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Analysis of multiple protein detection methods in human osteoporotic bone extracellular matrix: From literature to practice

Caterina Licinia, Giorgia Montalbano, Gabriela Cipetti, Giorgia Cerqueni, Chiara Vitale-Brovarone, Monica Mattioli-Belmonte

Department of Applied Science and Technology, Politecnico di Torino, Corso Duca degli Abruzzi 24, 10129 Torino, Italy
Department of Clinical and Molecular Sciences (DISCLIMO), Università Politecnica delle Marche, Via Tronto 10/A, 60126 Ancona, Italy
Laboratorio di Fisiopatologia Ortopedica e Medicina Rigenerativa, Istituto Ortopedico Rizzoli, IRCCS, Via di Barbiano 1/10, 40136 Bologna, Italy

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ABSTRACT

The punctual analysis of bone Extracellular Matrix (ECM) proteins represents a pivotal point for medical research in bone diseases like osteoporosis. Studies in this field, historically done to appreciate bone biology, were mainly conducted on animal samples and, up to today, only a few studies on protein detection in human bone are present. The challenges in bone ECM protein extraction and quantitation protocols are related to both the separation of proteins from the mineral content (i.e. hydroxyapatite) and the difficulty of avoiding protein denaturation during the extraction processes. The aim of the present work was to define appropriate protocol(s) for bone ECM protein extraction that could be applied to investigate both normal and pathological conditions. We compared and optimised some of the most used protocols present in the literature, modifying the protein precipitation method, the buffer used for resuspension and/or the volume of reagent used. Bradford and BCA assays and Western Blotting were used to evaluate the variations in the total protein recovery and the amount of selected proteins (Type I Collagen, TGF-β, IGF-1, Decorin, Osteopontin, Bone Sialoprotein-2 and Osteocalcin). Collectively, we were capable to draw-up two single-extract protocols with optimal recovery and ideal protein content, that can be used for a detailed analysis of ECM proteins in pathological bone samples. Time-consuming multi-extract procedures, optimised in their precipitation methods, are however crucial for a precise detection of specific proteins, like osteocalcin. As the matter of fact, also the demineralization processes, commonly suggested and performed in several protocols, could hinder an accurate protein detection, thus inherently affecting the study of a pathological bone ECM. This study represents a starting point for the definition of appropriate strategies in the study of bone extracellular matrix proteins involved in the onset and maintenance of bone diseases, as well as a tool for the development of customized scaffolds capable to modulate a proper feedback loop in bone remodelling, altered in case of diseases like osteoporosis.

1. Introduction

Bone is composed of 2 phases: approximately 75% wt. inorganic and 25% wt. organic. The inorganic phase contains water and mineral, whereby the main component is hydroxyapatite (HA) constituted by crystalline nanoplatelets containing Ca$^{2+}$ and PO$_4^{3-}$. The organic phase comprises cells and molecules of the extracellular matrix (ECM). Type I Collagen is the most abundant protein, constituting about 90% of bone ECM, whereas non-collagenous proteins (NCPs) represent the remaining part [1]. These latter molecules are different in function and chemical feature, and can be grouped in growth factors - like Transforming Growth Factor-beta (TGF-β) and Insulin-like Growth Factor 1 (IGF-1), glycoproteins - such as Osteopontin (OPN) and Bone Sialoprotein 2 (BSP-2), proteoglycans - like Decorin (DCN), and γ-carboxyglutamic acid-containing proteins - such as Osteocalcin (OCN) [2–7].

The ECM proteins exert an important role in normal bone metabolism and their unbalance could result in bone diseases like osteoporosis. Therefore, the accurate analysis of bone ECM protein content could represent a pivotal point for medical research in this field [8,9]. In the past, the studies that were considered central to the understanding of bone biology were mainly conducted on animals [10–12]. Currently,
few studies on protein detection in human bone have been published [13,14].

Protein extraction from bone ECM and the consequent analysis are challenging processes, and some issues, such as the separation of proteins from the HA nanocrystals and the ability to prevent their denaturation during the extraction processes, have not been completely solved [15–17]. Another issue that should be addressed is the complete removal of all soft tissues and cells, in order to avoid inaccurate results [18].

The study of proteins from decellularized ECM makes difficult the normalization of Western Blotting results, as the usual normalization process based on the expression of endogenous proteins from the cytoskeleton or cytoplasm (i.e. Actin, Tubulin, or GAPDH) is not appropriate in the decellularized matrix analysis [19].

A variety of reagents with different functions has been employed for protein extraction from the bone tissue. Reagents, such as ethylenediaminetetraacetic acid (EDTA) and hydrochloric acid (HCl), have been used to demineralize bone and recover proteins strictly bonded to HA [20–22]. Solutions containing guanidine hydrochloride (GuHCl) [10,20,21], ammonium phosphate and ammonium bicarbonate [9], glycerol [13,23], or surfactants and detergents, like Triton X-100 and sodium dodecyl sulphate (SDS) [13,23] have also been applied to extract and solubilize proteins from bone ECM.

The aim of this study was to define appropriate protocol(s) for bone ECM protein extraction that correctly analyse the bone protein content and could be applied to investigate a pathological condition such as osteoporosis. We, therefore, compared some of the most used protocols present in the literature in order to obtain 8 different proteins (Col1a1, Col1a2, TGF-β, IGF-1, DCN, OPN, BSP-2, OCN) that are implicated in osteoporosis onset and its management [24]. Changes in the protein precipitation method, the buffer used for resuspension and/or the volume of reagents used were tested to evaluate the impact on the total protein recovery and the amount of the selected proteins. Bradford and BCA assays and Western Blotting were performed to estimate the efficiency of each protocol.

2. Materials and methods

2.1. Sample preparation

Bone samples were obtained from one osteoporotic humeral head [25] discarded during prosthetic replacement of the shoulder prosthesis surgery carried out at Istituto Ortopedico Rizzoli. In accordance with the Local Ethical Committee guidelines and with the 1964 Helsinki declaration, informed consent was obtained. The patient was aware that the tissue used for the study represented a discard from the surgical procedure and voluntarily participated in the study (freedom from coercion or undue influence, real or imagined). The humeral head was mechanically deprived of the majority of the soft tissue, washed in PBS 1×, dried and stored at ~80 °C until use. Sample treatments were summarized in Fig. 1. Briefly, the bone was broken using a hammer and chisel and 1 g of the sample was collected for each protocol, except for protocol 5 where 100 mg of bone were used. The same starting weight for each sample was used to reduce variations that could influence the protein quantification. To degrease and remove the remaining soft tissues and cells, each sample was immersed in a saline solution at pH 7.2 (0.05 M NaCl, 0.02 M NaH₂PO₄, 0.03 M Na₂HPO₄) with protease inhibitors, sonicated in ultrasonic bath for 1 min (min) and interposed with 1 min in ice, for 5 cycles overall (modified from [18]).

Samples were then powdered in liquid nitrogen by mortar and pestle and the same weight of powder (i.e. 850 mg) was aliquoted in tubes.

2.2. Protein extraction

The obtained bone powder was suspended in solutions for each extraction protocol and processed as described below. Each protocol was tested thrice. All incubation and centrifugation steps during protein extraction were performed at 4 °C where not otherwise specified. Samples were resuspended in the same volume of the final solution. To determine protein concentration, the Bradford and BCA based Lowry assays were performed [26].

2.2.1. Protocol 1 (P1): G-E extracts protocol

G-E extracts protocol was modified from Termine 1980 [18] (Table 1) (Fig. 2). The bone powder was suspended in a G solution, constituted of 4 M Guanidine Hydrochloride (GuHCl) in 0.05 M Tris pH 7.4 and protease inhibitors (all from Sigma-Aldrich), and incubated for 72 h. The solution was then centrifuged at 800g for 20 min and the supernatant collected (G extract), whereas pellet was resuspended in an E solution containing 0.5 M EDTA (Sigma-Aldrich) in 4 M GuHCl and 0.05 M Tris pH 7.4 with protease inhibitors, and incubated for 72 h. This E extract was then centrifuged at 12,000g for 40 min and the supernatant was collected. Proteins in the G extract were obtained by Acetone precipitation, while proteins in E extract were harvested by Acetone or Trichloroacetic acid and sonication (TCA/sonication) precipitations, as described below. After precipitation, 7 M Urea (Sigma-Aldrich) or distilled water (H₂O_d) were used as buffers for resuspending. From P1 the following final extracts were obtained: 1G, 1E1, 1E2 and 1E3 (Table 1) (Fig. 2).

2.2.2. Protocol 2 (P2): G1-E-G2 extracts protocol

Protocol 2 was applied according to Goldberg 1988 et al. [27], who have modified the protocol from Termine 1980 et al. [18], to obtain three extracts (Table 1) (Fig. 2). The bone powder was incubated with the G solution for 48 h and then centrifuged at 1000g per 15 min. The supernatant was saved as G1 extract and the pellet was washed twice with 0.05 M Tris-HCl pH 7.4, before incubation with an E solution (0.5 M EDTA in 0.05 M Tris-HCl pH 7.4 containing protease inhibitors) for 48 h. This solution was then centrifuged, and the supernatant stored as E extract. The E pellet was suspended again in the G solution for further 48 h and, after centrifugation, the supernatant was collected as G2 extract. Proteins in the G1, E, and G2 extracts were obtained with Acetone or TCA/sonication precipitations, as described below, or concentrated by Amicon Ultra-4. The buffer used after precipitation and during concentration was 7 M Urea. From P2 it was possible to obtain the extracts 2G1, 2G2, 2E and 2G3 (Table 1) (Fig. 2).

2.2.3. Protocol 3(P3): HCl-SDS extracts protocol

HCl-SDS protocol was modified from Buckley 2010 and Craig 2002 [22,28] (Table 1) (Fig. 2). The bone powder samples were treated overnight with 5 or 20 ml of 0.6 M HCl at room temperature and then centrifuged at 7200g for 20 min. The supernatant was collected and the remaining pellet washed with H₂O_d and centrifuged thrice as above mentioned. The final pellet was then resuspended in 2% SDS (Sigma-Aldrich) for 48 h at room temperature. Proteins in HCl and SDS solutions were precipitated with TCA/sonication and EtOH precipitations respectively, as described below. Proteins from TCA/sonication precipitation were resuspended in 7 M Urea, while proteins from EtOH precipitation were resuspended in 2% SDS. From P3, the extracts 5-HCl, 20-HCl, 5-SDS, and 20-SDS were obtained (Table 1) (Fig. 2).

2.2.4. Protocol 4 (P4): NaOH-EDTA protocol

Protocol 4 was modified from Singh 2011 et al. [29] (Table 1) (Fig. 2). We performed this protocol to determine whether the EDTA solution, used in most protocols for collagen recovery, could hamper protein content, thus reducing their correct analysis and quantification. The bone powder was incubated in 0.1 M NaOH overnight and centrifuged at 3200g for 20 min and discarding the supernatant containing NCps. The pellet was washed twice with H₂O_d and then resuspended in 0.5 M EDTA pH 8 containing protease inhibitors for 72 h. The sample was centrifuged as described above and the supernatant was collected.
Two consecutive incubations with the EDTA solution were performed. EDTA solution extracts were then concentrated by Amicon Ultra 4 progressively changing the initial buffer with 7 M Urea (Table 1) (Fig. 2). From P4, the extracts 1-EDTA and 2-EDTA were obtained (Table 1) (Fig. 2).

2.2.5. Protocol 5 (P5): TRIzol reagent protocol

TRIzol protocol was conducted according to the manufacturer’s instruction (Table 1) (Fig. 3). Briefly, the bone powder (100 mg) was dissolved in 1 ml TRIzol reagent and chloroform (Sigma-Aldrich). After 2–3 min incubation at room temperature, the solution was centrifuged at 12,000g for 15 min and the aqueous phase containing RNA discarded. Absolute ethanol was then incorporated, incubated at room temperature and centrifuged at 2000g for 20 min, to obtain the protein phase as supernatant. To precipitate proteins, isopropanol was added, and the solution was centrifuged at 12,000g for 10 min. The obtained pellets were washed with 0.3 M GuHCl in 95% Ethanol, then centrifuged at 7500g for 5 min after incubation and the supernatant was discarded. This step was repeated for 3 times, before incubating with 100% Ethanol for 20 min, centrifuging at 7500g for 5 min and
discarding the supernatant. In the end, the pellet was resuspended in 200 μl of 1% SDS, the solution was centrifuged at 10000 g for 10 min to remove insoluble materials and the supernatant stored at −80 °C until use. From P5, T extract was obtained (Table 1) (Fig. 3).

2.2.6. Protocol 6 (P6): NET-Triton buffer protocol

NET-Triton buffer protocol was modified from Wang 2014 [13] (Table 1) (Fig. 3). The bone powder was incorporated in the NET-Triton buffer (0.01 M Tris-HCl 7.4, 1 mM EDTA, 0.1 M NaCl, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitors) and the solution was homogenized by Ultra-Turrax T8 (IKA-WERKE, Lille, France) for 1 min in ice interposed by 1 min of rest, for 4 cycles overall. The sample was centrifuged at 14,000 g for 10 min to remove debris and the supernatant was collected at −80 °C until use. From P6, N extract was obtained (Table 1) (Fig. 3).

2.2.7. Protocol 7 (P7): high concentration phosphate buffer protocol

High Concentration Phosphate protocol was modified from Cleland et al. 2015 [9] (Table 1) (Fig. 3). Concisely, the bone powder was incorporated in the NET-Triton solution containing 20 mM DTT was added to the pellet, incubating at −20 °C for 1 h and vortexing every 20 min, and then the solution was centrifuged at 13,000 g for 15 min. The supernatant was discarded, and the pellet was air-dried before resuspending in the selected buffer and sonicating for 10 s for 6 cycles overall. For Acetone precipitation protocol, 4 volumes of ice-cold acetone containing 20 mM DTT were added to 1 volume of protein solution. The solution was vortexed and incubated at −20 °C for 1 h. The supernatant was discarded after centrifuging at 15,000 g for 15 min, and the pellet was air-dried before resuspending in the selected buffer.

Ethanol precipitation was performed as previously described by Rajalingam 2009 and all the passages were performed at 4 °C where not otherwise specified [30]. Briefly, for TCA/sonication precipitation protocol, 1 volume of 20% TCA was added to 1 volume of protein solution and incubated at −20 °C for 1 h. The solution was centrifuged at 15,000 g for 15 min and the supernatant was removed. Ice-cold acetonitrile containing 20 mM DTT was added to the pellet, incubating at −20 °C for 1 h and vortexing every 20 min, and then the solution was centrifuged at 13,000 g for 15 min. The supernatant was discarded, and the pellet was air-dried before resuspending in the selected buffer.

Western Blotting was performed on the extracts obtained from three different reps of the above-mentioned protocols. All protein samples (50 μg of protein for each sample) were prepared using NuPAGE<sup>™</sup> LDS Sample Buffer according to manufacturer's instruction, fractionated in NuPAGE<sup>™</sup> 4–12% Bis-Tris Protein Gels and electrophoretically transferred to PVDF membranes (Millipore). Membranes were incubated with 5% milk in Tris-Buffered Saline with 0.1% Tween 20 (TBS-T) to block non-specific sites and then with primary antibodies in TBS-T at 4 °C overnight. After washes with TBS-T, the membranes were incubated with secondary antibody anti-mouse conjugated with horseradish peroxidase or secondary antibody anti-rabbit conjugated with horseradish peroxidase. Detection of antibody binding was performed with Pierce ECL Western Blotting Substrate and images were acquired with Alliance Mini HD9 (Uvitec, Cambridge, UK). Densitometric analysis was performed with ImageJ software (https://imagej.nih.gov/ij/download).

2.4. Western Blotting

Western Blotting

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No membrane was stripped between antibody incubations to avoid protein loss.

### 2.5. Key resources table

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**CHEMICALS**

- **Protease inhibitors**: N/A  N/A  S8820  Sigma-Aldrich
- **TRIzol reagent**: N/A  N/A  15596018  Invitrogen
- **Dithiothreitol (DTT)**: N/A  N/A  NP0009  Invitrogen
- **Bradford reagent**: N/A  N/A  B6916  Sigma-Aldrich

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**Protocol 1**

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**Protocol 2**

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**Protocol 4**

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Fig. 2. Schematic drawing of the steps of the multi-extract protocols (P1–P4). For each step of the protocols, starting work solutions, precipitation and/or concentration methods, final buffers employed for resuspension after precipitation or concentration and final extracts obtained are indicated.
3. Results

3.1. Macroscopic observations

Macroscopically, some solutions showed insoluble residues after protein extraction. The TS and N extracts, obtained from P8 and P6 respectively, exhibited a filamentous residue during the homogenization phase. The 1E2 extract, obtained in P1 from the resuspension in H2O after acetone precipitation, contained a copious amount of white residue, whereas the 1E3 extract from P1 gained in the same protocol after the resuspension in 7 M Urea after the TCA/sonication precipitation contained only a little insoluble white deposit.

3.2. Total protein amounts

Table 2 summarized the total protein recovery from each extract, comparing Bradford and BCA assays. The highest protein recovery detected by Bradford assay was in P5 and P2: the protein concentration of the T extract (P5) was 3.450 μg/μl from 100 mg of bone, while the 2G1 extract (P2) contained 15.613 μg/μl from 1 g of bone. A good protein recovery was also detected in the HCP extract from P7 (11.774 μg/μl), the 2G2 extract from P2 (9.625 μg/μl), and the N extract from P6 (8.239 μg/μl).

Further differences were found treating the same extract with different precipitation methods (i.e. 2G1 vs 2G2 extracts) and/or different buffer (i.e. 1E1 vs. 1E2 extracts from P1) or using a different volume of extraction solution (5-HCl vs 20-HCl extracts from P3). The highest protein content was detected in the T (P5) and the 2G1 (P2) extracts also by BCA assay; overall, this assay showed a lower protein recovery than Bradford one: the protein concentration for the T extract was 1.452 μg/μl from 100 mg of bone and for the 2G1 extract was 1.950 μg/μl. A good protein recovery was observed also in the P extract from P7 (1.150 μg/μl), in the 2G2 extract from P2 (1.460 μg/μl) and in the TS extract from P8 (1.015 μg/μl). We found differences in protein recovery comparing the same extract with different precipitation methods (i.e. 2G1 vs 2G2 extracts from P2) or using a different volume of extraction solution (5-SDS vs 20-SDS from P3). No proteins were detected in 1E3 extract (P1).

3.3. Protein staining analysis

Results of the densitometric analysis are summarized in Fig. 4. Detailed information are available as Supplementary files (Figs. 1S–4S and Table 1S). Only TS (P8) and T (P5) extracts showed staining for all the examined proteins. The T extract revealed the best staining for TGF-β at 25 and 50 kDa, DCN at 40 kDa, OPN at 55 kDa and BSP-2 at 55 and 80 kDa. The TS extract showed the highest intensity for Col1a1 at 140 kDa, IGF-1 at 7.6 kDa, OPN at 25 and 66 kDa and a similar intensity to the T extract for BSP-2 at 55 kDa. Furthermore, the N extract (P6) exhibited the highest amount of Col1a1 at 90 kDa, TGF-β at 12.5 kDa and BSP-2 at 35 kDa but did not show signals for Col1a2 and IGF-1.
proteins. Col1a2, TGF-beta, IGKF-1, DCN, and OCN were identified in the 5-SDS and 20-SDS extracts (P3). The 5-SDS extract showed also the highest staining for IGKF-1 at 22kDa. The 5-HCl extract (P3) exhibited staining for Col1a2, TGF-beta, OPN, OCN and the highest content of DCN at 120kDa, whereas the 20-HCl (P3) showed only the presence of DCN and OCN. The 1E1, 1E2 and 1E3 extracts (P1) contained Col1a2 and OPN in similar amounts, but the 1E1 extract contained also IGKF-1 and showed staining for OCN 4-fold more than 1E2 and 1E3. The 2E extract permitted the collection of Col1a2, IGKF-1, and OPN. The remaining extracts showed staining only for one protein: among these, the 2G1 extract (P2) showed the highest staining for OCN and the 2-EDTA one for Col1a2.

4. Discussion

Protein extraction from bone ECM is a complicated process

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Fig. 4. Schematic drawing to resume the results of densitometric analysis for protein expression of each extract (I: intensity/mm3). −: no staining; +/−: I < 10^6; +: 10^6 < I < 2.5 × 10^6; ++: 2.5 × 10^6 < I < 5 × 10^6; +++: 5 × 10^6 < I < 8 × 10^6; ++++: 8 × 10^6 < I < 1.2 × 10^7; +++++: I > 1.3 × 10^7.
influenced by several variables, such as the use of different reagents, the concentration methods and the resuspension buffers, which could hamper the accurate quantitation of many proteins involved in bone biology and pathologies.

In this study, we compared different protocols from published literature to identify the best method for the extraction of bone ECM proteins in a pathological condition (i.e. osteoporosis). Since the imbalance between bone resorption and formation affects the bone ECM protein content [8], the modification of current protocols in terms of total protein recovery and collection of peculiar proteins involved in osteoporosis could represent a valid tool in the study and/or management of this pathology.

The normalization of Western Blotting results was one of the main issues to address because the usual analysis of endogenous proteins cannot be useful in the examination of a decellularized bone ECM.

Therefore, in order to reduce variations that could influence the protein quantification, arrangements like the use of only one humeral head to obtain different samples (each protocol was tested thrice), the same starting weight and final volume of resuspension, an equal protein amount loaded in Western Blotting gels, and no membrane stripping were taken [32,33].

To load the same protein amount we performed Bradford and BCA assays. Matching the same extract, we found a higher protein quantitation with Bradford assay. In bone ECM, Type I Collagen usually represents 90% of the total protein content, and its chains contain arginine and lysine that preferentially bound to Coomassie in the Bradford assay [26,34,35]. The remaining 10% of bone ECM proteins is constituted by 180–200 NCPs [34,35]. Osteocalcin, the most abundant among them, and TGF-β are primarily revealed by BCA assay, whereas IGF-1, DCN, OPN and BSP-2 are preferentially identified by Bradford assay. Considering the protein detection amount and the affinity for the most proteins of interest, Bradford assay was the best for our aim.

Other useful arrangements to reduce influence in protein quantification, save for the suggested above, could be the use of Ponceau or Coomassie normalization for protein loading/transfer measure and the positive control loading for the analysed proteins [36,37].

Protocols P5, P6, P7 and P8, which are single-extract protocols, provide the proteins in a single extract without using precipitation or concentration methods and do not require > 3 days. The T extract from P5 showed both the higher total protein recovery and the detection of all proteins of interest, providing the best-tested ones. Also, P6 showed good results in terms of protein content, allowing the collection of most of the selected proteins. On the contrary, although the total protein recovery from P7 was high, the HCP extract contained only Type I Collagen and OCN. Additionally, the TS extract from P8 exhibited similar staining results obtained with P5, but with a 10-fold lower total protein concentration than the latter.

In the T (P5) and TS extracts (P8), that resulted as the best-tested protocols, Type I Collagen was the protein with the highest expression, but OCN staining was very low compared to all the other detected NCPs.

With the multi-step protocols, we obtained several extracts capable to provide different proteins according to their chemical and physical features. With the use of GuHCl buffers for the first step of P1 and P2, we expected to collect proteins from unmineralized tissue and osteoid layer, like proteoglycans and part of Type I Collagen, in the corresponding extracts (1G, 2G1 and 2G2) [18,20]. Nonetheless, the extracts were positive only for OCN which is strictly related to mineralized tissue [38], showing a high content of this protein.

Demineralizing solutions were employed in the first step (as in P3) or after (as in P1, P2 and P4). The extracts including EDTA or HCl contained OCN and other proteins associated with HA, like OPN and Type I Collagen, as expected [18,20,27,36–40]. Interestingly, we also observed the expression of growth factors (i.e. TGF-β and IGF-1) and proteoglycans (i.e. DCN). In the EDTA and HCl extracts derived from demineralizing solutions, we obtained a notable protein amount and variety, as observed also by Schroeter et al. [17]. Furthermore, in P3, the SDS extract, collected after the demineralizing step with HCl solution, exhibited only a low amount of Type I Collagen. Some protocols for protein extraction from bone, especially those employed for collagen collection, use a first demineralizing step on samples before the effective extraction procedures [41,42]. Our data suggest that the initial decalcification step causes loss of proteins, affecting the final protein recovery, and therefore should be avoided.

Lately, from the third step of P2, we recovered the residue Type I Collagen in the 2G3 extract, as expected [27].

Although each multi-step protocol provides two or more solutions for the obtainment of specific extracts, no tested protocol was per se useful to collect all the 8 proteins of interest. Additionally, these multi-step methods require more days and longer period of incubations than the single-extract ones, thus they are not time-efficient. With these protocols (from P1 to P4), just a few extracts exhibited a good protein recovery with a good or high expression of few proteins of interest. Among them, OCN showed a very high expression in the P1 and P2, greater than the single-extract protocols that thus revealed a limitation in the collection of this protein.

The possibility to investigate, within these protocols, different precipitation and/or concentration methods and different buffers for resuspension, could overall ameliorate protein recovery. The TCA/sonication precipitation and the subsequent buffer changing with 7 M Urea provided the best protein recovery and staining in G extracts, while acetone precipitation and 7 M Urea resuspension was the greatest for the recovery and staining in E extracts, even if in the 1E2 and 1E3 extracts (P1) an insoluble white sediment was observed. This deposit that we supposed to be mineral residues, was not evident in the 1E1 extract (P1) obtained with TCA/sonication precipitation.

Also, in the TS (P8) and N (P6) extracts from single step-protocols a filamentous residue, consisting probably of an insoluble fraction of Type I Collagen [18], was observed. Overall, these deposits, which probably contain not solubilized proteins, could affect the proper estimation of protein content in the tested protocol.

Another issue that needs to be addressed in the search of an optimal protocol is the volume of extraction solution. For instance, in P3, the extract from 5 ml HCl was better in quantity and quality than that from 20 ml HCl. Similarly, even if the SDS extracts contained the same proteins, yield and expression were higher in the extract from a pellet that was demineralized by a smaller amount of HCl.

In some extracts (i.e. 2G2 from P2 and HCP from P7), we observed differences between a high total protein recovery, detected by Bradford and BCA assays, and a very low protein expression, observed by Western Blotting. This discrepancy may be attributable to the fact that in these extracts there were other proteins beside the ones we investigated. Indeed, the investigated proteins relative to osteoporosis are few considering the very high number of NCPs in bone ECM [34]. Otherwise, also the buffer used for extraction and/or for resuspension could cause this discordance. Indeed, solutions containing a high salt amount, as the HCP extract from P7, could be not fully suitable for the SDS-PAGE system in Western Blotting, thus making the protein detection difficult [43]. For this reason, P7 was not optimal for Western Blotting analysis, but its high protein recovery makes it a good protocol to analyse proteins by other assays such as HPLC [9]. On the other hand, the solutions containing SDS, like those used in P3, P5, P6 and P8, were compatible with SDS-PAGE system and showed a wide range of proteins detectable.

5. Conclusions

Collectively, we drew-up two single-extract protocols (P5 and P8) with optimal recovery and ideal protein content for the main proteins involved in osteoporosis and that can be used for a satisfactory analysis of ECM proteins in pathological bone samples. These protocols need, however, to be supported by the multi-extract protocols for a punctual


