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Study of extraction procedures for protein analysis in plankton samples by OFFGEL electrophoresis hyphenated with Lab-on-a-chip technology

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ABSTRACT

Extraction procedures for protein analysis from plankton samples were studied. OFFGEL electrophoresis combined with Lab-on-a-chip technology has been applied for protein analysis in plankton samples. BCR-414 (plankton) certified reference material from the European Commission was used to evaluate the protein extraction procedures. Three protein extraction procedures were studied: (1) by using Tris–HCl buffer containing a protease inhibitor cocktail, (2) urea/triton X-100 buffer extraction, and (3) using the phenol/sodium dodecyl sulphate method after different washing steps with 10% trichloroacetic acid/acetone solution and methanol. The pellet of proteins obtained was dried and then dissolved in the OFFGEL buffer. Proteins were separated according to their isoelectric points by OFFGEL electrophoresis. This separation was performed using 24 cm, pH 3–10 IPG Dry Strips. The proteins present in each liquid fraction (24 fractions) were separated according to their molecular weight using a microfluidic Lab-on-a-chip electrophoresis with the Protein 80 LabChip kit. This kit allows for the separation of proteins with a molecular weight ranging from 5 to 80 kDa. Taking into account the intensity and the number of the protein bands obtained, the protein extraction procedure using the phenol/sodium dodecyl sulphate after different wash steps with 10% trichloroacetic acid/acetone solution was selected.

The developed method was applied for protein determination in a fresh marine plankton sample. The proteins found in this sample have a molecular weight ranging from 6.4 to 57.3 kDa, and the proteins with highest molecular weight were in the OFFGEL fractions with an isoelectric point ranging from 4.40 to 8.60. The concentration of proteins were calculated using external calibration with Bovine Serum Albumin, and the protein concentrations varied from 50.0 to 925.9 ng μL^{-1}.

1. Introduction

Plankton in the marine environment can be classified in two main groups, zooplankton (animal plankton) and phytoplankton (vegetal plankton) [1]. Plankton contains all the components (proteins, amino acids, lipids, carbohydrates minerals and vitamins) necessary for marine life [2,3]. Plankton can be considered as a primary source of marine proteins dissolved in open seawater. They are some of the most reactive substances and the key factor in the direct effect of dissolved organic matter in the global carbon cycle [4]. Therefore, the analysis of proteins in plankton samples is important for understanding the processes in the marine ecosystem.

Sample preparation is one of the most important steps in an analytical procedure. When protein analysis is performed, this step includes the protein extraction procedure as well as the elimination of matrix interferences such as pigments, polysaccharides, high levels of salts, etc. The presence of these substances can affect the protein resolution in the separation procedure. Furthermore, the protein extraction efficiency and the presence of matrix interferences can influence analytical sensitivity.

Different protein extraction procedures have been reported in the literature as a first step in proteomics studies. The protein extraction protocol must be selected in function of the sample matrix and must be optimized for each type of sample to obtain the highest extraction efficiency. Moncheva et al. [5] extracted the proteins in homogenized phytoplankton by using 20% (w/v) trichloroacetic acid (TCA) in acetone containing 0.1% 2-mercaptoethanol and 1% sodium dodecyl sulphate (SDS). This extraction procedure was repeated three times. The remaining pellet was then lyophilized and dissolved in phosphate buffer before the analysis. Barbarino et al. [6] evaluated different extraction procedures for extraction and quantification of proteins from marine macro- and microalgae. The protein extraction protocol selected by these authors involve the immersion of samples

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in 4.0 mL ultra-pure water for 12 h, followed by complete grinding of the samples with a Potter homogenizer and re-extraction with 1.0 mL 0.1 N NaOH with 0.5% β-mercaptoethanol (v/v). Protein precipitation was performed with 2.5:1 25% TCA:homogenate (v/v). Carpentier et al. [7] evaluated four extraction protocols for protein extraction from plant tissues (banana, potato and apple): (1) TCA precipitation, (2) TCA/acetone precipitation and fractionation, (3) no precipitation fractionation, and (4) phenol extraction methanol/ammonium acetate precipitation. The authors concluded that TCA/acetone precipitation and phenol extraction methanol/ammonium acetate precipitation were useful as standard methods for plant tissues. Wang et al. [8] proposed a simple and universal protocol for extracting proteins from recalcitrant plant tissues such as, leaves from bamboo, grapes, lemon, pine etc. and they concluded that the protein extraction efficiency depends on the sample disruption. Therefore, in the first step of the proposed protocol, the plant tissues are pulverized to a fine powder in a mortar under liquid nitrogen to minimize photolytic and other modes of degradation. The sample was first washed with TCA/acetone, followed by a second washing step with methanol and acetone to remove residual TCA and contaminants. Finally, an extraction with phenol/SDS mixture to extract proteins from the dry pellets was performed. Recently, Rodrigues et al. [9] proposed a simplified protocol (based on the Wang et al. [8] protocol) for protein studies of soybean roots. In this new procedure the three preliminary washing steps and the vacuum-drying step were eliminated, reducing reagents and time consumption. Maldonado et al. [10] compared three precipitation protocols of protein extraction for 2-DE proteomic analysis using *Arabidopsis* leaf tissue: TCA–acetone, phenol, and TCA–acetone–phenol. The protocols were compared taking into account the number of spots, image quality and reproducibility obtained in the analysis by two dimensional gel electrophoresis (2DE). The authors concluded that TCA–acetone–phenol protocol provided the best results in terms of spot focussing, resolved spots, spot intensity and reproducibility. Wang et al. [11] compared four protein extraction methods for proteomic study of dinoflagellate *Alexandium* sp. using two-dimensional electrophoresis: (1) Urea/triton X-100 buffer extraction with TCA/acetone precipitation, (2) TCA/acetone precipitation, (3) 40 mM Tris(hydroxymethyl) aminomethane buffer extraction, (4) 50 mM Tris–HCl/5% glycerol buffer extraction. The 2DE protein profiles showed that the urea/triton X-100 buffer extraction with TCA/acetone precipitation method was the best. This method removed all the interfering substances and salts efficiently and also offered a high quality in terms of resolution, number spots and spot intensity.

Recently, Jimenez et al. [12] evaluated different extraction, purification and concentration conditions for protein extraction from plankton, taking into account the protein concentration in the extract, the number of bands obtained by poliacrilamide gel electrophoresis separation (PAGE), as well as metal–protein binding before electrophoretic separation. The extraction procedure with 25 m mol L^{-1} Tris–HCl buffer and a protease inhibitor was the most efficient means of protein extraction. The presence of reducing agents (tris(2-carboxyethyl)phosphine hydrochloride, TCEP) or absorbents (phenylmethylsulfonyl fluoride, PMSF), for phenolic compounds, in the extracting solution did not improve protein and metal extraction efficiency.

Isoelectric foccussing (IEF) of proteins combined to a mass separation technique is the most commonly used method in the proteome analysis [11,13,14]. Although in-gel IEF is used routinely as the first dimension in 2D gel electrophoresis protein separation, this technique presents same drawbacks, such as low sensitivity and tedious sample preparation procedures to recover the protein from the gel for a further analysis (for example, with mass spectrometry). The latter stage increases therefore the risk of protein losses or sample contamination.

An alternative approach for the first dimension separation of proteins is the use of the OFFGEL isoelectric focusing. In this technique, proteins or peptides are separated according to their isoelectric point (pI), using immobilized pH-gradient gels (IPG) but working with a liquid phase. Fractionated proteins or peptides are easily recovered from the liquid phase to be separated in the second dimension, for example, by mass spectrometry or MS/MS. In recent years, different applications for protein identification using this fractionation technique combined with LC/MS or LC/MS/MS have been reported [15–17]. This methodology has been applied for protein fractionation and identification in different types of samples, such as extraocular muscle, plasma samples, cortical brain tissues, etc. This fractionation technique presents the same high resolution as IPG gels and offers several advantages such as: (i) low sample volumes; (ii) the procedure is less tedious than IPG gels fractionation because the sample is recovered in a liquid phase; (iii) there are commercial available IPG strips of various pI ranges; (iv) focused component are concentrated; and (v) the system provides information about the pI of proteins and peptides acting as a filter in further protein identification procedures [15,18–20].

Regarding quantitative and qualitative sizing of proteins, poliacrilamide gel electrophoresis (PAGE) [11,21,22,23] and high-performance liquid chromatography (HPLC) or HPLC-MS [17,24] are normally used. Capillary gel electrophoresis by using Lab-on-a-chip technology can be an alternative approach for assessing information concerning the second dimension. This technology provide some advantages such as: it uses small volumes of reagents and samples; it provides real-time data on composition, molecular weight and concentration for 10 samples in less than 45 min; it consists of an automatic analysis; the resolution, the precision and the sensitivity achieved are superior than those offered by the gel electrophoresis [25]. This methodology has been used by different authors for qualitative and quantitative protein determination in different matrices, such as soybean cultivars [25], tear fluid [26] and human granulocyte-colony [27].

The aim of this work is the study of different protein extraction procedures for protein extraction from plankton samples. The proteins extracted were analysed by the application of the new OFFGEL electrophoresis-Lab-on-a-chip technology.

2. Materials and methods

2.1. Reagents

The pH 3–10 IPG Dry Strips, IPG Buffer 3–10 and Plus One Dry Strip cover fluid used for protein fractionation were supplier by GE Healthcare Life Science (Uppsala, Sweden). Urea (electrophoresis grade), thiourea (analytical grade) ammonium acetate and phenol solution, equilibrated with 10 mM Tris–HCl (pH = 8.0, 1 mM EDTA) were provided by Sigma-Aldrich (St. Louis, MO, USA). 1, 4 Dithiothreitol (DTT, electrophoresis grade), methanol, Tris–HCl and glycerol solution were purchased from Merck (Darmstadt, Germany). Trichloroacetic acid and acetone were from Panreac (Barcelona, Spain) and 2-mercaptoethanol was from Fluka (Vancouver, Canada). Sodium dodecyl sulphate and sucrose were purchased from AppliChem (Darmstad, Germany). β-mercaptoethanol was from Fluka (Vancouver, Canada) and Bovine Serum Albumin from Sigma-Aldrich (St. Louis, MO, USA).

The Agilent protein 80 kit containing the protein chips, Protein 80 Gel-Matrix, Protein 80 Dye Concentrate, Protein 80 Sample Buffer and Protein 80 Ladder was supplied by Agilent Technologies (Waldbronn, Germany).

BCR-414 (plankton) certified reference material from the European Commission was used to develop this study.

Protein inhibitor cocktail (Complete lysis-y, EDTA free) was purchased from Roche Diagnostics (Mannhein, Germany).

2.2. Instruments

The 3100 OFFGEL fractionators and the Bioanalyzer 2100 used for the protein analysis were purchased from Agilent Technologies.

A Heidoph shaker, type Reax 2000 (Gemini B.V. Apeldoorn, Netherlands) was used to vortex the samples. A SIGMA 2K15 centrifuge (SIGMA Laborzentrifugen GmbH, Osterode amm Harz, Germany) was used in the protein extraction procedure.

A Branson digital Sonifer was from Branson Ultrasonics S.A. (Geneve, Switzerland) and the ultrasonic bath was from VWR International Eurolab, SL (Barcelona, Spain).

2.3. Protein extraction procedures

Different protein extraction procedures for protein extraction from plankton samples were evaluated in this work.

2.3.1. Procedure 1

In the first extraction procedure the proteins present in the plankton samples were extracted using a Tris–HCl buffer containing a protease inhibitor cocktail (Roche) (Solution A). Solution A was prepared by mixing 450 μL of stock solution of protease inhibitor cocktail with 2550 μL of Tris–HCl 25 mM (pH=7.5). A volume of 1200 μL of solution A was added to 0.1000 g of dry plankton sample and agitated vigorously for 24 h. The sample was then centrifuged at 2500 rpm for 30 min at 4 °C and the supernatant was then transferred to a new tube. The proteins were precipitated adding 2400 μL of ice cold acetone and stored at −20 °C for 120 min. The sample was then centrifuged at 2500 rpm for 5 min (4 °C) and the supernatant was discharged. The pellet obtained was then washed several times with ice-cold acetone. The pellet of proteins was dried and then dissolved in the buffer.

2.3.2. Procedure 2 (urea/triton X-100 buffer extraction)

The extracting solution used in this protein extraction procedure was prepared containing 7 M urea, 2 M thiourea, 2% SDS (w/v), 2% triton X-100 (v/v), 1% DTT (w/v) and 2% buffer pH 3–10. Therefore, 0.1000 g of dry plankton sample was mixed with 1200 μL of the extracting solution and then subjected to ultrasound energy. The uses of an ultrasound bath (for 1, 2 and 4 h, at room temperature) or an ultrasonic probe (for 30 and 60 s, applied at intervals of 10 s) were evaluated in this work. After the ultrasonic treatment, the sample was centrifuged at 4000 rpm for 30 min (4 °C) and the supernatant was transferred to a new tube. The proteins were precipitated adding 2400 μL of ice cold acetone and stored at −20 °C for 120 min. The sample was then centrifuged at 2500 rpm for 5 min (4 °C) and the supernatant was discharged. The pellet obtained was then washed several times with ice-cold acetone. The pellet of proteins was dried and then dissolved in the buffer solution.

2.3.3. Procedure 3 (TCA/acetone wash and phenol precipitation)

Proteins were extracted from the plankton sample using the extraction protocol proposed by Wang et al. [8]. Therefore, 0.3000 g of dry plankton sample was introduced into a 2 mL tube and filled with 10% TCA/acetone. The tube was vortexed and centrifuged at 10,000 rpm for 3 min at 4 °C; and the supernatant was then removed. Afterwards, the tube was filled with 80% methanol plus 0.1 M ammonium acetate, vortexed and centrifuged again at 10,000 rpm for 3 min at 4 °C, to remove the supernatant. The solid residue was then washed with acetone and dried for at least 10 min at 50 °C. After this treatment, the proteins were extracted and precipitated by adding 0.8 mL of 1:1 phenol (pH 8.0)/SDS buffer, by mixing thoroughly and incubating 5 min before centrifugation at 10000 rpm for 3 min. The upper phenol phase was then transferred into a new 2 mL tube, and the tube was filled with methanol containing 0.1 M ammonium acetate and stored at −20 °C from 2 h to 10 h. The sample was then centrifuged at 2500 rpm for 5 min (4 °C) and the supernatant was discharged. The protein pellet obtained was then washed once with 100% methanol and once with 80% acetone. The pellet of proteins was dried and then dissolved in the buffer solution.

2.4. Procedure of the OFFGEL electrophoresis

The proteins extracted from the sample were separated according to their isoelectric point (pI) using a 3100 OFFGEL fractionator. The separation was performed using 24 cm, pH 3–10 IPG Dry Strips following the manufacturer's instructions. The strips were rehydrated for 15 min in the 24-well assembled device with 40 μl of OFFGEL Rehydration solution. This solution was prepared by mixing 960 μL of Protein OFFGEL Stock solution with 240 μL of ultrapure water. The Protein OFFGEL Stock solution contains 25.2 g of urea, 600 mg of DTT, 6 mL of glycerol solution and 600 μL of OFFGEL buffer (pH 3–10) in a final volume of 50 mL with ultrapure water.

The pellet of proteins obtained following the procedure described in Section 2.3 was redissolved in 3000 μL of Protein OFFGEL Stock solution mixed with 750 μL of ultrapure water, and 150 μL of this solution was loaded in each well of the OFFGEL fractionator.

The proteins were then fractionated based on their pI using the conditions detailed in Table 1. The recovered fractions were stored at −20 °C until further analysis.

2.5. Procedure for the protein separation and quantification using the Agilent 2100 Bioanalyzer system

The proteins present in each fraction obtained in the fractionation were separated according to their molecular weight using Lab-on-a chip technology.

The Lab-on-a-chip separation was performed on the Agilent 2100 Bioanalyzer system in combination with the Protein 80 LabChip kit. This kit allows sizing and quantification of 10 protein samples in less than 30 min with a size resolution of approximately 10% or better. All chips were prepared according to the protocol provided with the Protein 80 LabChip kit.

The denaturing solution was prepared adding 1 μL of β-mercaptoethanol (BME) to an aliquot of 28.6 μL of sample buffer (provided in the kit). A portion of 4 μL of protein sample (isolated by electrophoresis) was then combined with 2 μL of denaturing solution in a 0.5 mL eppendorf tube. The molecular weight standard solution (6 μL) (ladder provided in the kit) was also pipetted in a 0.5 mL eppendorf tube. The samples tubes and the ladder tube were then heated for 5 min at 95–100 °C in a hot water bath and cooled down afterward. Ultrapure water (84 μL) was then added to samples and ladder and vortexed. The Gel–Dye Mix (G/D) and the destaining solution (DS) were prepared following the provider's instructions. A volume of 6 μL of each sample and ladder were then loaded onto the chip, previously filled with G/D and DS

Table 1
Instrumental conditions for OFFGEL fractionation.

	Volt. hour [KVh]	Voltage [V]	Current [μA]	Power [mW]	Time [h]
Focusing	64.0	4500	50	200	100
Hold		500	20	50	

solutions. The chip was placed in the Agilent 2100 Bioanalyzer and analysed immediately. Separated proteins were detected by laser-induced fluorescence. The Agilent 2100 Bioanalyzer was controlled by software that includes data collection, reporting and interpretation functions. Agilent 2100 Expert Software shows the results of the analysis as gel-like images, electropherograms as well as a Table with the molecular weight and the relative concentration of the proteins found.

2.6. Statistical analysis

Statistical analysis was performed using the Statgraphics Centurioh XVI Version 16.1.11 program (Stat Point Technology, Inc., Warrenton, Virginia, USA).

3. Results and discussion

The proteins extracted from the plankton samples were analysed in this work by OFFGEL electrophoresis combined with Lab-on-a-chip technology. With this methodology the proteins were separated in the first dimension, using OFFGEL electrophoresis, according to their isoelectric point. In the second dimension, the proteins were separated according to their molecular weight (kDa). This second dimension was performed on a chip and using the Agilent 2100 Bioanalyzer system. The results obtained with this technology are analogous to those obtained by SDS-PAGE [26,28,29].

For protein fractionation using the OFFGEL electrophoresis, the dried pellet obtained using the different extraction procedures proposed in this work was dissolved in 3000 μL of Protein OFFGEL Stock solution mixed with 750 μL of ultrapure water, prepared as described in Section 2.4. Portions of the dissolved pellet (150 μL) were then loaded in each well (24 wells) of the OFFGEL electrophoresis system. The proteins were fractionated based on their pI using the procedure detailed in Table 1 and with 24 cm, pH 3–10 IPG Dry Strips. This separation was performed in 38 hs and the recovered fractions were stored at −20 °C until further analysis. The pH corresponding to each well of the OFFGEL fractionator is shown in Table 2.

The proteins present in each fraction obtained in the OFFGEL fractionation were separated according to their molecular weight using Lab-on-a-chip technology following the procedure described in Section 2.5. The separation was performed using the Protein 80 LabChip kit. Migration time and fluorescence intensity are the parameters used for sizing and quantification of proteins. Sizing and analysis of proteins by LOC electrophoresis is performed with the Agilent protein 80 Kit Agilent Technologies (Waldbronn, Germany). This supply consists of: protein chips containing an interconnected set of gel filled microchannels that sieves proteins by size within the 5–80 kDa range (electrophoresis separation); Protein 80 Gel-Matrix and Protein 80 Dye Concentrate, which are used as a filling gel for chips; and Protein 80 Sample Buffer, which is used for preparing the denaturing solution. The kit also contains the Protein 80 Ladder (external standard), which consists of standard proteins with molecular weights of 6.5, 15, 28, 46 and 63 kDa (concentration of 600 ng μL^{-1} as a sum of all standard proteins) used for molecular weight assessment; and also, standard proteins coded as lower marker and upper marker (internal standard), at concentrations of 1 ng μL^{-1} and 60 ng μL^{-1}, respectively, used for protein quantification. The internal standard (upper marker) is a protein that is part of the sample buffer and is added to each sample in a defined ratio. Protein sizing and quantification is performed automatically by the Agilent 2100 Expert Software, based on a number of mathematical calculations and algorithms. For protein size determination, the retention times are compared to the ladder retention times. For protein quantification, the peak areas are compared to the internal standard proteins. One of the advantages of this technique is that the system can analyse and provide the results (molecular weight and concentration) for 10 samples in less than 45 min. Furthermore, the Agilent 2100 Expert Software shows the results of the analysis as gel-like images and electropherograms.

Table 2
pH ranges in each liquid fraction obtained in the OFFGEL fractionation.

Fraction	pH range	Fraction	pH range
1	3.35–3.61	13	6.50–6.76
2	3.61–3.88	14	6.76–7.03
3	3.88–4.14	15	7.03–7.29
4	4.14–4.40	16	7.29–7.55
5	4.40–4.66	17	7.55–7.81
6	4.66–4.93	18	7.81–8.08
7	4.93–5.19	19	8.08–8.34
8	5.19–5.45	20	8.34–8.60
9	5.45–5.71	21	8.60–8.86
10	5.71–5.98	22	8.86–9.13
11	5.98–6.24	23	9.13–9.39
12	6.24–650	24	9.39–9.65

3.1. Study of protein extraction procedures

As mentioned in the Section 1, different protein extraction procedures have been reported in the literature as a first step in proteomics studies. In this step, interfering compounds from the sample such as pigments, polysaccharides, high levels of salts, etc., should be eliminated. Therefore, one of the objectives proposed in this study was to compare different protein extraction procedures for protein extraction from marine plankton samples.

The study of these protein extraction procedures was performed using a BCR-414 (plankton) certified reference material for metal determination from the European Commission. The selected protein extraction procedure was then applied for the protein analysis of a plankton sample.

3.1.1. Procedure 1

In the first extraction procedure the proteins present in the plankton samples were extracted using a Tris–HCl buffer containing a protease inhibitor cocktail (Solution A) prepared as described in Section 2.3.1. This extraction solution was previously used by Jimenez et al. [12] for screening analysis of Zn and Cu-binding proteins in plankton samples.

The gel-like images obtained in the Bioanalyzer system using this protein extraction procedure are shown in Fig. 1A. Each line of the gel shows the protein sizing of one liquid fraction. The liquid fractions are named with a number from 1 to 20 and the pH values varies from 3 (fraction 1) to 10 (fraction 24) (Table 2). The gel-like images for off-gel proteins fractions from 21 to 24 are not shown in Fig. 1A, because proteins bands were not found in these fractions. As can be seen in Fig. 1A, bands of proteins are only detected in the off-gel fractions 2, 3, 4, 5, 6, 7 and 10. The isoelectric point for the proteins present in these fractions varies from 3.61 (in fraction 2) to 5.98 (in fraction 1) (see Table 1). Proteins with a molecular weight of 27.5 kDa were found in fractions 2, 3 and 4, with molecular weight of 26.8 kDa in fractions 5 and 6, and proteins with a low molecular weight (6.5 kDa) were detected in fraction 10.

3.1.2. Procedure 2

The second protein extraction procedure is a modification of the procedure proposed by Wang et al. [11]. In this case the extracting solution was prepared containing 7 M urea, 2 M thiourea,

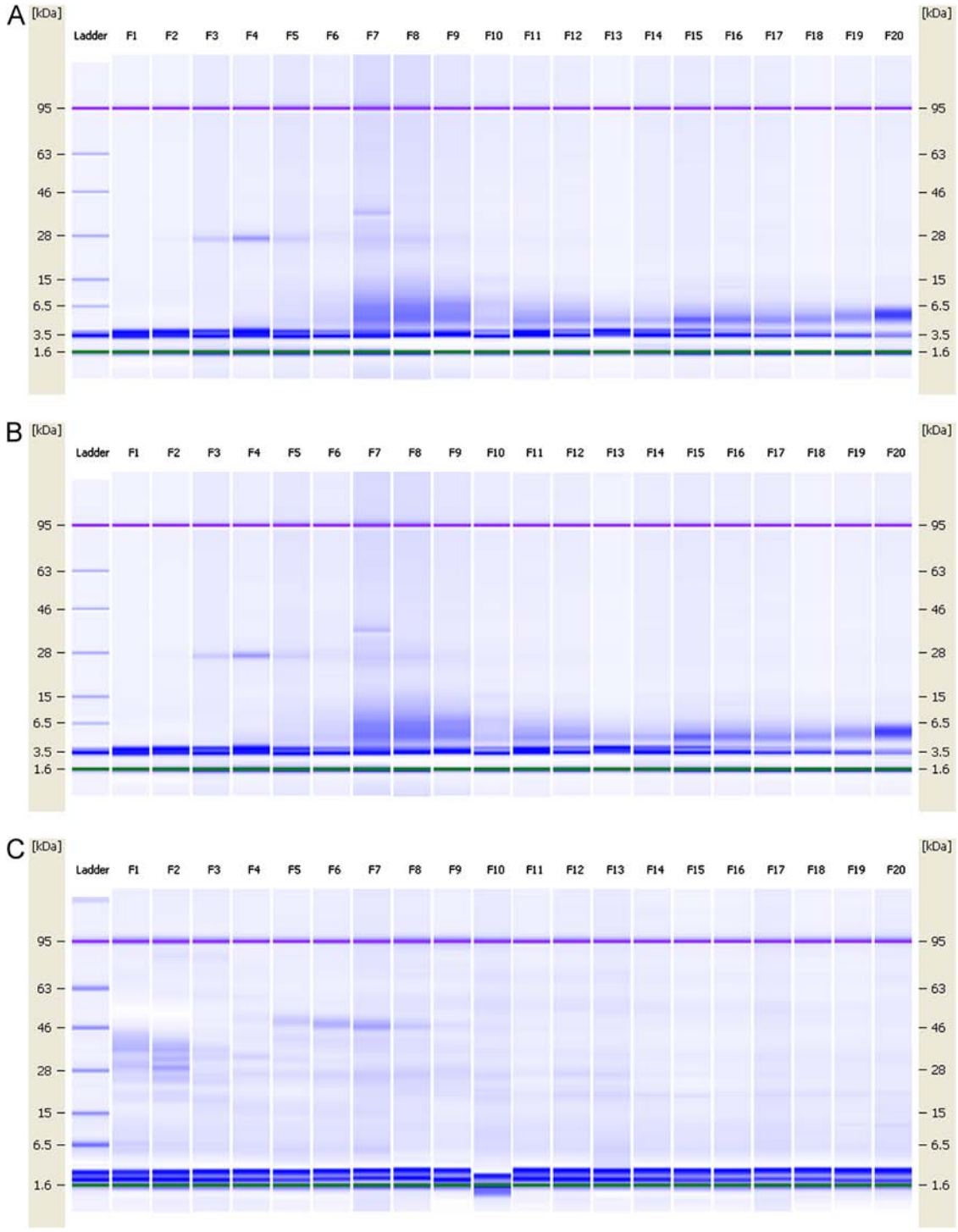

Fig. 1. Gel-likes images obtained for off-gel fractions from 1 to 20 using 0.1000 g of plankton sample (BCR-414). (A) Procedure 1, (B) procedure 2 and (C) procedure 3.

2% SDS (w/v), 2% triton X-100 (v/v), 1% DTT (w/v) and 2% buffer pH 3–10. The difference between this procedure and the proposed method is that we used SDS instead of CHAPS. This second extraction procedure is described in Section 2.3.2.

The first experiments were performed subjecting the sample for 1, 2 and 4 h to ultrasound energy by using an ultrasonic bath and working at room temperature. The results obtained in the three experiments were comparable and an increment of the intensity at longest times was not observed. With the aim of increasing the extraction efficiency, experiments were then performed using an ultrasonic probe. Sonication times of 30 and 60 ss (applied at intervals of 10 s) were studied, and during this time the sample was introduced into an ice bath to avoid sample heating. The results obtained using an extraction time of 30 s are shown in Fig. 1B. The results obtained with this extraction procedure were similar to the results obtained with the extraction procedure 1. The only difference was shown in fraction 4. The protein band with a molecular weight of 28 kDa shows higher intensity using procedure 2 than with procedure 1, and relative concentrations of 146.6 and 174.6 ng μL^{-1} were obtained for this protein band using procedure 1 and 2, respectively. Therefore, we concluded that the extraction efficiency was not improved using

this second protein extraction procedure and using ultrasound energy.

3.1.3. Procedure 3

In the third protein extraction procedure applied in this study, proteins were extracted from the plankton sample using the extraction protocol proposed by Wang et al. [8] and described in Section 2.3, by using TCA/acetone washing and phenol precipitation. These authors proposed this rapid protocol for protein extraction for recalcitrant plant tissues for proteomic analysis, and they applied the protocol for analysis the proteins present in different plant tissues, such as lemon leaves, aged olive leaves and bamboo leaves.

The protocol proposed by Wang et al. [8] requires a mass sample range from 0.1–0.3 g for the extraction procedure. In the first experiments, 0.1000 g of plankton sample was subjected to this protein extraction procedure. The proteins pellet was dried, dissolved in the buffer solution, fractionated by electrophoresis and then separated according to molecular weight of proteins by using the experimental conditions described above. The gel-like images obtained using this third protein extraction procedure is shown in Fig. 1C. Results indicate that the intensity of the protein bands was higher with this method (procedure 3) than with the procedures 1 and 2. Furthermore, with procedure 3, the number of proteins bands increased and new protein bands were observed in the other fractions such as fraction 8. Therefore, procedure 3 was selected as the best extraction procedure for protein extraction from plankton samples.

3.2. Selection of the sample plankton mass

Taking into account the results obtained with extraction procedure 3, and with the objective of increasing the intensity of the protein bands, a new experiment was performed using 0.3000 g of plankton sample. The gel-like images obtained using this third protein extraction procedure are shown in Fig. 2. The gel-like images and the electropherograms obtained for the OFF-GEL fractions 2 and 6, using 0.1 g and 0.3 g of sample are shown in Fig. 3. As can be seen in the figures, the intensity was increased with the mass of plankton used in the extraction procedure. As an example, fraction 2 shows proteins with molecular weights of 29.3 kDa and 36.8 kDa, and with relative concentrations of 69.6 ng μL^{-1} and 70.5 ng μL^{-1}, respectively, using 0.1 g of sample; whereas, relative concentrations of 167.6 and 251.0 ng μL^{-1} were obtained when using 0.3 g of sample. In fraction 6, the protein with a molecular weight of 47.4 kDa showed relative concentrations of 79.3 and 265.2 ng/μL when using 0.1 and 0.3 g of sample, respectively. Taking into account the results obtained in this study, the mass of plankton sample was fixed at 0.3000 g for further experiments.

3.3. Protein quantification in the plankton sample.

The proteins present in the liquid fractions obtained from the plankton sample (extraction procedure 3 with 0.3000 g of sample) were quantified using the internal calibration described above. As can be seen in Fig. 2, the proteins with the highest molecular weight are present in the fractions with pI lower than 6, and they offered the highest concentrations in fractions 2, 3, 5, 6, 7 and 8. Fraction 2 presents proteins with relative concentrations from 7.5 to 251.0 ng μL^{-1}. The highest concentration was found for proteins of 36.5 kDa (251.0 ng μL^{-1}), followed by proteins of 28.9 kDa (167.6 ng μL^{-1}). Fraction 3 contains proteins with a molecular size between 6.4 to 36.9 kDa and relative concentrations from 5.9 ng μL^{-1} to 110.4 ng μL^{-1} (19.5 kDa). The molecular weight found in Fraction 5 varied from 6.5 kDa to 48.9 kDa, with relative concentrations lower than 83.5 ng μL^{-1}. Fraction 6 is the fraction with the highest relative concentrations. The proteins sizes varied from 6.3 to 47.6 kDa and the highest relative concentrations are for the sizes of 26.4 kDa (128.0 ng μL^{-1}), 40.4 kDa (105.9 ng μL^{-1}), and 47.6 kDa (265 ng μL^{-1}). The proteins present in this fraction have a pI ranging from 4.66 to 4.93. Fraction 7 also showed high relative concentrations of proteins. The proteins sizes vary in this fraction from 6.0 to 46.7 kDa, with concentrations of 168.7 and 221.9 ng μL^{-1} for proteins with a size of 26.6 and 46.7 kDa, respectively. Proteins with a size between 6.4 to 46.4 kDa were found in Fraction 8, with the highest relative concentration of 133.3, 68.7 and 49.1 ng μL^{-1} for proteins with a size of 26.7, 46.4 and 36.8 kDa, respectively. The concentration of proteins present in the OFFGEL fractions decreased at highest pH.

3.4. Precision in the protein sizing analysis

The precision in the analysis using the Agilent 2100 Bioanalyzer system was evaluated. For this purpose, eight replicate analysis of the ladder (standard) were performed using the operating conditions shown in Section 2.5. This standard contained proteins with molecular weights of 6.5, 15.0, 28.0, 46.0 and 63.0 kDa. The gel-like images obtained in this experiment are shown in Fig. 4. The experimental values obtained were 6.6 ± 0.14, 15.1 ± 0.10, 28.01 ± 0.16, 46.05 ± 0.17, and 62.9 ± 0.075 kDa, which offered

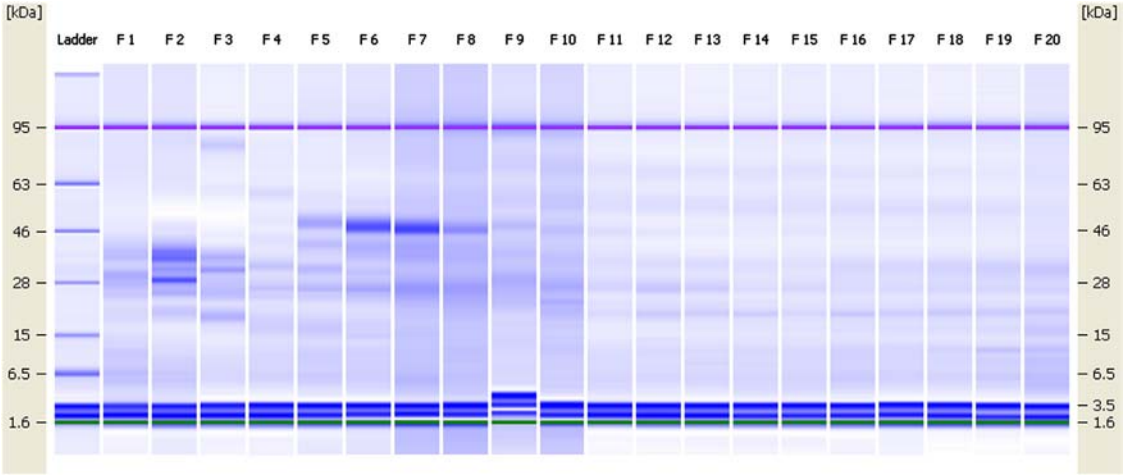

Fig. 2. Gel-likes images obtained for off-gel fractions from 1 to 23 using 0.3000 g of plankton sample (BCR-414) and using the protein extraction procedure 3.

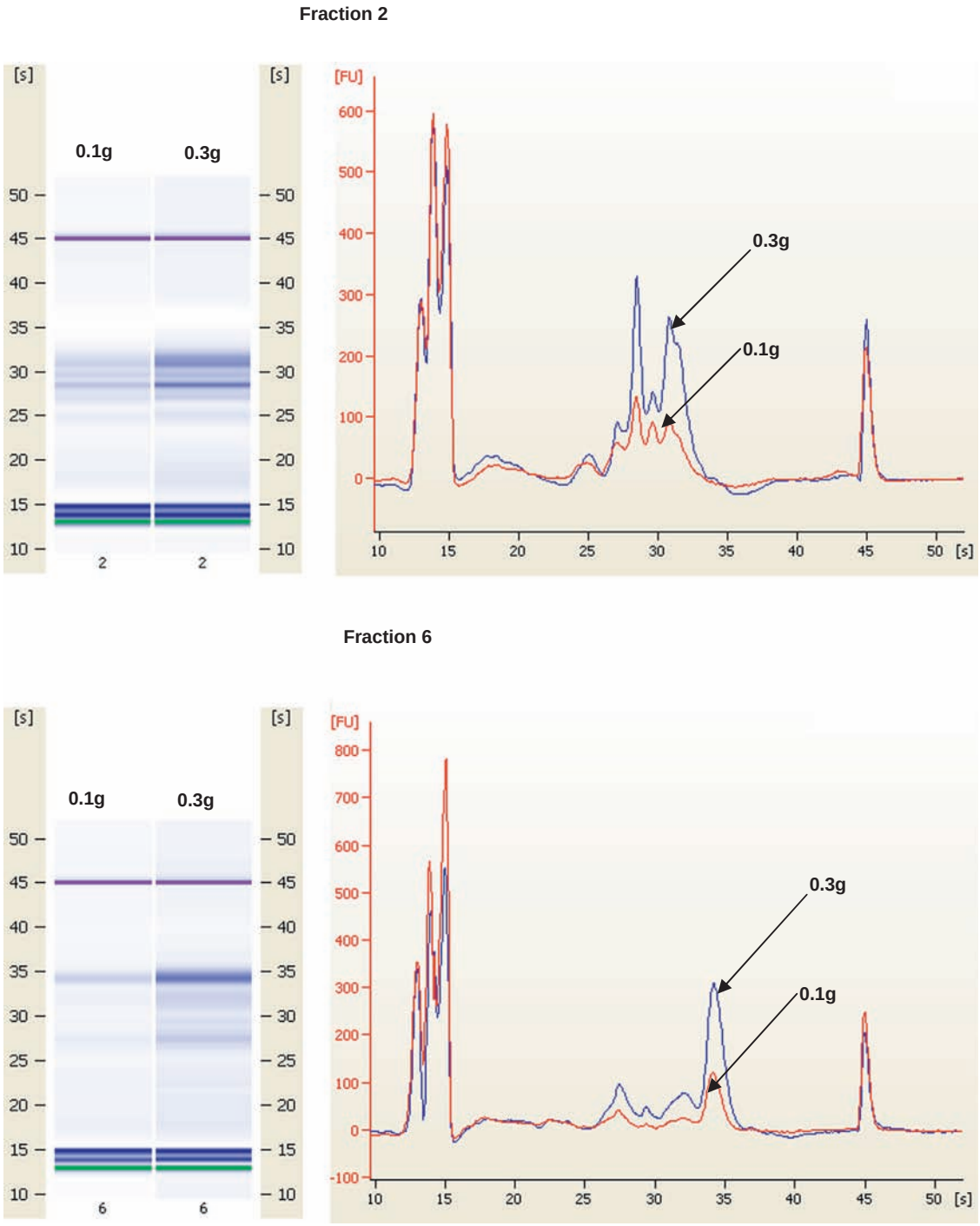

Fig. 3. Gel-like images and electropherograms obtained for fraction 2 and fraction 6, using 0.1 and 0.3 g of sample in the protein extraction procedure.

RSD values of 2.1, 0.7, 0.6, 0.4, and 0.1%, respectively. Results show that the analysis performed with this system offers good precision.

4. Application to a real fresh marine plankton sample

The method developed in this work was applied for protein sizing and quantification in a fresh marine plankton sample. This marine plankton sample was collected from a feeding tank in a clam hatchery located in the Ria de Arousa (North-western of Spain) in pre-cleaned 5 L bottles. After collection, plankton was concentrated by centrifugation at 4000 rpm for 15 min at 20 °C, and the supernatant was removed. A portion of wet plankton sample was first ground in a mortar under liquid nitrogen; and portions of 1.0800 g, 1.0050 g and 1.0010 g of the ground sample were then subjected to the protein extraction procedure 3. The proteins pellets obtained were redissolved in 3000 μL of Protein OFFGEL Stock solution mixed with 750 μL of ultrapure water, and 150 μL of these solutions were loaded into each well of the OFFGEL fractionator. The proteins were then analysed in each liquid fraction by using the Agilent 2100 Bioanalyzer system in combination with the Protein 80 LabChip kit, which was described in Section 2.5. Proteins were quantified using external calibration with Bovine Serum Albumin (BSA) in a concentration ranging from

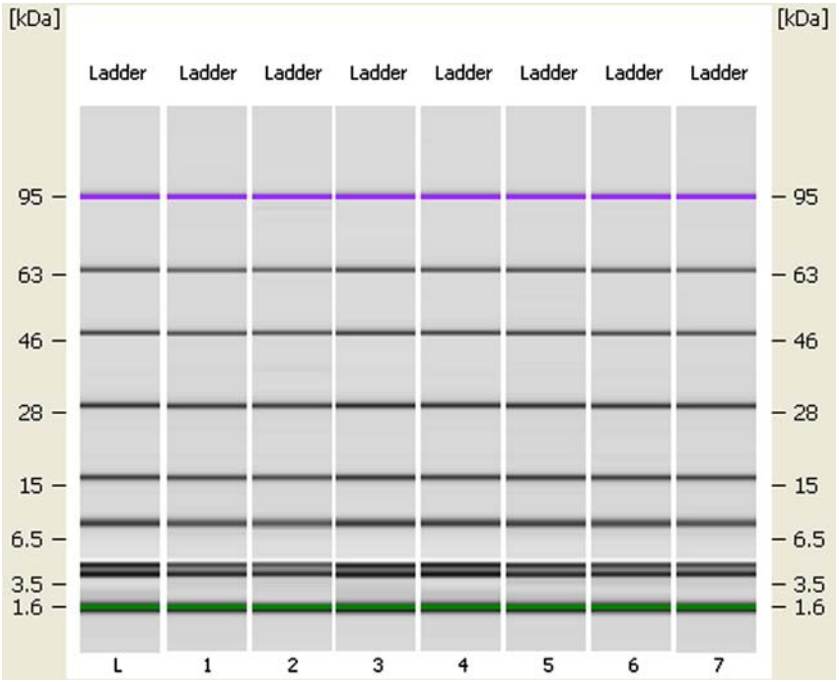

Fig. 4. Gel-like images obtained using the Agilent 2100 analyser for the separation of proteins present in the ladder.

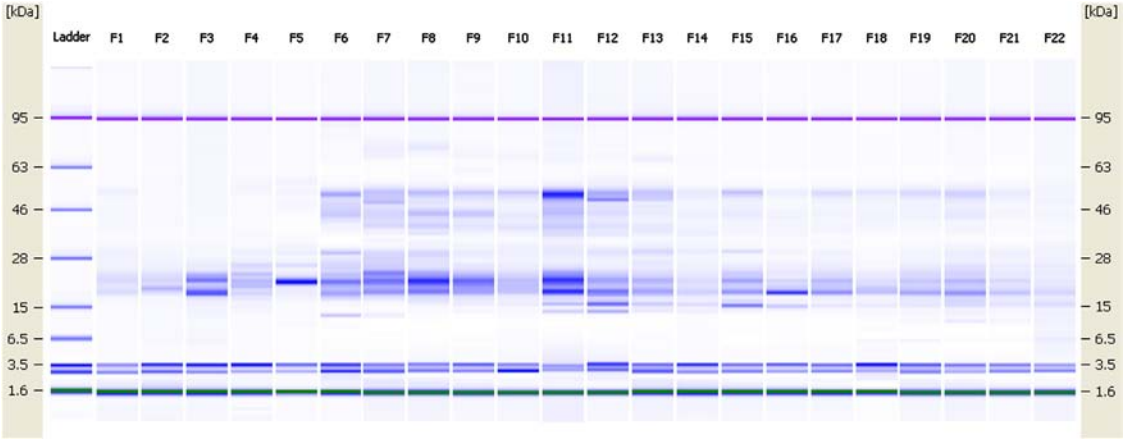

Fig. 5. Gel-likes images obtained for off-gel fractions from 1 to 24 using 1.0800 g of fresh plankton sample and using the protein extraction procedure 3.

50 to 1000 ng μL^{-1}. The gel-like image obtained, for one replicate, is shown in Fig. 5, and the concentrations of proteins found in each liquid fraction are shown in Table 3. As can be seen, the proteins molecular weight (MW) varies from 6.4 to 57.3 kDa, and the proteins with the highest MW are present in fractions 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 18, 19 and 20. These fractions contain proteins with pIs from 4.40 to 8.60. The highest protein concentrations were found in fractions 5, 6, 7, 8, 11, 12 and 15, and the proteins with the highest concentration were in fraction 5 (22.0 kDa, 925.9 ng μL^{-1}), fraction 8 (22.2 kDa, 459.9 ng μL^{-1}), fraction 11 (19.4 kDa, 394.0 ng μL^{-1}; 22.5 kDa, 380.0 ng μL^{-1}; 52.7 kDa, 409.9 ng μL^{-1}) and fraction 16 (19.1 kDa, 492.8 ng μL^{-1}). As an example, Fig. 6 shows the electropherograms obtained for fractions 5, 6, 11 and 12.

The precision of the method was studied taking into account the results obtained for three replicates. As an example, the gel-like images obtained for fractions 6 corresponding to each extraction replicate (6.1, 6.2 and 6.3), are shown in Fig. 7. The relative standard deviations for the proteins of Fractions 6, with molecular weight of 13.1, 19.0, 22.0, 30.5 and 52.6 kDa, were 12, 2, 8, 12 and 9%, respectively. Taking into account the RSDs values obtained and the number of steps in the procedure, we consider that the method is precise.

5. Conclusion

Three protein extraction procedures for protein extraction from plankton samples were studied. OFFGEL electrophoresis combined with Lab-on-a-chip technology was applied for protein analysis in a plankton sample. Taking into account the intensity and the number of the protein bands obtained, the protein extraction procedure using phenol/sodium dodecyl sulphate after different washing steps with 10% trichloroacetic acid/acetone solution, was selected as the best extraction procedure for this type of sample. The method was applied for protein analysis from a fresh plankton sample. The proteins found in this sample had a molecular weight ranging from 6.4 to 57.3 kDa and the proteins with the highest molecular weight were in the OFFGEL fractions with an isoelectric point from 4.40 to 8.60. The protein concentrations varied from

Table 3
Molecular weight and concentration of proteins present in each liquid fraction obtained with the OFFGEL fractionation for a fresh marine plankton sample.

F1		F2		F3		F4		F5		F6		F7		F8		F9		F10	
MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}
19.4	54.1	20.3	121.6	18.9	352.8	18.9	84.4	19.3	59.9	13.1	115.2	19.3	225	19.5	284.7	19.6	202.3	19.7	149.8
22.6	56.5	22.5	66.8	22.6	291.3	21	142.3	22	925.9	19	195.1	22.3	302.1	22.2	459.9	22.3	317	21.3	149.5
						22.2	152.4	26.5	127.4	22	304.1	24.4	256.9	27.4	50	44.8	113.2	22.4	151.2
						24.2	129.9	30.6	57.3	25.9	90.5	27.9	84.3	30.4	71.8	53.3	116.4	39.9	56.9
						26.4	78.9			30.5	128.2	40.8	74.9	40.4	90.7			53.3	128.4
										44.6	108.9	45.1	82.6	44.9	133				
										52.6	196.9	49.4	142.5	49.4	48.6				
												51.5	110.2	53.3	129.1				
												53.5	117.2						

F11		F12		F13		F14		F15		F16		F17		F18		F19		F20	
MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}
14.1	72.5	14.1	166.8	16.1	117	15.9	77.8	15.6	321.8	15.4	121.7	15.6	51	19.4	167.5	15.8	53.7	16.8	89.2
16.1	114.6	16.1	336.9	19.5	202.9	19.4	101.5	19.3	243.4	19.1	492.8	19.1	268.9	22.3	79.5	17.2	51.4	19	269.8
19.4	394	19.5	306.5	22.4	172.1	22.3	78.8	22.3	220	22.2	98	22.2	159.2	53.7	58.5	18.9	224.4	22.1	174.6
22.5	380	22.6	194.9	31.2	78.3			24.2	109.9			24.2	79			22.2	141.7	23.9	97.6
40.3	82.3	45.3	62	50.8	101			31.1	78.5							24	90	52.9	100.5
45.4	113.9	50.4	270	53.4	124.8			53.7	139.9							53.3	86.8		
52.7	409.9	53.1	174																

F21		F22	
MW kDa	Conc. ng μL^{-1}	MW kDa	Conc. ng μL^{-1}
16.1	61.7	16.1	65.4
19.1	147	19	87.8
22.2	93.5		
24	51.7		

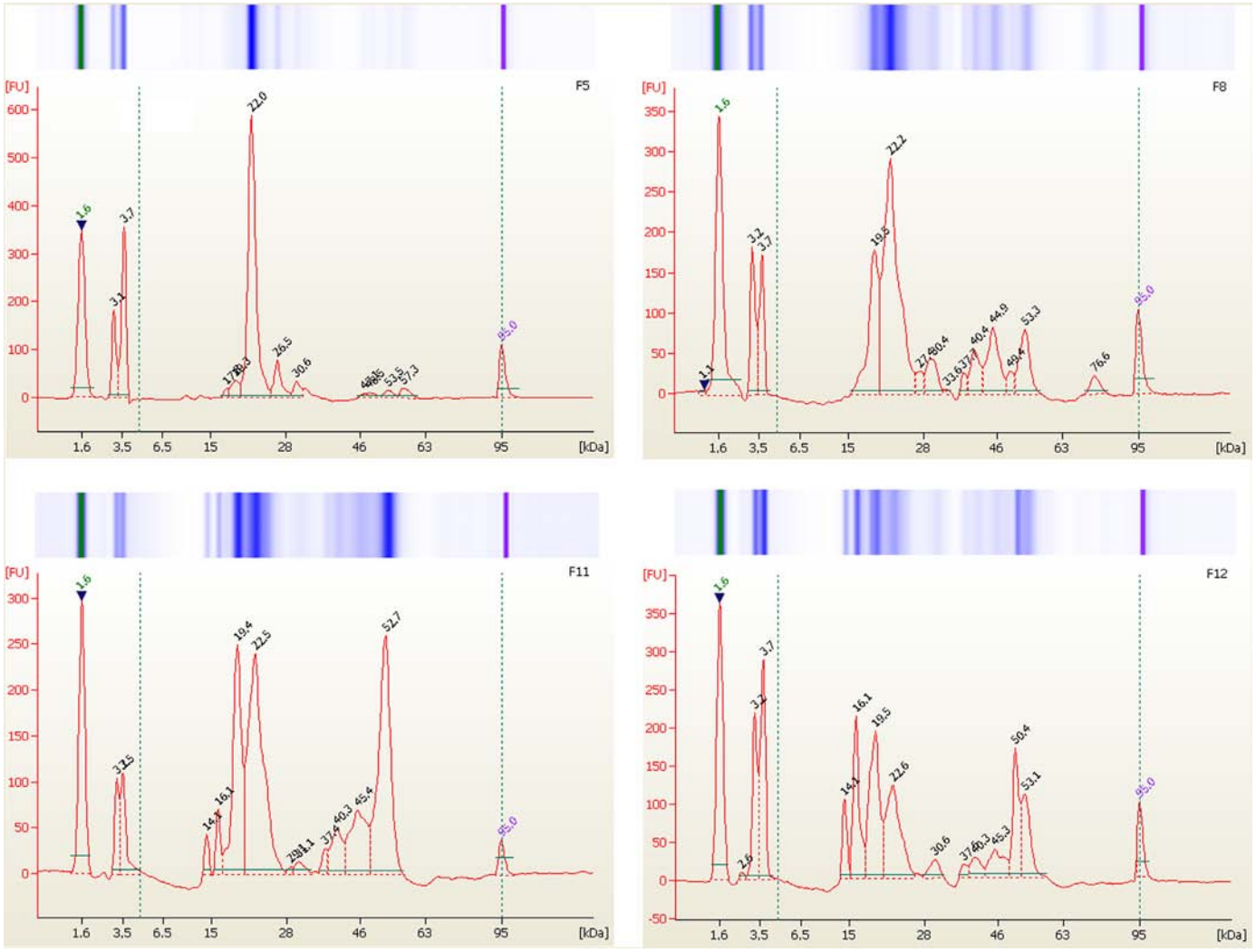

Fig. 6. Electropherograms obtained for the OFFGEL fractions 5, 8, 11 and 12 obtained from a fresh marine plankton sample and using the protein extraction procedure 3.

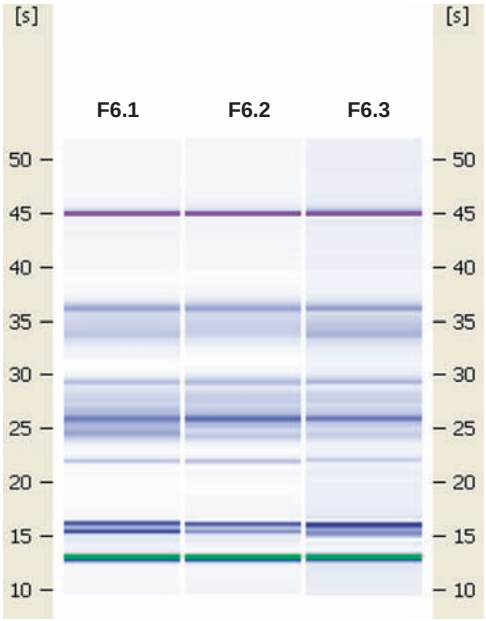

Fig. 7. Gel-like images obtained for fractions 6 corresponding to each extraction replicate (6.1, 6.2 and 6.3).

50.0 to 925.9 ng μL^{-1}. The method was precise, obtaining RSDs values lower than 12%.

The proposed method offers some advantages, such as low sample consumption, less tedious procedures, good repeatability and shorter analysis time. Further studies will be performed to identify the separated proteins by proteomics techniques.

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