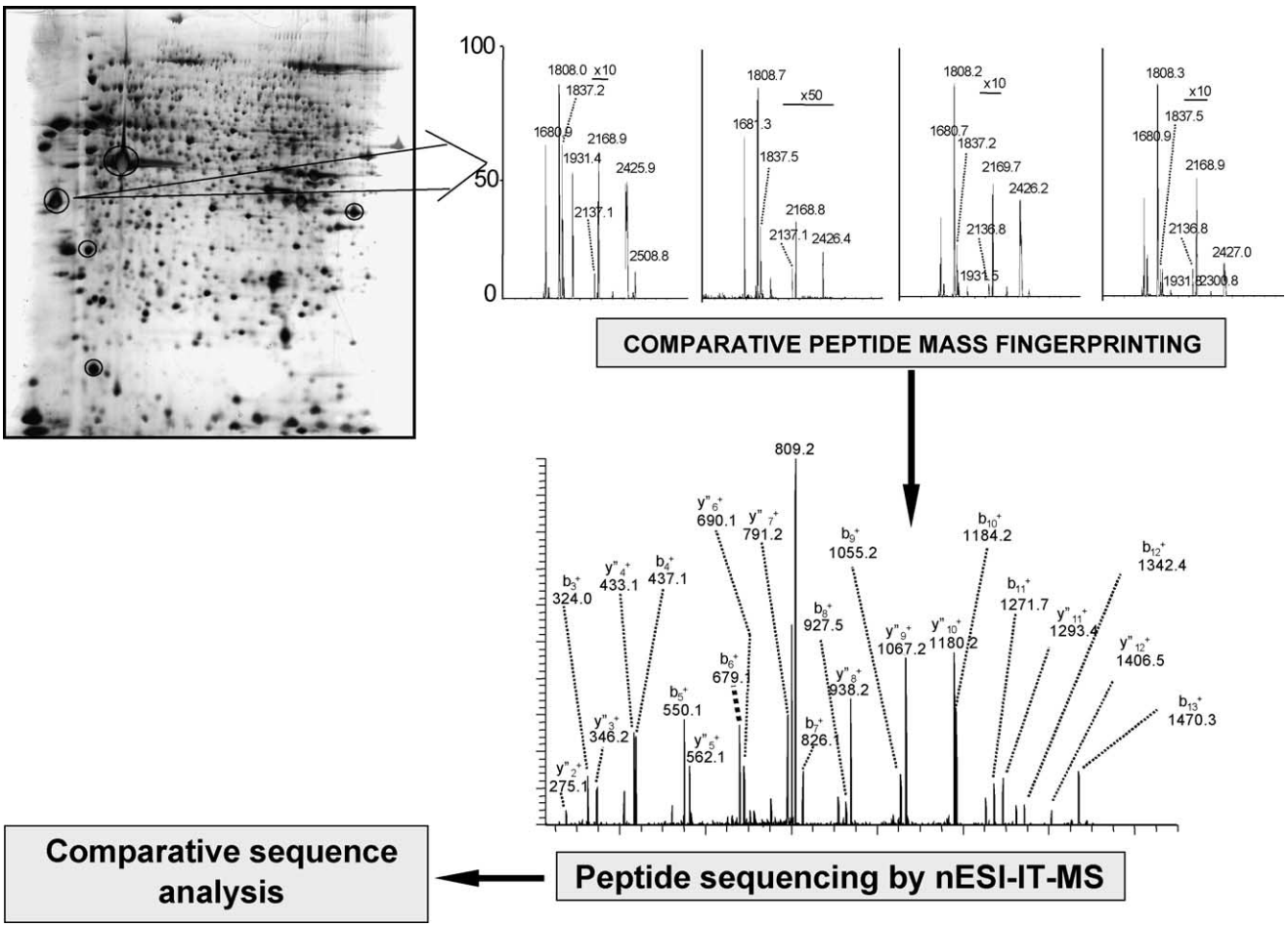

Fig. 7. A schematic overview of comparative analysis of species-specific peptides from prominent protein spots "not" showing apparent alterations in 2-DE behaviour.

species in a highly selective and specific manner is possible by tandem HPLC–ESI–IT MS using the "selected ion monitoring" (SIM) configuration [127]. Fig. 7 summarises the different steps taken in this approach.

Finally, in López et al. [127] a sequence comparison analysis of all tropomyosins present in databases was performed. As shown, the sequence of the *M. trossulus* peptide was unique for all species. This peptide can therefore be used as a specific marker for this species provided that no novel species having the same sequence are discovered. These results supported the validity of our approach for detecting species-specific peptide sequences.

This last strategy may be potentially applied for the routine identification of species-specific peptides by arbitrarily choosing to analysis several of the most abundant proteins from any species, without any previous information about protein variability or any clue about the existence of potential protein markers. Although it is not possible to foresee how many proteins are, in general, needed for the identification of species-specific peptides, the high-throughput and speed of analysis of the modern MALDI–TOF mass spectrometers may easily allow the extension of this kind of comparative study to hundreds and even thousands of proteins from a large number of individuals, making the identification of peptide markers highly likely. In this step no information from databases is needed. Once potential peptides are identified, then lower throughput MS techniques, such as nanoESI-IT MS, may be used to perform a more detailed characterisation of these markers. HPLC-tandem MS, focused on the peptide markers, may then be used for a fast and highly accurate confirmation of specificity. Since this last technique is strictly quantitative, it may be also used as a routine technique for species identification. In addition, and since the analysis may, in principle, be focused on highly abundant proteins, it would allow an easy development of anti-peptide antibodies to detect the presence of proteins containing the specific peptides in crude tissue extracts. Furthermore, this procedure is suitable for possible phylogenetic studies.

4. Conclusions

Apart from the studies that use DNA, allozymes, etc., that allow the analysis of interpopulational, interindividual and interspecific genetic variability, there are also classical and recent studies using 2-DE. All these techniques allow the construction of phylogenetic trees that reflect the relationships among populations and species. However, a proteomic analysis by 2-DE and MS, as described in this review, allows not only to find relationships and differences among populations and species but also to explain these relationships from a biochemical, physiological and ecological point of view. The better the two-dimensional gels are, the greater will be the number of loci studied and the greater the amount of data obtained. Therefore, the correct selection of the tissue to study, the sample preparation and the resolution of the gels is very important. In organisms like *Mytilus*, that present a high degree of genetic interindividual polymorphisms, it is necessary to sensibly modify the study strategy to obtain reliable results. Moreover, mussels, like many other interesting species, are poorly represented in databases, adding yet another obstacle. Therefore, many times it is necessary to recur to homologies with other species or try to overcome these limitations by developing new strategies. The search for proteins that are common and abundant, with slight changes in mobility in the gels, has allowed in some cases to detect interspecies differences. Then, the random selection of abundant and common proteins among individuals and species, not showing apparent alterations in 2-DE behaviour, has also been useful. Differences in mass maps of common proteins have been found. Therefore, it will not be necessary to identify the proteins in databases to be able to carry out the comparative study. The possibilities are endless. The enormous potential of proteomic methodologies should be exploited to design new strategies.

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