Talanta 119 (2014) 90–97

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A comprehensive factorial design study of variables affecting protein extraction from formalin-fixed kidney tissue samples

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ARTICLE INFO

Article history:
Received 17 August 2013
Received in revised form
3 October 2013
Accepted 15 October 2013
Available online 5 November 2013

Keywords:
Formalin-fixed
Protein extraction
Ultrasound
Ultrasonic
Tissue proteomics

ABSTRACT

Formalin-fixed tissues are an important source of biological samples for biomedical research. However, proteomics analysis of formalin-fixed tissues has been set aside by formalin-induced protein modifications, which reduce protein extraction efficiency. In this study, a two level full factorial experimental design (2^4) was used to determine the effects of the extracting conditions in the efficiency of protein recovery from formalin-fixed kidney samples. The following variables were assessed: temperature of extraction, pH of extraction, composition of the extracting buffer and the use ultrasonic energy applied with probe. It is clearly demonstrated that when hating and ultrasonic energy are used in conjunction, a 7-fold increase ($p < 0.05$) in protein extraction is obtained if compared to extracting conditions for which neither heating nor ultrasonic energy are used. The optimization study was done following the amount of protein extracted by UV (Nanodrop® technology, protein ABS at 280 nm) and by 1D SDS-PAGE. Extracts obtained with the optimized conditions were subjected to LC-MALDI MS/MS. A total of 112 proteins were identified.

1. Introduction

Formalin-fixed tissues are an important source of biological samples for clinical research, since formalin allows tissue long-term stability and it preserves tissue architecture [1–3]. This procedure ensures good quality for histopathological analysis. However, the reaction of formaldehyde with proteins or peptides involves the formation of an unstable methylol group through the reaction of an amino or thiol group of lysine, histidine, arginine or cysteine residues. Then the methylol group reacts with lysine or tryptophan to yield Schiff bases. The Schiff bases may induce the formation of stable intra- or inter-chain methylene bridges with amino acids such as arginine, asparagine, glutamine, histidine, tryptophan and tyrosine residues [4]. Such protein cross-linking makes protein extraction and identification from formalin fixed tissues difficult. And yet, the first and most important step in the analysis of formalin-fixed tissues using proteomics is protein extraction [4]. Literature dealing with the extraction of proteins from formalin-fixed tissues is rather confusing and somewhat contradictory [5–9]. Although there is agreement about the variables that influences the extraction process, namely extraction buffer composition (pH and glycerol), temperature of extraction and application of focused ultrasounds, there is no agreement about the levels to which those variables must be set [5,6,10].

It is well recognised that ultrasonic energy promotes solid disruption aiding to increase the solid surface in contact with the extracting solution, thus helping to increase the extraction efficiency of proteins from tissues [3]. However, ultrasonic energy has been scarcely used in works dealing with extraction of proteins from formalin-fixed tissues [3,6,10–12].

Univariate optimization is the most common way to develop a set of experiments in research. However, when a high number of variables are investigated, univariate optimization is time consuming because it requires intensive sample handling, as a high number of experiments are done. In addition, it is expensive, as a large number of experiments demand a proportional amount of reagents and standards. Moreover, no interaction between variables can be estimated. Furthermore, the access to large amount of sample is constrained by the size of biopsies. The aforementioned problems make difficult the development of univariate optimizations when the number of variables to be investigated is large. Factorial design replaces univariate optimization as a way

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http://dx.doi.org/10.1016/j.talanta.2013.10.019

to overcome the aforementioned drawbacks. For a factorial design, the variables assessed are set to two levels, one maximum (named as "+") and one minimum (named as "−"). The number of experiments to be done is reduced to 2^x, where x is the number of variables under observation [13]. In addition, interaction between variables is estimated, helping to better understand the system under study.

In this work a comprehensive study of the interactions between variables affecting the extraction of proteins from formalin-fixed tissues is presented through a full factorial design. The following variables were studied: temperature of extraction, buffer composition (pH and glycerol) and ultrasonic energy provided by an ultrasonic probe.

2. Experimental

2.1. Material and methods

All reagents used were HPLC grade or higher. Albumin, from bovine serum (BSA), (N,N,N′,N′-tetramethylethylene-diamine (TMED), glycine, β-mercaptoethanol, glycerol 86–88%, bradford reagent, coomassie blue R-250 (CBB), DL-dithiotreitol (DTT), iodoacetamide (IAA), trypsin sequencing trifluoroacetic acid (TFA), and acrylamide/bis-acrylamide 30% solution (37.5:1) were purchased from Sigma-Aldrich (Basel, Switzerland). Formic acid, ammonium bicarbonate (Ambic), ammonium persulphate (APS) were purchased from Fluka (Basel, Switzerland). Hydrochloride acid (HCl), glacial acetic acid, tris–base, sodium dodecyl sulfate (SDS), methanol, acetonitrile were purchased from Panreac (Barcelona, Spain). Bromophenol blue was from Riedel-de Haën (Basel, Switzerland). Molecular weight marker for gel electrophoresis PageRuler Prestained Protein Ladder was purchased from Fermentas (St. Leon-Rot Germany). α-Cyano-4-hydroxycinnamic acid puriss for MALDI-MS from Fluka was used as MALDI matrix.

2.2. Sample pre-treatment

Bovine kidney purchased in the local market was used as a tissue model. The kidney was cut in small pieces of 0.2 cm^3 approximately. Tissue fixation was carried out by incubation of the tissue in formalin-fixative containing 50% (v/v) ethanol, 10% (v/v) formaldehyde, 7% (v/v) acetic acid and 0.9% picric acid [14,15]. Tissue fixation was carried out for 48 h at room temperature. Then, tissue samples were washed with 50% (v/v Ethanol), 70% (v/v), and absolute ethanol to remove the excess of fixation solution. The tissue was allowed to dry 30 min in a fume hood to eliminate the remaining ethanol and then the fixed samples were snap frozen with liquid nitrogen and finely grinded to obtain a homogeneous powder prior to protein extraction.

2.3. Two-level factorial design (2^4)

Factorial designs at two-levels involve relatively few experiments for each variable, and unlike univariate optimization they allow to detect and estimate interactions between variables [13,16]. The variables studied as well as their values for each (+ representing the maximum and − the minimum) are shown in Table 1.

2.4. Protein extraction

The tissue sections were prepared as described in Section 2.2. A total of 16 randomized experiments corresponding to four variables ($2^4=16$) were carried out in triplicate. Replicates were prepared in different days. To 16 ± 2 mg of tissue was added 100 µL of the appropriate extraction buffer, as described in Table 1. In this work were tested four different extraction buffers as follows:

(i) 20 mM Tris–HCl, pH 7.4, 1% (w/v) glycerol, 2% SDS (w/v) and 6% (v/v) β-mercapthoethanol, was used in experiments 1, 5, 9 and 13;
(ii) 20 mM Tris–HCl, pH 9, 1% (w/v) glycerol, 2% SDS (w/v) and 6% (v/v) β-mercapthoethanol, was used in experiments 2, 6, 10 and 14;
(iii) 20 mM Tris–HCl, pH 7.4, 10% (w/v) glycerol, 2% SDS (w/v) and 6% (v/v) β-mercapthoethanol, was used in experiments 3, 7, 11 and 15 and
(iv) 20 mM Tris–HCl, pH 9, 10% (w/v) glycerol, 2% SDS (w/v) and 6% (v/v) β-mercapthoethanol was used in experiments 4, 8, 12 and 16.

Then, samples number 1–4 and 9–12 were left at room temperature for 140 min and samples number 5–8 and 13–16 were heated in a dry-bath first to 100 °C for 20 min and then during 2 h to 60 °C. Afterwards, samples were allowed to cool down and then, experiments 9–16 were sonicated 4×10 s using an ultrasonic sonotrode UP100H (100 W, 30 kHz) from Dr. Hielscher (Teltow, Germany; operating at 100% ultrasonic amplitude; Volume sonicated: 0.1 mL; sonotrode diameter: 0.5 mm). The remaining samples, 1–8 were not sonicated.

The supernatants were harvested by centrifugation at 14000*g* for 20 min at room temperature and then collected for a new Lo-bind tube. The remaining pellet was washed with 50 µL of extraction buffer and centrifuged again at 14000*g* for 20 min. The resulting supernatant was combined with the previous one. Protein extraction efficiency was assayed with Nanodrop® technology (Thermo scientific, USA, protein absorbance at 280 nm) and by SDS-PAGE.

Table 1
2^4 factorial design experimental matrix.

Exp. number	Exp. order	Variables			
		A	B	C	D
1	8	−	−	−	−
2	2	+	−	−	−
3	10	−	+	−	−
4	4	+	+	−	−
5	15	−	−	+	−
6	9	+	−	+	−
7	1	−	+	+	−
8	13	+	+	+	−
9	16	−	−	−	+
10	5	+	−	−	+
11	11	−	+	−	+
12	14	+	+	−	+
13	3	−	−	+	+
14	12	+	−	+	+
15	6	−	+	+	+
16	7	+	+	+	+

A: pH "−"=7.4; "+"=9 [6]; **B**: % Glycerol w/v "−"=1%; "+"=10% [6]; **C**: heating "+"=20 min 100 °C, then 2 h at 60 °C [18,19]; "−"=21 °C; **D**: ultrasonic energy ("+"=4×10 s, 100% ultrasonic amplitude, 0,5 mm sonotrode, 100 µL volume sonicated; "−"=no ultrasound).

2.5. One dimensional SDS-PAGE

SDS-PAGE was performed by the method of Laemmli using a separating gel containing 12% (w/v) acrylamide/bis-acrylamide (37.5:1) and 1 mm thickness. To 7.5 µL of the protein extract were added 7.5 µL of sample buffer (5 mL of Tris–Base 0.5 M, 8 mL of

SDS 10% (w/v), 1 mL of β-mercaptoethanol, 2 mL of glycerol, and 4 mg of bromophenol blue in a final volume of 20 mL in water). The samples were loaded on the SDS-PAGE gel and proteins were separated at 200 V and 400 mA until the blue line of bromophenol blue reached the 0.5 cm from the bottom of the gel.

2.6. Gel staining and image analysis

Gels were fixed for 30 min with 40% (v/v) ethanol and 10% (v/v) acetic acid and then stained overnight with coomassie brilliant blue. Gels were destained with 40/10 (% v/v) methanol/acetic acid until a clear background was observed. Gel imaging was carried out with a ProPicII-robot using 14 ms of exposure time and a resolution of 70 μm.

2.7. In-gel protein digestion

After excision, gel bands were transferred to 0.5-mL Lo-bind tubes. Gel pieces were washed with water and then with 50% acetonitrile/25 mM Ambic until the blue color disappear. For protein reduction, gel bands were incubated for 60 min with 10 mM dithiothreitol in 25 mM Ambic at 37 °C followed by alkylation at room temperature in the dark with 55 mM iodoacetamide in 25 mM Ambic. Prior to trypsin digestion, gel bands were washed with 25 mM Ambic and dehydrated with acetonitrile. Then, 15 μL of trypsin (0.02 μg/μL in Ambic 12.5 mM/9% acetonitrile) was added to the gel pieces and incubated for 60 min on ice. After this time, gel bands were inspected and all the trypsin solution not absorbed into the gel was removed and the gels were covered with 25–50 μL of 12.5 mM Ambic depending on the band size. The samples were incubated 12 h at 37 °C. Then 25 μL formic acid 5% (v/v) was added to quench enzymatic activity. The supernatant was transferred to new lo-bind tube and the peptides were further extracted from the gel with 50% acetonitrile/5% formic acid. Samples were dried-down and stored at −20 °C until MS analysis. Before analysis, samples were resuspended in 10 μL of formic acid 0.3% and 0.5 μL of sample was hand-spotted onto a MALDI target plate (384-spot ground steel plate) then 1 μL of a 7 mg/mL solution of a-cyano-4-hydroxycinnamic acid matrix in 0.1% (v/v) TFA and 50% (v/v) ACN was added and allowed to air dry.

2.8. In-solution protein digestion

The solution containing the extracted proteins (approximately 150 μL) was ultra-filtrated with a 3 kD cut-off membrane to remove the extraction buffer. The ultra-filtrate, approximately 25 μL, was recovered to a new Lo-bind tube and the ultra-filtration membrane was washed twice with 25 μL of 12.5 mM Ambic/2% (v/v) acetonitrile. The ultrafiltration step was done in duplicate. Protein concentration was determined using the Nanodrop® function of protein Abs 280 nm (21.1 ± 0.4 and 20.5 ± 0.3 μg/μL). For in-solution digestion, samples were diluted with 12.5 mM Ambic/2% (v/v) acetonitrile to a final protein concentration of 1 μg/μL. To 20 μL of this solution, it was added 2 μL of DTT 110 mM in Ambic 12.5 mM and the samples were incubated for 45 min at 37 °C. Then protein alkylation was carried out by the addition of 2 μL of IAA 400 mM in Ambic 12.5 mM and incubated at room temperature protected from light for 35 min. After reduction and alkylation, samples were diluted with Ambic 12.5 mM/2% acetonitrile to a final volume of 100 μL. To the samples were then added 5 μL of a trypsin solution 0.1 μg/μL in Ambic 12.5 mM/2% (v/v) acetonitrile to obtain a protein:enzyme ratio of 1:40. Trypsin digestion was carried out at 37 °C for 12 h. Past due the solution were acidified to 2.5% (v/v) formic acid. Samples were evaporated to dryness, and previous to LC peptide separation, samples were resuspended in 100 μL of 3% (v/v) acetonitrile 0.05% (v/v) TFA.

2.9. LC-MALDI-TOF MS/MS

The LC instrument used was the Accela 600 (Thermo Scientific) consisting of a quaternary pump, an auto-sampler and a PDA detector. The LC system was equipped with a flow-splitter with a restriction of 1:40. The mobile phases used for the reverse phase separation were: Buffer A: 0.05% (v/v) TFA/3% (v/v) acetonitrile and Buffer B: 100% Acetonitrile/0.05% (v/v) TFA. The peptide digest samples were loaded onto a BioBasic-18 column 30 mm × 0.18 mm C18 reverse phase trapping column and then eluted into the BioBasic-18 150 mm × 0.18 mm 5 μ particle size C_{18} analytical separation column. The separation was performed across a gradient of 0–40% eluent B in 60 min with a flow rate of 3 μL/min. The peptides eluted off the analytical column were mixed with HCCA-matrix using a MALDI Spotter SunCollect SunChrom (Napa, CA, USA) and spotted every 10 s onto a MTP AnchorChip™ 384 TF from Bruker (Bremen, Germany). Matrix solution syringe pump was settled with a flow rate of 3 μL/min. Matrix solution was prepared as follows: a stock solution of 5 mg/mL of HCCA in Ethanol: Acetone 2:1/0.8 mM of $NH_4H_2PO_4$ was diluted with the same solvent to a final concentration of 0.45 mg/mL HCCA.

2.10. MALDI-TOF-MS/MS analysis

A MALDI mass spectrometer Bruker model Ultraflex was operated in positive ion mode using reflectron technology, and thus, spectra were acquired in the *m/z* range of 600–3500. A total of 500 spectra were acquired for each sample at a laser frequency of 50 Hz. External calibration was preformed with the $[M+H]^+$ monoisotopic peaks of bradykinin 1–7 (*m/z* 757.3992), angiotensin II (*m/z* 1046.5418), angiotensin I (*m/z* 1296.6848) substance P (*m/z* 1758.9326), ACTH clip 1–17 (*m/z* 2093.0862), ACTH18-39 (*m/z* 2465.1983) and somatostatin 28 (*m/z* 3147.4710). Peptide mass fingerprints (PMF) were searched with MASCOT search engine with the following parameters: (i) SwissProt Database2012_04 (535698sequences; 190107059 residues); (ii) molecular weight of protein: all; (iii) one missed cleavage; (iv) fixed modifications: carbamidomethylation (C); (v) variable modifications: oxidation of methionine and (vi) peptide tolerance up to 50 ppm after close-external calibration. Tandem MS spectra were searched with MASCOT search engine using the same parameters as for de PMF, and for fragments tolerance it was used 0.5 Da. The significance threshold was set to a minimum of 95% ($p \leq 0.05$).

3. Results and discussion

3.1. Preliminary experiments

Literature dealing with the extraction of proteins from formalin-fixed tissues provides a large number of levels for each variable that influences the extraction process [4,6]. Therefore, before proceeding to develop the two level full factorial design, it was decided to assess some levels for each variable, according to data retrieved from literature. Variables identified as influencing the extraction process, were temperature of extraction, composition of extracting buffer and ultrasonic energy [4,6,10–12]. As far as the temperature is concerned, heating the tissue in the extraction solution helps to remove the intra- and inter-molecular cross-linking of proteins, making this way easier protein dissolution. Guo et al. proposed heating tissues during 2 h at 70 °C whilst Shi et al. and Fowler et al. proposed heating tissues, first at 100 °C during 20 min and then at 60 °C during 2 h [17–20]. Both methods were reproduced and the final extracts were run in 1D gel, as it is shown in Fig. 1A. Apparently, the bands belonging to the tissues heated at 100 °C during 20 min followed by 2 h at 60 °C present the higher

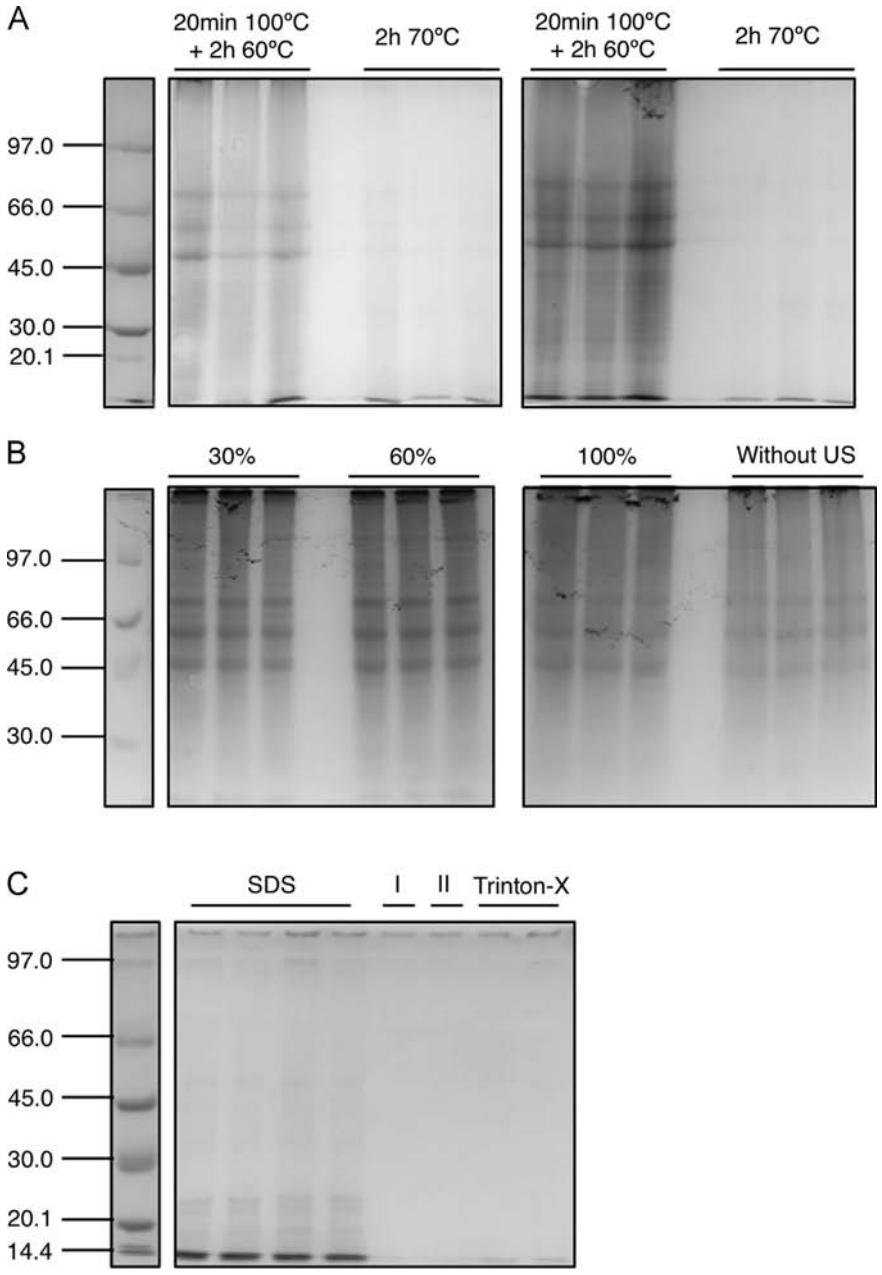

Fig. 1. A – Representative 1D SDS-PAGE of the proteins extracted using temperature (20 min at 100 °C followed by 2 h at 60 °C or 2 h at 70 °C). Samples were extracted in 20 mM Tris, pH 9, 10% (w/v) glycerol, 2% (w/v) SDS and 6% (v/v) β-mercaptoethanol. B – Representative 1D SDS-PAGE for the optimization of extraction using US. 30%, 60% and 100%). Samples were extracted 20 min at 100 °C following 2 h at 60 °C in 20 mM Tris, pH 9, 10% (w/v) glycerol, 2% (w/v) SDS and 6% (v/v) β-mercaptoethanol and then sonicated 4 × 10s using an ultrasonic probe using 30%, 60% or 100% of ultrasonic amplitude. Control experiments, without ultrasonication were also done. C- Representative 1D SDS-PAGE for the selection of extraction detergent, 2% (w/v) SDS or 1% (w/v) Triton X-100. Samples were extracted 20 min at 100 °C following 2 h at 60 °C in 20 mM Tris, pH 9, 10% (w/v) glycerol, 6% (v/v) β-mercaptoethanol containing 2% (w/v) SDS or 1% (w/v) Triton X-100.

amount of protein. Such result is consistent with the amount of protein extracted for each sample, as calculated by absorbance at 280 nm (data not shown). Therefore, the optimum heating method selected was the one proposed by Shi et al. and Fowler et al.

Ultrasonic energy applied through the use of an ultrasonic probe may be done at three levels of energy: low, medium and high. As the energy delivered is proportional to the ultrasonic amplitude, we selected the following amplitudes of sonication 30% (low energy), 60% (medium energy) and 100% (high energy). Once the sample treatment was finished, the extracts were run in 1D gel, which are shown in Fig. 1B. The intensity of the gel bands suggests that there are no differences between the ultrasonic amplitudes assayed in terms of extraction. And yet, the best results in terms of (i) amount of protein extracted and (ii) precision of replicates (%RSD) were attained with the amplitude set at 100% (data not shown). Therefore, this was the amplitude chosen as optimum for further experiments.

As far as the buffer composition is concerned, the use of detergents in the extracting solution is recommended in literature dealing with formalin-fixed tissues to aid in the solubilisation of proteins [21]. Based on such recommendations we assessed two different reagents, SDS (2% w/v) and triton X-100 (1% w/v). The detergents were prepared in solutions containing β-mercaptoethanol, as this reagent helps to shorten the extraction time as well as it helps to avoid the degradation of the extracted proteins [22]. The final extracts of this set of experiments were also run on a 1D gel. The gel lanes corresponding to this set of experiments are presented in Fig. 1C. As may be seen, the best results in terms of

extraction efficiency were obtained using the solutions containing SDS. This finding may be linked to the fact that SDS is a powerful anionic surfactant due to its high denaturing effects over proteins. In addition, SDS helps to promote cross-link reversal on the proteins present in formalin-fixed tissues [6]. Triton X-100 is an excellent detergent but it works as a weak protein-denaturing agent [17]. Therefore, it was decided to use SDS for further experiments. It is advised to use SDS at concentrations below 4% w/v, because higher ones hamper solubilisation of hydrophilic proteins. In addition, SDS interferes also in the analysis done by MALDI. If trypsination or MALDI analysis is going to be done after protein extraction, we recommend the purification of the extracted proteins using ultrafiltration or dialysis to avoid the drawbacks linked to the use of SDS [5,6]. The buffer of extraction most widely reported in literature is Tris at different pH, ranging from 4 to 9 [4,6]. However, it is claimed in literature that neutral or alkaline pH seems to give a higher yield of proteins recovery. Most likely, higher pH facilitates the breaking of methylene bridges, thus facilitating protein release [4]. Based on the aforementioned information, pH 7.4 and 9 were selected as the minimum and maximum temperature values for the two level full factorial design.

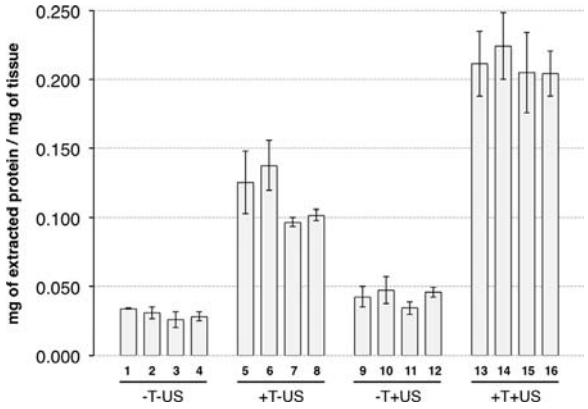

Fig. 2. Representation of the amounts of extracted protein/mg of tissue for the 16 experimental conditions tested. Error bars represent the standard deviation of 3 replicates.

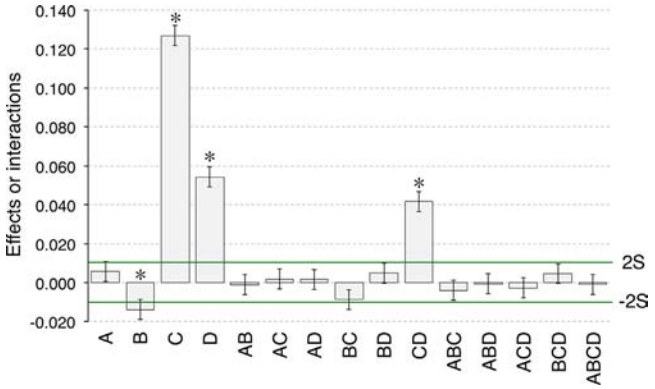

Fig. 3. Estimated effects or interaction. ∗ Significant effects or interactions. Significant effects or interactions were considered when they were greater than 2S.

Based on the above detailed preliminary experiments, the maximum (+) and minimum (−) levels set for each variable in each single experiment, as well as the order in which the experiments were done, are shown in Table 1.

3.2. Two level full factorial design (2^4).

Fig. 2 shows the amount of the protein extracted for each one of the 16 experiments that were carried out (for further details see Table 1 of supplementary material, Table 1SM). This amount was calculated normalizing the protein content as a function of the mass of the tissue treated. From Fig. 2 may be easily seen that conditions of experiments number 13, 14, 15 and 16 are the ones with which the higher amounts of protein are extracted.

Main effects for each variable (i.e. A) as well as the interactions between two (i.e. A × B), three (i.e. A × B × C) and four variables (A × B × C × D) were calculated as indicated in Ref. [6]. The table presenting the signs used to calculate the effects for the factorial design is presented in Table 2SM.

Twice the standard deviation of an effect or interaction (2S) was used to assess whether they were statistically significant as described in Refs. [13,16]. The first step to do so, is to calculate the combined variance (s^2) of all experiments, which can be obtained from the following equation:

$$s^2 = \frac{\upsilon_1 s_1^2 + \upsilon_2 s_2^2 + \ldots + \upsilon_g s_g^2}{\upsilon_1 + \upsilon_2 + \ldots + \upsilon_g} \quad (1)$$

where, $\upsilon_i = n_i - 1$ degrees of freedom and s_i is the standard deviation of each experiment. The variance of an effect or interaction (V) can be calculated as follows:

$$V(\text{effect of interaction}) = \left(\frac{1}{16} + \frac{1}{16}\right) s^2 \quad (2)$$

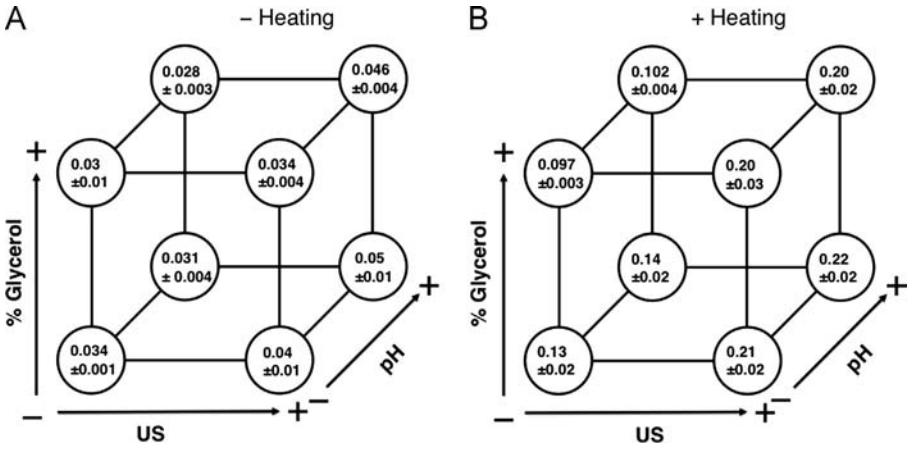

Fig. 4. Geometric diagram showing the amount of extracted protein/mg of tissue using minimum conditions of heating (no heating on the left) and using maximum conditions of heating (20 min at 100 °C followed by 2 h at 60 °C on the right).

Therefore, the standard deviation of an effect or interaction (S) is

$$S = \sqrt{V} \quad (3)$$

The main effects, interactions and variances obtained are presented in Fig. 3. The standard deviation of an effect of a single variable or the standard deviation of an interaction between two or more variables is used to ascertain which effect or interaction is statistically significant. The main effect of a variable must be individually considered only when interactions do not occur. Interactions prevail over effects. The only interaction found as significantly affecting the extraction efficiency was the one for heating and ultrasonication. This result highlights that the interaction between heating and ultrasonication is the key to enhance protein extraction efficiency. The effect of ultrasonication and heating over the extraction process can be easily understood and linked with the other two variables by looking at the geometric diagrams shown in Fig. 4A and B. Those diagrams represent ultrasonication (US, X-axis), % of glycerol (Y-axis) and pH (Z-axis). Fig. 4A, was constructed with the lower level used for heating (21 °C) and Fig. 4B with the maximum level used for heating (200 min at 100 °C plus 2 h at 60 °C). Fig. 4A shows that when pH, % of glycerol and US are set in their lowest value the amount of protein extracted is c.a. 0.030 mg for each mg of sample treated. Interestingly, if the level of glycerol is set in its maximum value whilst the other variables remains unchanged, the amount of protein extracted does not change. This finding would indicate that glycerol is not important, in terms of extraction efficiency. However, a deep analysis of Fig. 4B reveals that when heating is set in its maximum level and pH and US are set in their minimum levels, the amount of protein extracted varies from 0.13 ± 0.02 to 0.097 ± 0.003 mg of protein/mg of sample when glycerol is set from 1% to 10% (w/v). In other words, glycerol has a negative effect on extraction efficiency. This conclusion is confirmed by the estimated effect for the variable B (% of glycerol), which is -0.014 ± 0.005 (Fig. 3) thus, suggesting a negative effect over protein extraction when the amount of glycerol in the extraction buffer increases from 1% to 10% (w/v).

As far as the effect of pH concerns, it was found a negligible influence on the extraction efficiency, as the total amount of protein extracted was the same regardless of the pH of the extraction buffer, 7 or 9. This finding is in agreement with the estimated value for this effect, 0.006 ± 0.005 (Fig. 3).

Undoubtedly, the variables that most influence the extraction efficiency are ultrasonic energy and heating. If heating and US are set in their maximum levels, the amount of protein extracted reaches the maximum regardless of the levels set for the pH and the glycerol. To obtain a deep understanding of the overall process, the extracts obtained for experiments 13, 14, 15 and 16 were run by 1D SDS-PAGE. The gel is shown in Fig. 5. As may be seen, the

Table 2
Summary of the identified proteins by 1D-SDS-PAGE and MALDI-TOF/TOF MS.

#	Accession Number	Protein Name	Extraction condition			
			13	14	15	16
6	Q9TU23	Centrosomal protein of 290 kDa	✓	✓	✓	✓
	P02769	Serum albumin	✓	✓	✓	✓
	E1BM70	Coiled-coil domain-containing protein 39	✗	✓	✓	✓
	P46194	Cytochrome P450 19A1	✗	✓	✗	✗
7	P02769	Serum albumin	✓	✓	✓	✓
8	P60712	Actin, cytoplasmic 1	✓	✓	✗	✓
	P63258	Actin, cytoplasmic 2	✓	✓	✓	✓
	P68138	Actin, alpha skeletal muscle	✓	✗	✓	✓
9	Q2KJ32	Selenium-binding protein 1	✓	✓	✓	✓
	Q3MHM5	Tubulin beta-4B chain	✓	✓	✓	✗
	P00829	ATP synthase subunit beta, mitochondrial	✗	✓	✓	✓
	Q6B856	Tubulin beta-2B chain	✓	✓	✓	✓
	Q2KJD0	Tubulin beta-5 chain	✓	✓	✓	✓
	Q2KJD0	Tubulin beta-6 chain	✗	✗	✓	✗
10	Q2HJ74	Glycine amidinotransferase, mitochondrial	✓	✓	✓	✓
	Q3SZM7	Dipeptidase 1	✓	✓	✓	✓
11	P60712	Actin, cytoplasmic 1	✓	✓	✓	✓
	P63258	Actin, cytoplasmic 2	✓	✓	✓	✓
	Q3ZC07	Actin, alpha cardiac muscle 1	✗	✗	✗	✓
	P68138	Actin, alpha skeletal muscle	✓	✗	✓	✓
12	Q3T0S5	Fructose-bisphosphate aldolase B	✓	✓	✓	✓
14	Q32LG3	Malate dehydrogenase, mitochondrial	✓	✓	✓	✓
17	P02070	Hemoglobin subunit beta	✓	✓	✓	✓
	P0C0S9	Histone H2A type 1	✓	✓	✓	✓
18	O77834	Peroxiredoxin-6	✓	✓	✓	✓
	Q3T149	Heat shock protein beta-1	✓	✓	✓	✓
	P12378	UDP-glucose 6-dehydrogenase	✗	✓	✗	✗
19	Q3T149	Heat shock protein beta-1	✓	✓	✓	✓
20	O77834	Peroxiredoxin-6	✓	✗	✓	✗
	P37141	Glutathione peroxidase 3	✗	✓	✗	✓
21	P02510	Alpha-crystallin B chain	✓	✓	✓	✓
	P02070	Hemoglobin subunit beta	✗	✓	✓	✗
24	P02070	Hemoglobin subunit beta	✓	✓	✓	✓
	P62803	Histone H4	✓	✓	✓	✓
	P01966	Hemoglobin subunit alpha	✗	✓	✓	✗

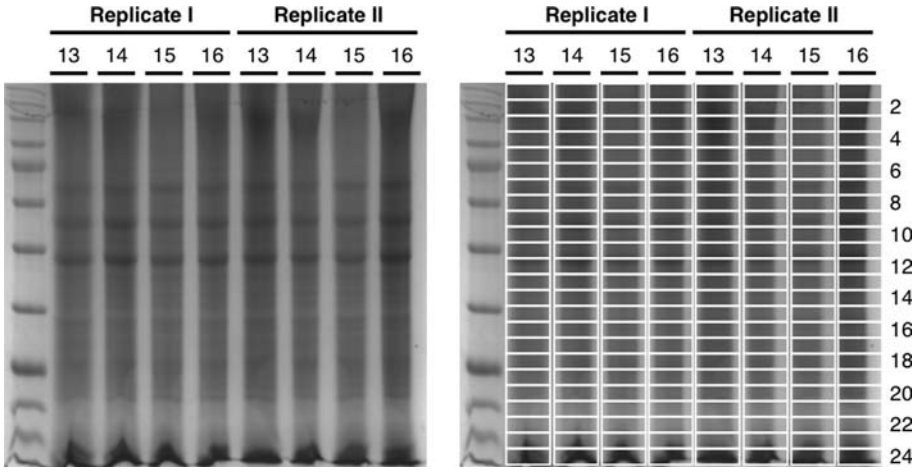

Fig. 5. Representative 1D SDS-PAGE of the best conditions 13–16 (on the left). On the right is shown the same gel indicating the bands processed for in-gel digestion and protein identification by MALDI-TOF/TOF MS.

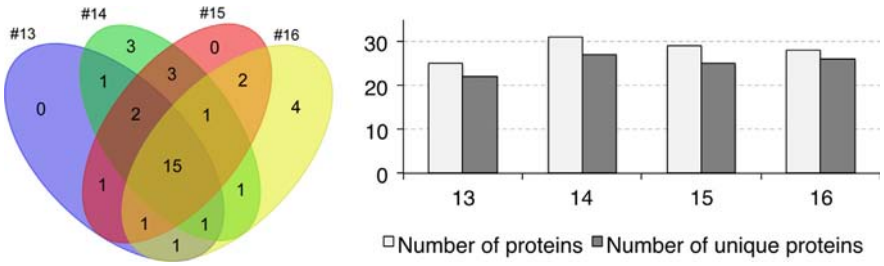

Fig. 6. Venn diagram comparing the proteins identified in each of the four conditions tested (#13–#16). On the right are represented the number of proteins identified for each condition. Samples were separated by 1D SDS-PAGE and the gel-lanes were excised as shown in Fig. 5, and submitted to in-gel digestion. Then samples were analysed by MALDI-TOF/TOF MS.

extraction of proteins is consistently achieved when temperature and ultrasonication are set in their maximum value, regardless of the levels chosen for pH and glycerol. Another potential advantage of this procedure is related to the fact that high temperatures and pressures are achieved by the cavitation promoted by the ultrasonic wave crossing through the liquid media. High temperatures and pressures can reverse the formation of some formaldehyde adducts and cross-links that fixation with formalin promotes over some amino acids [17,18,23].

3.3. *Identification of proteins in formalin-fixed kidney*

3.3.1. *Proteins separated by 1D SDS PAGE*

As shown in Fig. 2, the higher amount of protein extracted was achieved with the conditions set for the experiments 13–16. Once the extraction process was completed, the proteins were separated using 1D SDS-PAGE. After coomassie blue staining, 24 gel bands were excised, as shown in Fig. 5, and the proteins were in-gel digested using the protocol described in Section 2. The list of proteins is presented in Table 2 (for further details see Table 3SM of supplementary material). The number of identified proteins for the conditions tested were: (i) 13: 25 proteins, 22 unique proteins; (ii) 14: 31 proteins, 27 unique proteins; (iii) 15: 29 proteins, 25 unique proteins and (iv) 16: 28 proteins, 26 proteins unique (see Fig. 6). These results confirmed that the levels set for each variable for experiments 13, 14, 15 and 16 lead to comparable results in terms of total protein extracted and number of proteins identified. It was decided to choose the conditions of experiment 14 as the optima (see Figs. 2 and 6).

3.3.2. *LC-MALDI-TOF MS/MS*

The protein extracts obtained using conditions 14 were ultrafiltrated and digested as described in Section 2. The resulting pool of peptides was separated by reverse phase (RP)-HPLC on-line coupled to a MALDI spotter. The peptides separated by RP were sequenced by MS/MS and the proteins were identified using MASCOT search engine. In a first trial, RP peptide separation was done using a 30 min gradient from 0% to 40% of buffer B (100% Acetonitrile/0.05% (v/v) TFA) obtaining 45 unique proteins. However, increasing the gradient time to 60 min (0–40% of buffer B) a total of 112 unique proteins identified (for further details see Table 4SM of supplementary material).

4. Conclusions

It has been demonstrated that if heating (20 min at 100 °C followed by 2 h at 60 °C) is used in combination with ultrasonic energy (volume sonicated, 0.1 mL; 30 kHz at 100% amplitude; sonotrode diameter, 0.5 mm) neither pH nor glycerol influences the total protein extracted nor the amount of proteins identified. As a matter of fact 7-fold increase in protein extraction is achieved when heating and ultrasonication are used together. This result is independent of the pH and % of glycerol values tested in this work. Following protein extraction with the set of best conditions found using the two factorial designs, a total of 112 unique proteins were identified using LC-MALDI-TOF MS/MS.

Acknowledgments

J. E. Araújo acknowledges Grant form *Fundación Renal Íñigo Álvarez de Toledo*. E. Oliveira and H.M. Santos, acknowledge the post-doctoral Grants SFRH/BPD/72557/2010 and SRFH/BPD/73997/2010 respectively, provided by the Portuguese Foundation of Science and Technology (Fundação para a Ciência e a Tecnologia – Ministério da Educação e Ciência) – FCT. J.L. Capelo and C. Lodeiro are grateful to the *Xunta de Galicia*, Spain for financial support provided under Projects 09CSA043383PR and 10CSA383009PR. Scientific Association ProteoMass is acknowledged for the financial support. Authors thanks to REQUIMTE-FCT PEst-C/EQB/LA0006/2013.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.10.019.

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