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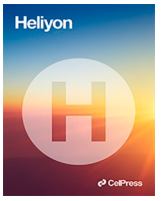

Research article

Comparative analysis of electrophoresis and interferometric optical detection method for molecular weight determination of proteins

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ABSTRACT

Analytical detection methods play a pivotal role in scientific research, enabling the identification and quantification of specific analytes in various disciplines. This scientific report aims to compare two very different methodologies for determining the Molecular Mass (MM, also known as Molecular Weight, MW) of proteins: electrophoresis gel and the Interferometric Optical Detection Method (IODM). For this purpose, several proteins with different MM were selected.

The electrophoresis technique was employed to validate the structure and MM of different parts or fragments of the Matrix Metallopeptidase 9 antibody (anti-MMP9), antibody against S100 calcium binding protein A6 (anti-S100A6) and Cystatin S4 antibody (anti-CST4) by examining the presence of bands with expected sizes. The IODM was applied to study the above-mentioned proteins (part of the antibodies) together with the protein G, as a reference to correlate the MM and protein sizes with the measured signal.

We report the evidence of IODM as a competitive analytical approach for the determination of the MM of proteins for the first time. This innovative method allows for accurate MM determination using minimal sample volumes and concentrations, employing a simple experimental procedure that eliminates the requirement for protein denaturation.

1. Introduction

The scientific disciplines such as chemistry, environmental science, pharmaceutical analysis, biochemistry, and forensic science based most of their results on analytical detection. Thus, many applications including scientific research, environmental monitoring, healthcare quality control, and pharmaceuticals depend on the process of identifying and quantifying the presence of a specific analyte

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(target compound or substance) within a sample. For this reason, a wide variety of analytical detection methods have been developed since the early 19th century. Nowadays the most widely employed methodologies can be classified into different categories: chromatographic and spectroscopic techniques, electromigration methods, immunoassays, electrochemical methods, and molecular biology-based approaches. According to the nature of the analyte, its concentration range, and the complexity of the sample matrix among other factors the selection of a convenient detection method can be performed.

Attending the methods based on electromigration, one of the most popular is the electrophoresis technique, which applies an electric field to separate charged particles such as proteins, nucleic acids, and carbohydrates. Thus, several data can be obtained from electrophoresis since it provides the size, charge, structural components, and relative abundance of biological molecules, particularly proteins and nucleic acids [1]. Particularly, electrophoresis gels allow the separation of complex mixtures of biomolecules into individual components based on their size and charge. It can be also used to estimate the Molecular Mass[1] (MM), commonly named Molecular Weight[2] (MW) although the International System of Units recommends using the term MM, of unknown molecules, as well as the shape or conformation of a protein since it affects its mobility through the above-mentioned gel. Eventually, the level of the absorbance produced, visually observed by bands or spots, on the electrophoresis gel, could be used as a semi-quantitative measure of the abundance of different molecular species in each sample. However, this technique has its own constraints such as a limited resolution when dealing with complex mixtures or closely related analytes, the matrix sample itself may interfere with the analyte migration leading to distorted results or reduced separation efficiency and the quantification results from the molecular concentration based on the band absorbance intensity might be not accurate enough [2,3]. Moreover, in order to achieve good results, high concentrations of molecules are needed. Therefore, electrophoresis becomes an expensive technique and in terms of operability, it is a complex and time-consuming procedure that requires qualified personnel.

In addition, within the group of optical techniques based on interferometry for analytical detection, it can be found in the scientific literature, the Interferometric Optical Detection Method[3] (IODM) [4,5]. The basic principle of the IODM is to amplify the readout signal change generated when the analyte interacts with the sensing element, since it causes a change in the optical interferometric signals produced for changes such as refractive index, optical thickness, or light scattering, resulting in a measurable signal. This technique applied to globular conformational particles, can provide accurate information on the MM and concentration of the unknown molecular samples. The key advantage of the IODM is its ability to enhance the signal-to-noise ratio and improve the detection limits of analytes without the need to denature the proteins [6]. By amplifying the relative change in optical power, even small concentrations of analytes can be detected with higher sensitivity and accuracy. Besides the complexity of the carefully designed technological system, the handling of the equipment is very simple, and the detection can be performed for any unqualified person with a very low volume/concentration of sample.

Although in their origins both, electrophoresis and IODM, were techniques with different purposes, in this work we aim to show for the first time the usefulness of using IODM as an alternative technique for obtaining the MM in a faster, simple and efficient manner, using a very low volume, and concentration of sample and thus reducing the cost of the process.

2. Material and methods

2.1. Electrophoresis gel

Electrophoresis is a common and widely used technique for separating proteins based on their Molecular Weight for complex samples. It operates by applying a constant electric field to a medium, causing ionized molecules to migrate at a certain velocity (v). This velocity is determined by the mobility constant (m) associated with each particle under specific electrophoresis conditions. The mobility constant relies on factors such as particle size, shape, and charge. Among the various electrophoresis methods available, this work focuses on Zone Gel Electrophoresis or PAGE, which employs a Polyacrylamide gel as the medium [7,8].

$$v = mE \quad \text{(eq. 1)}$$

The Polyacrylamide gel (PAG) serves as a physical porous barrier, allowing proteins to travel through it based on their size and the applied electric field. The PAG is an acrylamide gel that polymerizes in a specific porous size thanks to the Tetramethylethylenediamine (TEMED) catalyzer resulting in two phases with different pore sizes. The first phase (concentrator) has larger pores to concentrate all proteins at the interphase position before the electric field is applied. The second phase (spacer) has smaller pores, causing molecules to move at different velocities depending on their sizes when the electric field is introduced.

To ensure protein migration in the second phase of the Polyacrylamide gel, the sample is pretreated with a buffer that denatures the proteins and imparts a negative charge to them. Therefore, proteins such as antibodies are separated into subunits such as the represented in Fig. 1 [9]. The buffer also contains bromophenol blue (~669 Da), which acts as an indicator. Although the migrating molecules are not visible to the naked eye during electrophoresis, due to the bromophenol blue indicator it is known when the end is reached indicating when the process should be stopped. Additionally, a calibration curve with stained particles of known Molecular Mass is run alongside the sample. After staining the gel with Coomassie blue, which stains all proteins in the Polyacrylamide gel, the Molecular Mass of the particles can be estimated by directly comparing their migration distances to the calibration curve [10]. It is also

[1] MM: Molecular Mass.
[2] MW: Molecular Weight.
[3] IODM: Interferometric Optical Detection Method.

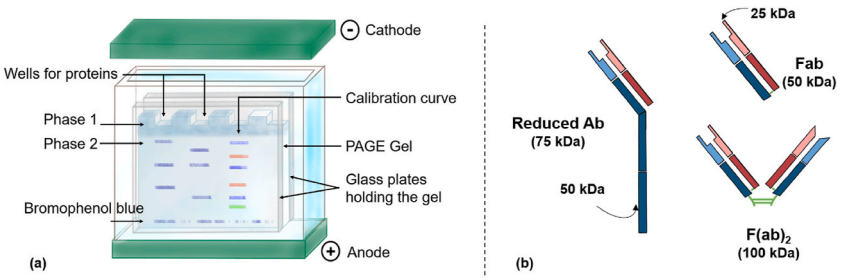

Fig. 1. (a) Electrophoresis gel layout where all components involved in the process are specified, (b) antibodies fragments with their corresponding MM [9].

possible to calculate the molecular weight through the retention factor[4] (Rf). By knowing the total distance traveled by the bromophenol blue indicator front and the partial distances from the calibration curve, an exponential equation is obtained that serves to estimate the molecular weights of proteins based on their Rf values [11–13] (see Fig. 1).

2.2. Interferometric optical detection method (IODM)

This detection method is based on the comparison of the information acquired from two interferometric signals originating from two interferometers as biotransducers (namely the reference channel and biosensor channel, wherein each one is placed an optical Fabry-Perot sensor based on interferometric thin films) which allows obtaining a unique response (see Fig. 2). Through this approach, a distinctive and highly responsive output is achieved, specifically attuned to variations in the optical properties exhibited by the biosensor used for the analytical detection [4,5].

For this purpose, two optical Fabry-Perot based biosensors made by interferometric layersare used, one as a reference and the other one as a biosensor where the interaction with biomolecules takes place. These interferometers can be composed of interferometric layers in order to work as a simple optical Fabry-Perot [14]. Its configuration is adapted to the IODM read-out method to obtain the highest sensitivity and therefore an improved Limit of Detection and a maximum dynamic range for the sensor. This adaptation involves a correct selection of the interferometric layer according to the optical interrogation band used [4,15].

As the biosensor transducer performs the analyte detection (a biolayer is immobilized and therefore the Fabry-Perot increases its thickness), its signal (I_{bio}) will change compared with the reference channel signal (I_{ref}). The difference between the reference signal and the biolayer thickness immobilized in I_{bio} is described by the Increased Relative Optical Power (IROP). This technique allows the monitoring of biofunctionalization and recognition processes (step by step) and permits the obtaining $\Delta IROP(\%)$ magnitude correlated with analyte concentration (proportional to biolayer thickness) [14].

2.3. Optical biosensor

The biosensor employed in conjunction with the IODM (Interferometric Optical Detection Method) technique is an optical Fabry-Perot (FP) transducer that has been utilized by many authors for multiple applications [16]. This FP sensor has been specifically designed and fabricated to optimize performance parameters, including signal intensity, the limit of detection, and dynamic range. To ensure compatibility with the intended wavelength range of the read-out instrument, the interferometer layer is constructed using SU8, a biocompatible resist, with a carefully chosen thickness [17]. In this particular study, because the lasers used as light sources, the optical interrogation band falls within the wavelength range of 800–860 nm, while the SU8 layer has a thickness of 495 ± 10 nm in order for its spectral response to match the considered interrogation band. To enable the simultaneous execution of three assays, a silicon substrate serves as the foundation for the biosensor, upon which three SU8 independent cells, each of them with a diameter of 100 μm, are fabricated, employing a SiO_2 antireflective layer.

To minimize the consumption of reactive substances and reduce economic costs, a PDMS (polydimethylsiloxane) container was employed (110 μm thickness film from Gel-Pack®). The container was fabricated to match the desired shape and size of the SU8 cells using a plotter machine, and its integrity was assessed through resistance testing. The results of this evaluation demonstrated that the container achieved a complete seal, enabling stable incubation of volumes up to 2 μL overnight.

2.4. Characterized proteins

This study aims to compare the electrophoresis gel technique with the Interferometric Optical Detection Method by evaluating proteins with varying Molecular Mass and sizes. The proteins selected for analysis were obtained from Cloud-Clone Corporation and Merck Life Science S.L.U., and a summary of their key characteristics is presented in the following table.

The Molecular Mass of the proteins is known from the data sheets provided by the providers. Regarding the sizes of the molecules,

[4] Rf: Retention factor.

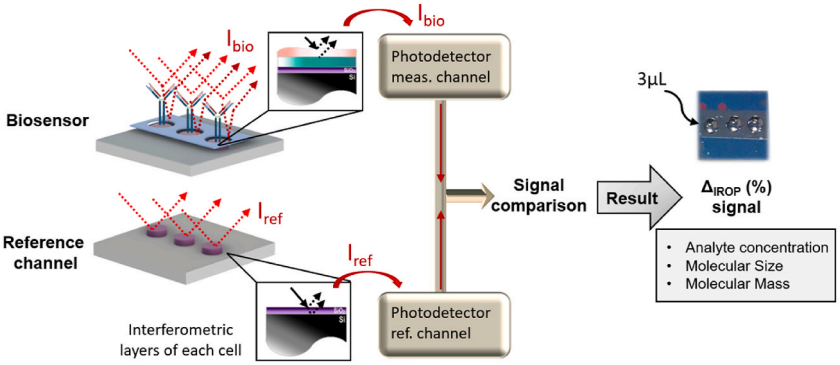

Fig. 2. A schematic description of the IODM where each input light (from biosensor and reference channel) strikes on the transducers placed on each channel, goes to each of its Optical Power detectors and through the information recorder it can be obtained the analyte concentration, Molecular Size or Molecular Mass. Enclosed are the interferometric layers that form each cell of the transducer. On the right top three wells PDMS container is used for the recognition stages capable of incubating 3 μL per cell.

deep studies can be found in the literature or web pages in function of their Molecular Mass (MM) or number of amino acid residues [18,19]. The equations below show how Erickson estimates the minimum radio of a protein assuming that it has a simple sphere shape. And this is the basis used for protein size calculation in the third column of Table 1. It should be mentioned here that no information is available for the CST4 antibody, and therefore it will be treated as the problem molecule that will serve to contrast the results obtained through electrophoresis and IODM.

3. Experimental section

3.1. Molecular Mass determination by electrophoresis gel

In this study, the Molecular Mass of various antibodies was determined using the electrophoresis gel method and compared with a commercially available calibration curve (Ref.: 11832124, PageRuler™ Plus Prestained 10–250 kDa Protein Ladder from Thermo Scientific™).

The electrophoresis protocol began with the denaturation of proteins. This was achieved by mixing the lyophilized antibodies (with a minimum amount of 50 μg per antibody) with a buffer solution composed of beta-mercaptoethanol (a reducing agent that disrupts disulfide bridges in the quaternary structure), urea 8M (unfolding the amino acid chains and disrupting the tertiary structure), SDS 8 % (an anionic detergent that imparts negative charge to the proteins), TRIS 0.08M, and HCl (to maintain protein stability at a pH of 6,8). Additionally, bromophenol blue (a low Molecular Mass protein) was added to serve as an indicator to determine the completion of electrophoresis.

The denatured antibodies were loaded onto a pre-prepared polyacrylamide gel (PAG) along with labeled control proteins of known Molecular Mass, each labeled with a different color. The entire setup was immersed in an anionic buffer, and a constant current of 20 mA was applied, resulting in a potential difference of 220 mV. The electrophoresis process lasted for 1,5 h. Once the bromophenol blue marker reached the bottom of the gel, the current was halted, and the gel was developed by immersing it in a diluted solution of Coomassie blue for 40 min. Subsequently, the gel was washed, allowing the Coomassie blue dye to stain the proteins and enabling the visualization of their respective molecular sizes. It should be noted that when having denatured particles, as a result of electrophoresis, more than one band can be obtained due to the different partitions of the molecules, and therefore the total size of it will be the sum of the retention factor (Rf) of those bands [20,21].

3.2. Molecular Mass determination by IODM

As previously stated, the alterations in the ΔIROP (%) signal are directly associated with changes in the thickness of the interferometer. Exploiting this characteristic, it becomes possible to determine the size of the immobilized protein. To achieve this, it is crucial to establish a uniform monolayer of the protein ensuring the full sensor surface coverage. Failure to attain a uniform monolayer may result in a lower signal variation, thus providing an inaccurate measurement of the molecule's true size.

To achieve a stable protein monolayer on the surface coverage of the SU8 100 μm diameter cell, an immobilization protocol was implemented, employing a conservative approach that involved high volumes and extended incubation times. The protocol began with the activation of the SU8 surface through acid catalysis using H_2SO_4 for 15 s, followed by rinsing with mQ water. Subsequently, a volume of 13 μL per cell of 1 μg/mL protein (approximately 10^{-3} μg of protein) was incubated for 3 h in a humid atmosphere at a temperature of 37 °C. After incubation, the biosensor was washed with PSB-T and mQ water. Before conducting the read-out, the biosensor was thoroughly dried.

To ensure the acquisition of accurate and dependable results, a total of 12 distinct SU8 cells were utilized for the immobilization of each protein type. The subsequent monitoring of these immobilized proteins was conducted utilizing the Interferometric Optical

Table 1
Purchased reagents from Cloud-Clone Corp. and Merck Life Science S.L.U. *NA: Not Available.

Protein	Reference	Molecular Mass (kDa)	Radio (nm)	Ab isotype
Protein G	P4689	32	2,09	–
anti-MMP9	MAA553Hu22	84	2,89	IgG2b Kappa
anti-S100A6	MAB769Hu22	16	1,42	IgG2b Kappa
anti-CST4 from ELISA Kit	PBJ3244Hu01	NA*	NA*	NA*

Detection Method.

4. Results

The electrophoresis technique induces the denaturation of proteins by disrupting their quaternary structure. The experimental results for each antibody are reflected in the form of absorbance bands. From there, the retention factors (Rf) are obtained for the calibration proteins, and an exponential fitting is performed as shown in Fig. 3-right. Using this exponential curve and the Rf values obtained for the antibodies, their Molecular Masses are calculated. As shown in Fig. 3-left, both the two calibration curves and the bands show significant variations in their migration distances. For example, for the 15 kDa band on the left calibration curve, there is an absolute displacement of 10.44 mm, while on the right curve, it is 4.39 mm. This variation reveals the imprecision of this technique, as stated in the literature. However, the values obtained for the antibodies are close to the expected structure and Molecular Mass. Therefore, for the anti-MMP9[5] antibody, two bands close to 25 kDa and 55 kDa were observed, corresponding to the variable regions of the immunoglobulin molecule (Fab) and the constant region (Fc), respectively. According to the sum of the Molecular Masses obtained from their Rf values, the anti-MMP9 is estimated to be 79 kDa. This confirms that the antibody is a reduced IgG, as specified by the supplier. On the other hand, the anti-S100A6[6] molecule only exhibits a band between 15 and 25 kDa, and its Rf reveals a Molecular Weight of 23 kDa, corresponding to a Fab portion, which is consistent with the specifications provided by the supplier.

Lastly, the antibody from the ELISA kit was used to analyze the anti-CST4[7]. Electrophoresis revealed a Molecular Mass of 58 kDa for this antibody. Although this MM could not be directly compared with the manufacturer's specifications, it aligns with the results obtained through the IODM technique.

In addition to the analyzed antibodies, the IODM was employed to study proteins with varying MM too. The calibration curve was determined by evaluating the ΔIROP signal obtained from known concentrations of anti-MMP9, anti-S100A6, and Protein G. This approach allowed for the establishment of a linear curve based on the Molecular Mass provided by the manufacturer, which aligned with the results obtained from electrophoresis gel. Based on the linear fitting conducted, the molecular weight (MW) estimation for the anti-CST4 antibody is determined to be 48 kDa. Notably, this method offers the advantage of not requiring protein denaturation. Furthermore, a dimensional scale representing the equivalent thickness of the biofilm layer (the optical thickness provided by a monolayer of protein) is located on the right axis. It is observed that the obtained equivalent thickness values closely align with the estimated values for the molecule size (Table 1).

As a result, the obtained signal is directly proportional to the MM of the protein. The error bars represented in Fig. 4 indicate the standard deviation for each average value These error bars serve as tolerance limits for each antibody during the biofunctionalization stage, ensuring the formation of a uniform monolayer.

Finally, a comparison between both techniques is performed by illustrating the MM obtained from the calibration curves derived from electrophoresis and IODM. Fig. 5 demonstrates a linear correlation between the two methods.

A linear correlation is evident among all parameters, affirming the strong concordance between the measured values and the specifications of the proteins. The red areas shown in Figs. 4 and 5 represent the predicted molecular weight (MW) values and the corresponding confidence levels at 90 %. The reported MW values within the graph correspond to those provided by the protein's suppliers.

5. Conclusion

Analysis detection methods are key in various scientific fields to identify and quantify specific analyses in samples. Thus, this study aimed to compare two methodologies by determining the Molecular Mass of various antibodies. More precisely this is performed between the electrophoresis gel method together with a commercially available calibration curve and the Interferometric Optical Detection Method.

Electrophoretic technology is used to verify the structure and molecule mass of antibodies, identifying expected sizes and charges. This technique approximates the MM estimation of the MMP9 antibody fragments 20 kDa and 59 kDa, which corresponds to the Fab and Fc regions of the antibody. The anti-S100A6 molecule has only one band and its Rf-based Molecular Mass is 24 kDa, which corresponds to part of the antibody's Fab region. Anti-CST4 antibodies are estimated to be 58 kDa.

[5] MMP9: Matrix Metallopeptidase 9.
[6] S100A6: S100 calcium binding protein A6.
[7] CST4: Cystatin S4.

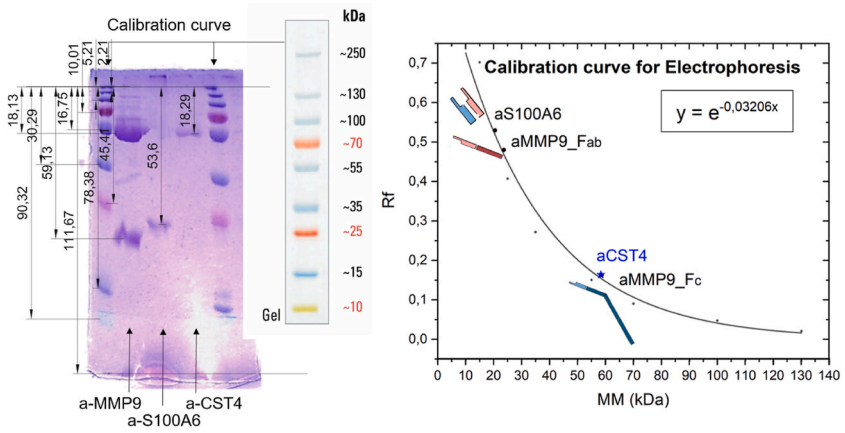

Fig. 3. On the left the obtained electrophoresis gel with the loaded antibodies (anti-MMP9, anti-S100A6, and anti-CST4) and the calibration proteins on both sides. The migration distance is shown on the described dimensions in order to obtain the Retention factor (Rf). On the right, is the calibration curve from the electrophoresis gel. From the calibration curve, the MM for the antibodies is determined: 24 kDa, 58 kDa, and 79 kDa for aS100A6, aCST4, and aMMP9 correspondingly.

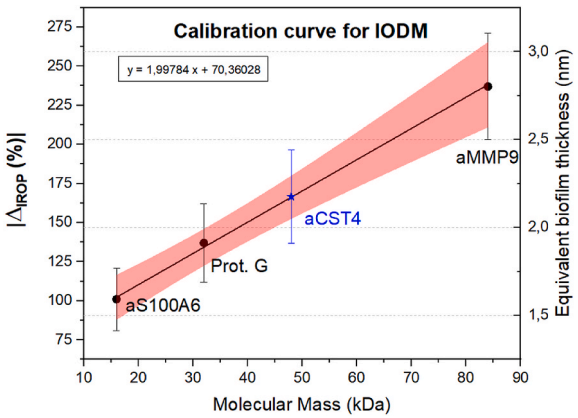

Fig. 4. Average absolute signals for the IODM obtained for aS100A6, Protein G, and aMMP9 whose Molecular Masses are known. The linear fitting is performed in those known Molecular Mass (kDa). From the calibration curve, the MM for the aCST4 is determined (48 kDa). The estimated equivalent layer of biofilm when a complete monolayer of the protein is achieved is on the right axe.

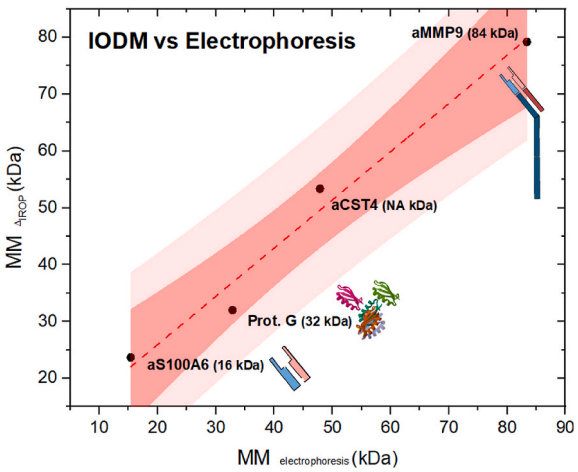

Fig. 5. Comparison of the Molecular Masses obtained from IODM on the y-axe and electrophoresis on the x-axe.

The IODM technique was also used to study proteins with different Molecular Mass, generating a calibration curve that correlates MM and protein sizes with the measured signal. The method demonstrated the accurate determination of protein sizes and mass without the need for denaturation. In addition, Fig. 4 showed a linear correlation among parameters, indicating a strong agreement between measured ΔIROP values and protein specifications (size). The error bars represent the standard deviation, ensuring the formation of a uniform monolayer during the biofunctionalization stage.

In summary, this work highlights the importance of analytical detection methods, comparing two of them: electrophoresis and the Interferometric Optical Detection Method, for determining the Molecular Mass and concentration of analytes. Although electrophoresis is a valuable technique to obtain information about proteins, it has certain limitations. These limitations include the requirement for protein denaturation, multiple preparation steps, and the need for large volumes and concentrations of proteins (a minimum of 50 μg per protein to be evaluated), which can increase the total cost of the analysis. In contrast to electrophoresis, the IODM does not require the proteins to be denatured, which can alter their structure and properties. This makes IODM a gentler technique and is less likely to damage the proteins. In addition, the IODM technique offers advantages in terms of simplicity, sensitivity, and cost reduction (only a few ng of proteins is needed for detection performance). This could state a simpler, cheaper, and faster alternative to the current electrophoresis technique (based on a subjective comparison against a visual calibration curve) used for protein mass determination.

CRediT authorship contribution statement

B. Santamaría: Conceptualization, Formal analysis, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing, Methodology. **A. L. Hernandez:** Supervision, Validation, Writing – review & editing. **M.F. Laguna:** Conceptualization, Methodology, Validation, Writing – review & editing. **M. Holgado:** Formal analysis, Methodology, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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