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Electrophoresis-Based Proteomic Meat Animal Research

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Summary

In this review, some of the limitations and issues related to current popular proteomic approaches will be discussed and a case will be made for the benefits of using one-dimensional electrophoresis-based proteomics for agricultural study setups. One-dimensional electrophoresis-based proteomics has an important advantage over conventional two-dimensional proteomic technologies because they are statistically robust and cost effective. The incorporation of greater numbers of samples can help alleviate the inherent variability of agricultural models that can adversely bias and influence the results.

Key words: one-dimensional electrophoresis, two-dimensional electrophoresis, proteomics, meat animals

Advances in Proteomic Research

Genome/RNA-based techniques have been increasing our knowledge of transcriptional events of growth and development in animals. However, it is ultimately the transcribed proteins, the proteome, that give rise to the phenotypes responsible for their variability. Over the past 15 years the study of the proteome has become a frontline tool to elucidate some of the vast amount of information gathered by functional genome characterization (*1*,*2*). Although the genetic information, in terms of the sequence of nucleotides, within an animal remains relatively unchanged throughout its life, the transcriptional patterns of genes into mRNAs, which are ultimately translated into proteins, are influenced by developmental and environmental factors (*3*). Absolute quantification of gene expression events, based on transcriptional events, is affected by post-translational modifications of proteins (*4*). Proteomic methodologies attempt to translate this diverse and ambiguous genomic information into a tangible and quantifiable description of the protein biological systems that are carrying out physiological functions.

The most common proteomic technologies available are based on two-dimensional electrophoresis (2-DE) (*5*), in which proteins are first resolved based on their isoelectric point then subsequently resolved based on their relative molecular size. This method has been further refined into a technique termed differential image gel electrophoresis (DIGE) that compares electrophoretically resolved spots (proteins) from two samples in which each of two 2-DE gels has been stained with differently colored fluorescent dyes (*6*,*7*). Although this methodology can theoretically resolve more than a thousand proteins based on their individual physicochemical properties, the method is limited by issues of relatively high cost, spot recognition, difficulties in quantification, low throughput, and low reproducibility (*8*,*9*).

Non-gel based methodologies such as isotope-coded affinity tags (ICAT) (*10*) and isobaric tag for relative and absolute quantitation (iTRAQ) (*11*) are techniques used for quantitative proteomic analysis and are extremely efficient but are unsuitable for large experimental setups, because of the vast amounts of information generated. Innovative combinations of proteomic methodologies are being developed constantly, mostly because of the necessity to adapt current technologies to specific research scenarios to bypass specific hurdles and to improve the efficiency of current methods. A recent innovative method has combined iTRAQ labeling and 2-DE to quantify an artificial mixture of various different proteins, which demonstrates the potential applicability of this method for quantitative mass-spectrometry proteomics (*12*).

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Another study performed 2-DE immuno-blotting of brain, myelin fraction and myelin-axolemmal proteins from biotinylated cerebrospinal fluid derived from multiple sclerosis patients. Proteins that reacted with the biotinylated sera were further analyzed by a matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF-TOF). This method allowed for the identification of potential biomarkers without the need to pool or concentrate samples (*13*). The hurdles encountered in proteomic studies are often unique to the individual fields of research.

In meat animal-based research, variability is major concern. Meat animal studies are subject to a multitude of external factors that are hard to identify, isolate, and control. These factors include temporal and spatial events during growth and development, season, and geography. Environmental factors such as nutrition, pollution, husbandry practices (*14,15*), and genetic variability are important factors that need to be taken into account in meat animal studies (*16–18*). In summary, in order to implement proteomic studies in meat animal studies, it is important to develop proteomic tools that can accommodate large sample sizes and have high reproducibility.

Meat Animal Research

Phenotypic variations within muscle tissue and between similar muscle tissues from animals with different genetic and environmental backgrounds are important in meat science because it is these variations that comprise the elements that will ultimately determine meat quality. Meat producers, processors and customers have identified inconsistency in meat quality, as defined by a lack of uniformity/consistency in marbling and tenderness, as one of the most important economic factors negatively impacting the meat industry (*19,20*). Meat quality parameter variation has been successfully analyzed using high throughput 1-DE-based proteomic studies where meat tenderness was predicted from the band intensity of electrophoretic bands (*21,22*). In addition, 1-DE-based proteomics has also been used for describing the differences in thermally induced meat gels (*23*). Other areas have also been explored such as the proteomic characterization of yellow perch in relation to fish mass and length (*24*).

The *post-mortem* mechanisms that occur during the 'aging' process that gives rise to meat tenderization have remained unsolved (*25*). To elucidate the mechanisms of meat tenderization, a multitude of studies using both genomic and proteomic approaches have been performed. Investigators using a transcriptomics-based study using RNA-based microarray technology suggested that the expression of a heat shock protein, which had been previously described as having an anti-apoptotic role, accounted for 60 % of the variability in meat tenderness in the genetic line investigated (*26*). However, the expression of mRNA is not always directly related to protein concentration (*4*), although, on average, the correlation between a population of genes and protein abundance are in substantial agreement (*27*). In a few instances, phenotype variation is related to the mutation of a single gene, like the structural differences in pig muscle due to the RN allele (*28*), double muscling in cattle due to mutations in the myostatingene (*29*), pale soft exudative syndrome in pigs due to a mutation in the ryanodine receptor (*30*), or the structural differences in callipyge lamb muscle (*31*).

Ultimately, it is the proteins that underlie the function of the cell, tissue and organ. For example, it has been shown that RNA degradation is not extensive enough to be the limiting factor for *post-mortem* protein synthesis (*32*). Regardless of RNA viability, energy availability required for protein synthesis plus the decrease in the functionality of protein synthesis machinery by the low pH abolishes the *de novo* protein synthesis (*33*). Thus, several investigators have suggested that proteomic pattern alteration, in *post-mortem* skeletal muscle, is mostly the result of *post-mortem* protein modification and proteolysis (*25,34–44*).

Food authentication has become a major concern for consumers in the recent years. Consumers are concerned about labeling of products in terms of origin, meat substitution, meat processing treatment and non-meat ingredient addition. There are several DNA-based protocols currently used to identify the species, sex or even the breed of animals present in meat products. There are also several technologies that can identify country of origin, rearing and feed intake based on specific markers using ELISA, chromatography, DNA, microscopy and spectroscopy. However, there are currently no quantitative methodologies available for the identification of meat cuts (*45*). The value of fresh meat is predicated on the meat cut. Processors and retailers, including restaurants, charge based on the meat cut being supplied. Meat cut differences can be identified during a visual inspection by trained personnel and some general aspects of their differences can be spotted by consumers. Meat quality parameters have been characterized for individual muscles in the carcass of an animal. For instance, inherent differences in meat quality parameters have been shown to be associated with muscle types in lamb (*46*), pork (*47*) and beef (*48*). Meat cut differences are phenotypic, that is they are based on the inherent muscle structure within the cut of meat at both the macroscopic (or visual) and the cellular level.

After water, muscle is composed predominantly of protein. Therefore, it is reasonable to hypothesize that proteomic technologies could be used to differentiate muscle groups or individual muscles based on protein abundance patterns unique to each of the muscle groups, and thus authenticate the cut of meat. In addition, the proteomic technologies would also be capable of differentiating meat cuts even after meat processing treatment including cooking.

The impact of nutrition on meat quality is an active area of research. Over the past decade, consumers have shown an increased interest for natural and organically grown meat. There is interest in the benefits of grass-fed beef, which is viewed a more natural and healthy, compared to grain-fed beef, which is the most common meat industry feeding practice (*49*). The same phenomenon affects the preference of grass-fed over grain-fed lamb (*50*). Proteomic analysis was used to characterize the differences between grass-fed and grain-feed beef (*51*). These investigators concluded that a muscle fiber type conversion to oxidative (slow-twitch) from glycolytic (fast-twitch)

was associated with the feeding regime. They suggested that the conversion was related to a change in the energy metabolic enzyme balance that is motivated by grazing in the latter fattening period.

Understanding the mechanisms of skeletal muscle growth and development has the potential of increasing the economic value of meat animals and is therefore an active focus in meat science research. Growth and development studies have focused on the participation of anabolic steroids and growth factors. For example, IGF-1 has been shown to play a predominant role in promoting normal muscle growth by increasing protein synthesis and inhibiting protein degradation by the ubiquitin-proteasome system (*52*). The use of hormone implants to promote animal growth has been subject to extended research (*53*). For example, a study in steers showed the benefits of animal hormone implantation with trenbolone acetate and estradiol on feedlot performance, carcass characteristics and carcass composition (*54*). The role of muscle specific growth promoters has been widely studied. Myostatin, a TGF-β protein family member that inhibits muscle differentiation and growth, has been intensively studied and its signaling pathways are well characterized in non-economically important species (*55*). In addition, developmental gene pattern determination has been successfully characterized in common animal models like the fruit fly (*56*). Unfortunately, no developmental gene pattern studies exist for animals of agricultural importance. At this point, this suggests that the use of proteomic studies for agricultural research is necessary.

Understanding the growth and developmental patterns of animals of economic importance is valuable not only because it has the potential to improve production and quality parameters but because it will likely have broader impacts by generating insights into human muscle growth, development and disease. In complex systems involving animals, it is important to develop technologies that permit animal scientists to accommodate factorial designs in their proteomic studies where the effects of several variables over multiple levels need to be evaluated.

Current Proteomic Research Challenges in Meat Animal Studies

There is a need for improved or more robust approaches to experimental design and analyses. Proteomic studies, which are founded on data generated by mass spectrometry, are often hindered when researchers are faced with vast amounts of data, derived from downstream mass spectrometric methodologies, which need to be assembled into a coherent explanation of a biological mechanism responsible for the observed phenomenon. Specifically, putting mass spectrometry data into a coherent and relevant picture is daunting because of the difficulty of interpreting mass spectrometry results. The interpretation of mass spectrometry results is confounding because the role of the individual proteins in the biological phenomenon is not straightforward. The interpretation of the mass spectrometry results has been facilitated recently by the implementation of protein sorting tools such as the KEGG pathway/module database (*57*,*58*), which can segregate the identified proteins into the pathways in which they participate. However, this method has limited reliability for proteins involved in major physiological pathways such as energy metabolism. For example, creatine kinase, a critical energy metabolism enzyme in skeletal muscle, is also associated with a number of cellular functions, including the regulation of calcium homeostasis (*59*,*60*).

Protein abundance estimation in a comparative study can be erroneous because of variations in the efficiency of sequence-dependent peptide ionization, the suppression of neighboring signals by dominant peptides, and missing MS/MS observations of peptide peaks due to algorithm and threshold limit settings that limit the detection (*61*). This is a general limitation to 'omics' studies. Some of these technical limitations have been improved with the development and integration of newer mass spectrometry technologies including LTQ/Orbitrap, which is capable of higher mass resolution, mass accuracy, a wider mass/charge range and a wider dynamic range compared to the conventional LTQ ion-trap or TOF detectors (*62*).

Breakthroughs in mass spectrometry have greatly surpassed many of these limitations of protein abundance estimation by offering new evaluation tools and more accurate technologies. A recent study using *Leptospira interrogans* demonstrated a novel methodology that permitted the abundance estimation and fold difference comparison of more than a thousand proteins (*63*). This technology consists in a combination of an isotopic labeling of a selected number of proteins, an average estimation of the three best detected peptides for each protein and spectral counting-based quantification. However, this technology is limited for the study of eukaryotic cells where multiple protein isoforms are generated as a result of gene splicing. Also, even though the methodology could potentially be adjusted to any experimental design, as the authors assert, it is still unlikely that it would have routine application in agricultural studies due to the large number of samples generally used in animal studies. It is likely that the analyses of multiple samples by this technology would also pose funding limitations. Another limitation of proteomic studies is the ability to segregate relevant proteins away from the irrelevant proteins. That is, electrophoretically resolved bands and spots potentially contain more than one protein/peptide species in which it is likely that only one or two of the protein/peptides is causal or integral in the biological difference observed and the other non-causal or integral protein/peptides are simply co-migrating because of limited resolving power. Emerging technologies to solve this in both 1-DE- and 2-DE-based proteomic analyses will be discussed later under statistics.

1-D and 2-D Electrophoresis and Mass Spectrometry Limitations and Accomplishments in Meat Animal Research

For comprehensive proteome analysis by 1-DE or 2-DE, pre-fractionation is essential because the need to reduce the complexity of each compartment is dramatically decreased by facilitating spot or band identifica-

tion and quantitative analysis. In 2-DE, there is a bias towards abundant proteins, thereby preventing the identification of low-abundance proteins. Because of the lower resolution of 1-DE compared to 2-DE, there is a greater tendency for the presence of co-migrating proteins/peptides in a given band. Pre-fractionation enriches low-abundance proteins and reduces the number of identically migrating proteins. Since the amount of any given protein that can be resolved is limited, pre-fractionation allows the proteins present in a particular fraction to be loaded at high levels, further increasing the representation of low abundance proteins.

It is widely perceived that each spot in a 2-DE gel corresponds to an individual protein and thus the number of spots identified in a gel is the same as the number of proteins that can be identified after mass spectrometry, but that is not the case. It is very common in studies using 2-DE-based technologies to report incomplete sequencing results because of the spots that yielded no identifiable proteins after the mass spectrometry sequencing analysis. For example, a study published in 2010 investigating yeast meiosis, using DIGE, a 2-DE-based methodology, identified 590 spots in the matched gels, from which 79 spots showed significant differences. From those 79 spots only 66 could be cut from the gel for downstream analysis. From those 66, only 48 spots gave protein fragment identifications (*64*). These technical issues are present to some extent in most, if not all the proteomic technologies. An older comparative study published in 2009, comparing plasma from dengue fever patients with healthy subjects, using DIGE, identified 359 spots based on Cy-5 staining (*65*). However, only 336 were still detectable after colloidal Coomassie post staining. From the initial 359 spots, 73 spots were found to be significantly different but only 65 could be visualized after colloidal Coomassie post-staining. All 65 spots were cut from the gel but only 37 yielded positive identifications that corresponded to 14 differentially expressed proteins (*65*). In another comparative study, published in 2004, using DIGE to investigate the effect of retinoic acid-induced differentiation of human leukemia cells, 32 significantly different spots were identified from an undisclosed number of spots. From those 32 spots, only 22 yielded positive protein identifications (*66*). These studies indicate that proteomic limitations have persisted for nearly a decade. However, the use of high throughput 1-DE-based proteomics based on a large number of replicates coupled with a robust statistical analysis enables the reduction of technical issues of protein identification associated with 2-DE-based systems. It is important to note that the advantage of 1-DE-based proteomics over 2-DE comes at the expense of some resolution as bands from a 1-DE gel suffer more from co-migration issues than spots from a 2-DE gel. This is exemplified by a study in 2004 (*67*) in which the investigators found degradation of actin *post-mortem* in a 2-DE gel analysis, a result which could not be obtained with the 1-DE gels used by other investigators (*68,69*).

Resolution limitations

Although it is widely perceived that 2-DE has a greater resolving power than 1-DE and that each spot in a 2-DE gel corresponds to an individual protein, in contrast to 1-DE which can suffer from co-migrating protein/peptides, there are limitations associated with the resolving dynamics of 2-DE gels. Two-dimensional gels are, generally, only capable of resolving proteins/peptides within the molecular mass range from ~20 to 100 kDa and isoelectric points within the pH range from 3 to 10. These ranges can be extended by using specialized low or high pH range isoelectric focusing strips and specialized gel compositions that allow the separation of proteins with low or high molecular mass. A brief review of the current literature reveals that the use of these strips and specialized gels in proteomic studies with a non-selective shotgun approach is not a common practice. A recent proteomic study with the objective to identify tumor-associated proteins as biomarkers in human esophageal squamous cell carcinoma by a 2-DE approach used isoelectric focusing in the pH range from 3 to 10 in the first step and a molecular mass from ~14 to 97 kDa in the second step (*70*). In another example, a study characterizing ovine *corpus luteum* protein abundance patterns by a 2-DE approach performed isoelectric focusing in the pH range from 3 to 10 and a molecular mass range from ~5 to 95 kDa (*71*).

One-dimensional electrophoresis has a clear advantage over two-dimensional systems in the dynamic range of its molecular mass resolution capability. Sample fractionation and wide dynamic range of the resolving power of 1-DE gel characteristics can improve the detection of proteins outside of the analysis range of 2-DE gels. For example, a study of plasma proteome analyzed the low-molecular mass protein fraction obtained by centrifugal ultrafiltration, this fraction was followed by 1-DE and LC-MS/MS (*72*). Proteins with molecular masses outside of the range of 2-DE can be separated effectively by 1-DE as demonstrated in a characterization study of high molecular mass wheat glutenin subunits that range from 70 to 140 kDa. These proteins were separated by 1-DE and then analyzed by MALDI-TOF-MS (*73*). High molecular mass proteins can be separated sufficiently to allow isoform characterization as demonstrated in a study that used 4 % glycerol added to acrylamide gels to separate myosin heavy chain isoforms. The method was able to resolve myosin heavy chain isoforms type 2A, 2X, 2B and 1 from each other and allowed the investigators to estimate isoform ratios (*74*).

Two of the major advantages of 1-DE are high throughput and reproducibility. In an early study (*21*) using 1-DE and linear regression analysis the authors were able to develop a model for predicting the carcass tenderness of beef cattle to an R^2 of 0.82. This was one of the first studies to report the *post-mortem* proteolysis of the large molecular mass myofibrillar protein, myosin. The identified proteolytic fragment had a molecular mass of 153 kDa, a protein fragment unlikely to have been identified using 2-DE. Another more recent study using 1-DE and 70 fish (*24*) identified 18 proteins associated with and predictive of body length and mass of yellow perch. As a further example of the high throughput and reproducibility of 1-DE in meat animal proteomic research, investigators (*75*) used 93 muscle samples to identify protein indicators of pork aging and water-holding capacity. Investigators using whole muscle in a 1-DE-based proteomic study of the muscle of cod were able to analyze proteins

>200 and <20 kDa, and find 446 unique proteins from the cod EST database and 3924 proteins from the zebrafish protein database (*76*). This study shows that 1-DE is capable of a wide dynamic range of protein molecular mass resolution and was the basis of the identification of nearly 4000 proteins, which is in the range of 2-DE-based proteomic analysis.

Other researchers (*67*) reported on the combined use of 1-DE and 2-DE to identify myofibrillar substrates for the proteolytic enzyme, μ-calpain, the enzyme considered by many investigators to be responsible for generating the *post-mortem* meat characteristic of tenderness. In another study investigators subjected all animals in their study to 1-DE and subsequently used 2-DE to analyze a subset of 8 animals to identify zones within sarcoplasmic and myofibrillar protein fractions affected by the meat quality defect, PSE (*77*).

Besides isoelectric focusing and molecular mass range limitations, 2-DE systems are not exempt from co-migration issues. Co-migrating proteins can confound the detection of important causal proteins and the interpretation of results (*78*). In the development of a proteome map of maize rachis by 2-DE and LC-MS/MS, it was found that 416 spots represented 517 distinct proteins. From those 416 spots, 143 (34 %) contained multiple proteins and 103 proteins (19.9 %) were present in multiple spots, likely due to isoform ambiguity and post-translational modifications (*79*). The authors suggested that a combination of methods be used to validate the results of 2-DE-based proteomic analysis.

Statistical methods to resolve co-migration

Despite the advantages of high throughput, high reproducibility and relative low cost, the major drawback of 1-DE-based proteomics is the confounding effect of the number of protein(s)/peptide(s) co-migrating in a given band (*21,22*). Protein ID interpretation is difficult because there is uncertainty resulting from the presence of a mixture of proteins that may participate in the studied biological mechanism and irrelevant proteins that likely make up the background (*22,24*).

The incorporation of protein abundance estimators has the potential of sorting out the co-migration issue associated with 1-DE-based proteomics (*80*). Protein abundance estimation methods like the exponentially modified protein abundance index (emPAI) (*81*), or the absolute protein expression (APEX)-based fragment count estimations from MS/MS data (*82*), or the quantification of peptides of interest by comparison with a calibrated isotope labeled reference (*83*), or by the use of a modified spectral count index (mSCI) derived from the incorporation of mRNA and protein expression data (*84*), or even a combined method (*63*) that uses multiple estimators can be incorporated into the analysis of 1-DE bands. The incorporation of abundance estimators can provide precise results that can sort the relevant proteins out of a mixture of confounding background proteins.

One of the key issues in label-free or electrophoresis-based proteomic research based on tandem mass spectrometry (MS/MS) is the identification of protein species and the characterization of their expression changes in normal and treated samples. Three analysis techniques are often required in any MS/MS study: identification, characterization or primary sequence determination, and quantification of expressed protein/peptide (*85*). The estimation of the abundances of hundreds or thousands of fragment ion spectra generated that may be present as part of a cadre of co-migrating bands or spots is a great statistical challenge (*86*). Recent advances in the estimation of the abundances of fragment ions have been made through the method of spectral counting by Lee *et al.* (*87*), who report on a novel spectral counting method to estimate peptide abundance by counting MS/MS spectra, comparing and clustering all experimentally observed spectra. The advantage of this approach is that it appears to be applicable to any tandem MS/MS-based analysis including electrophoresis-based 'omics'.

The reliability of mass spectrometry data and the protein identifications derived from it is generally accepted by the proteomic community. However, an additional issue associated with 2-DE gel resolution resides in the ability of any software to recognize a spot consistently across several gels. In theory 2-DE has the ability to separate more than a thousand spots, but the reproducibility of that separation across technical and biological replicates is not consistent. Spot recognition and matching is performed with the aid of software which relies on complicated algorithms that are not perfect. A study compared the efficiency of three popular DIGE analysis software packages: DeCyder v. 6.5 (GE-Healthcare, Piscataway, NJ, USA), Progenesis SameSpots v. 3.0 (Nonlinear Dynamics, Durham, NC, USA), and Dymension 3 (Syngene, Fredrick, MD, USA) in the analysis of two cancer cell lines. The study revealed differences in each of the software's capability to detect spots. Fold changes were substantially different across the different software packages, which indicates that spot quantification was software-dependent despite the use of internal standards (*9*). Gel analysis in a 2-DE experiment is very subjective and carries operator bias. Automation and standardization efforts to eliminate user bias are desired but are not always achieved (*88*). These investigators highlight the potential that a set of gels matched in one laboratory by a specific operator will not be matched in the same way by a different laboratory with a different operator. It is likely that even within the same laboratory differences will exist. The low reproducibility inherent in 2-DE-based separations makes the matching required for the analysis of 2-DE gels more complex than the matching required for the analysis of 1-DE gels. In the study of differential muscle protein concentrations associated with and predictive of tenderness determined that there was no statistical difference between the multiple 1-DE gel experiments performed over a period of several days (*21*). Thus, the matching required for the analysis of 2-DE gels is more complex and potentially less reliable than the matching required for the analysis of 1-DE gels.

Statistical analysis limitations

Proteomic studies handle vast amounts of data, thus the likelihood of reporting false discoveries is large. In a mass spectrometry protein identification, fragment ion spectra are assigned to peptide sequences by using database search engines like Mascot (Matrix Science Inc., Boston, MA, USA), SEQUEST (Thermo Scientific, San

Jose, CA, USA), or X!Tandem (Global Proteome Machine Organisation). Then protein identifications are inferred by assembling the identified peptide sequences into proteins (*89*). Neither database entries, nor sequencing results are perfect. Therefore, it is important to control the reliability of protein identifications. Proteomic studies expect to identify proteins that are truly differentially expressed due to environmental and/or genetic influence, but the large number of comparisons encountered in a typical proteomic study makes statistical multiple testing issues a major concern (*90*). While performing a large number of independent statistical tests at a certain confidence level, the likelihood of observing false differences is proportional to the sum of the error for each test (*91*). This means that an experiment performing 500 independent tests at a 99 % confidence level will likely contain 5 erroneous tests (0.01 times 500). If the confidence level is dropped to 95 %, the experiment will likely contain 25 erroneous tests (0.05 times 500). The most popular method available for the control of false discovery is known as the false discovery rate correction. This method uses the outputs of MS/MS search engines to calculate q-values for each comparison made. The q-value is the expected proportion of false positives incurred when a significant difference (*91*) is found through the use of a p-value. The p-value is a measure of significance in terms of the false positive rate of an individual test. However, the q-value is a measure in terms of the false discovery rate which is dependent on the entire number of individual tests performed in the study. Thus, by using this method, to declare a significant difference, the test must pass the p-value and the q-value criteria (*90*). To illustrate this point, a study that evaluated the response to mobile phone radiation of two types of human primary endothelial cells by DIGE, which subsequently used a *t*-test approach with a 99.99 % confidence level and a false discovery rate correction, found that from the 1746 spots analyzed in the gels, 368 spots were found to differ between the two cell types, which was expected. However, when analyzing each of the cell types separately to evaluate the effects of mobile phone radiation researchers found, using independent *t*-tests at a 95 % confidence level and no false discovery rate correction, that one cell type displayed 35 significantly different protein spots compared to its control, while the other cell type displayed 2 significantly different protein spots compared to its control. When the false discovery rate was implemented, all the significantly different spots detected previously were recognized as false positives (*92*). In a separate study investigating plasma biomarkers in pediatric patients undergoing cardiopulmonary bypass by 2-DE-DIGE (*93*), 556 protein spots in all gels were identified. Out of those 556 spots, 175 were significantly changing according to independent *t*-tests at a 95 % confidence level. The results were grouped by protein name and a total of 25 proteins were identified. In a very interesting step, the results were reevaluated by applying a Bonferroni correction for multiple comparisons and the protein list was reduced to 17 proteins identified after grouping. In an alternative approach, samples were subject to a multianalyte profile assay where the samples are challenged against 90 different human antigens related to tissue damage, inflammation and other pathologies. The multianalyte profile assay showed that 49 out of the 90 proteins assayed changed significantly at a 95 % confidence level; however, after a Bonferroni adjustment the number of proteins assayed that were significant were now 21. Even when the authors of that study did not comment on the discrepancies as the study is merely designed as exploratory, it is evident to the reader that inference based on proteomic results without an experiment-wise error correction can be risky, based on the observation that a large number of significant proteins are lost after an experiment-wise correction. Conclusion discrepancies due to the use of an experiment-wise error correction are rarely reported, thus its impact cannot be reliably estimated from a survey of the literature. Because of this, a formal proteomic study was performed in which the performance of the significance outcome obtained by independent uncorrected *t*-tests was compared to the significance outcome obtained using Bonferroni and false discovery rate adjustments (*94*). In this study, 1-DE fractionated microbial protein extracts of cultures grown at 10 °C *vs.* 30 °C. The 1-DE fractions were further analyzed by LC-MS/MS, which consistently found 954 proteins across the two treatments. Out of those 954 proteins an uncorrected *t*-test identified that 325 were differentially expressed, while only 56 were identified by Bonferroni and 272 were identified by false discovery rate. All tests were performed at a 95 % confidence level. It was concluded that the Bonferroni method offered results that were far too conservative for proteomic application. The false discovery rate method identified more than four times the number of proteins than Bonferroni but was more conservative and offers more confidence information than the uncorrected *t*-tests.

The use of methods that correct for experiment-wise error such as Bonferroni's adjustment or the false discovery rate method can improve the overall confidence of a study by offering results with less uncertainty. It is true that the use of experiment-wise error correction can eliminate a large number of proteins that could be related significantly to the biological question, but it is relevant to question if those proteins are really lost using these statistical analyses. The discovery of potential biological participants is ultimately related to chance since every discovery made has a potential of being true or false because it is associated with the experiment characteristics and the statistical analysis used. Therefore, it is not possible to ascertain the validity of a discovery with 100 % certainty. However, the inference that can be made based on statistical methods that yield more confident results leads to more trustworthy conclusions.

Conclusion

The application of 1-DE does not replace more refined technologies, because it lacks the level of resolution offered by 2-DE methodologies. Low-cost high-throughput proteomic methods can provide an opportunity to research fields where the search for biomarkers and limited funding are common through the use of large number of samples. One-dimensional electrophoresis has the advantages of being high throughput, highly reproducible and low cost. Thus, 1-DE-based proteomics can be

very appropriate in low-cost exploratory studies that can be used to guide investigations using less reproducible, lower throughput but higher resolution methodologies.

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