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By

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Title of the STM programme

Application of membrane technology for the recovery of functional and bioactive peptides obtained by the hydrolysis of protein recovered from animal by-products (bovine bones)

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1. Introduction

Membrane processes have an important role in the separation industry. Nowadays, they are a dominant technology in fields such as food and beverage, pharmaceutics, environment and biomedicine. Membrane processes mostly operate without heating, and therefore use less energy than conventional thermal separation processes such as distillation, sublimation or crystallization. The separation process is purely physical and both fractions (permeate and retentate) can be used. Also, membranes can enable separations that would be not possible to occur by thermal separation methods (Drioli & Giorno, 2010).

Most common membrane processes are those using hydraulic pressure as a driving force and are called ultrafiltration (UF), microfiltration (MF), nanofiltration (NF) and reverse osmosis (RO), according to membrane pore size and operating conditions (Table 1) (Bourseau *et al.,* 2009).

Table 1. Pressure-driven membrane operations

Pore size	Molecular mass	Process	Pressure	Removed compounds
>10 µm		Common filter		
>0.1 µm	> 5000 kDa	Microfiltration	< 2 bar	larger bacteria, yeast, particles
100-2 nm	5-5000 kDa	Ultrafiltration	1-10 bar	bacteria, macromolecules, proteins, larger viruses
2-1 nm	0.1-5 kDa	Nanofiltration	3-20 bar	Virus, valent ions
< 1 nm	< 100 Da	Reverse Osmosis	10-80 bar	Salts, small organic compounds

Protein separation is an important component of the ultrafiltration segment. Ultrafiltration is used for protein concentration, desalting, clarification and fractionation (i.e. protein–protein separation). Concentration, desalting and clarification are technologically less demanding and are broadly used in the bioprocess industry. Currently, UF is extensively applied in the dairy industry and mainly in the processing of cheese whey to obtain whey protein concentrate (WPC) and lactose-rich permeate (Arunkumar & Etzel, 2013; Cheang & Zydney, 2004). As an example, a UF process is able to concentrate the whey 10-30 times the feed in a single stage (Tamime, 2013).

Apart dairy industry, other proteins can be are recovered from animal by-products and fractionated by using membrane technology (Centenaro *et al.*, 2014; Ferraro *et al.*, 2013). Those proteins exhibit functional and bioactive properties when hydrolysed through various enzymes and according to their molecular weight (Ferraro *et al.*, 2013). The aim of this study was to investigate the possibility to fractionate hydrolysed bovine

bone protein by ultrafiltration membrane operation, and separate functional hydrolysates (bigger fraction) from bioactive peptides (smaller fraction).

2. Material and Methods

Bovine bone hydrolysed proteins (hereafter abbreviated as BBHP) have been obtained by acid extraction from femur and tibia of a young animal (< 4 years). The proteic extracts have been then hydrolysed by pepsin (Sigma-Aldrich) at pH 2.2–2.3. Hydrolysates particle size and shape have been characterised by the Sysmex FPIA3000 equipment (Malvern) (at INRA-QuaPA, Clermont-Fd, France), which uses the dynamic flow particle imaging technology to detect particles in the size range 0.8 – 300 µm. In Figure 1 and Figure 2, are reported particle size and shape of BBHP from femur and from tibia, respectively. All measurements were done in triplicate.

To perform BBHP fractionation by ultrafiltration, the original hydrolysates have been diluted down to 1 g/L with a solution of 6 g/L acetic acid and 1 g/L hydrochloric acid (Sigma-Aldrich) to maintain the original pH, which has been thereafter increased at 3.0 by 1 M NaOH (Sigma-Aldrich) to fit membranes compatibility. A two-stage ultrafiltration process has been accomplished by using a 100 kDa cut-off first and a 30 kDa cut-off after. Membranes used were regenerated cellulose (Millipore) for 100 kDa cut-off, and SEPA *UltraFillic* (polyacrylonitrile polymer) (General Electric) for the 30 kDa cut-off. Filtration was accomplished in the cross-flow (or tangential flow) configuration through the system reported in Figure 3. Operating conditions were 0.5 bar for the 100 kDa cut-off and 0.6 bar for the 30 kDa cut-off. To assess presence and entity of membrane fouling by samples, permeability through ultrapure water has been determined before and after each filtration step. The parameter has been obtained by plotting flow *versus* pressure, where it represents the slope. Flow has been measured over time and at least 10 points have been recorded.

Fractions recovered were characterised by the particle size analyser ZetaSizer Nano S90 (Malvern) (at the CNR-ITM, Rende, Italy). For comparison, also the BBPH have been analysed by the ZetaSizer equipment available at the CNR-ITM. All the measurements were done in triplicated with 11 runs for each replicate.

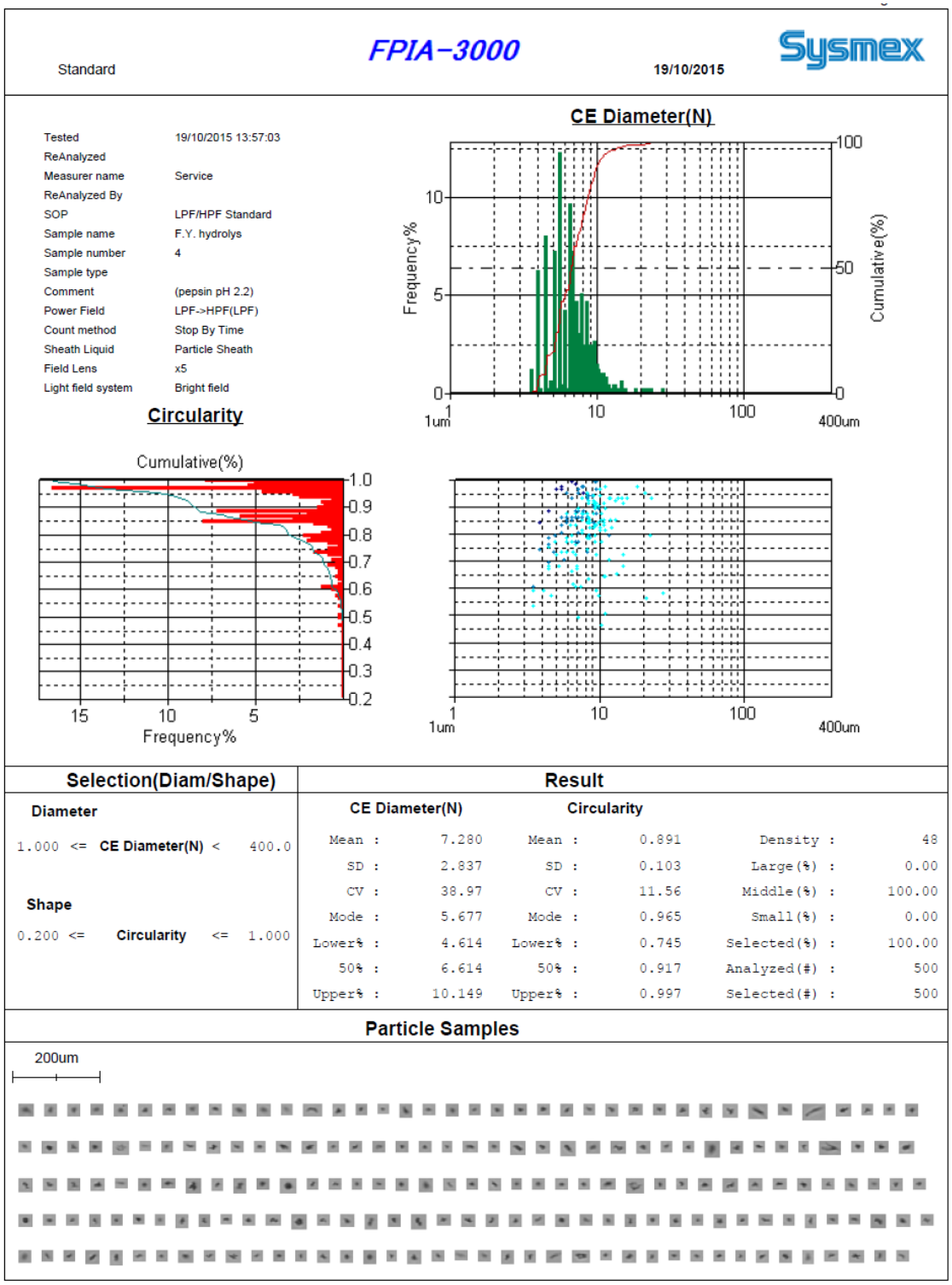


Figure 1. Particle size and shape characterisation for femur BBHP

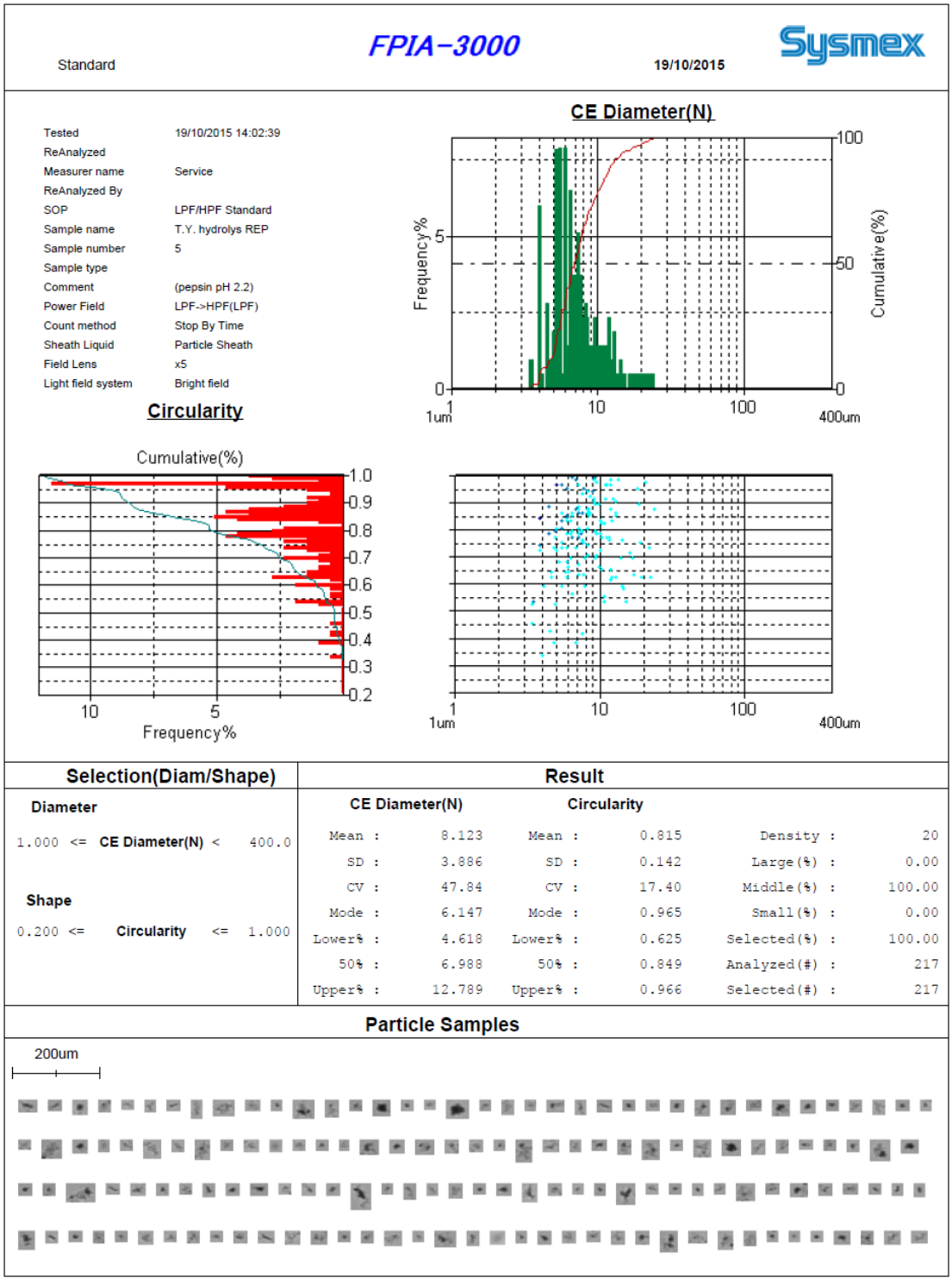
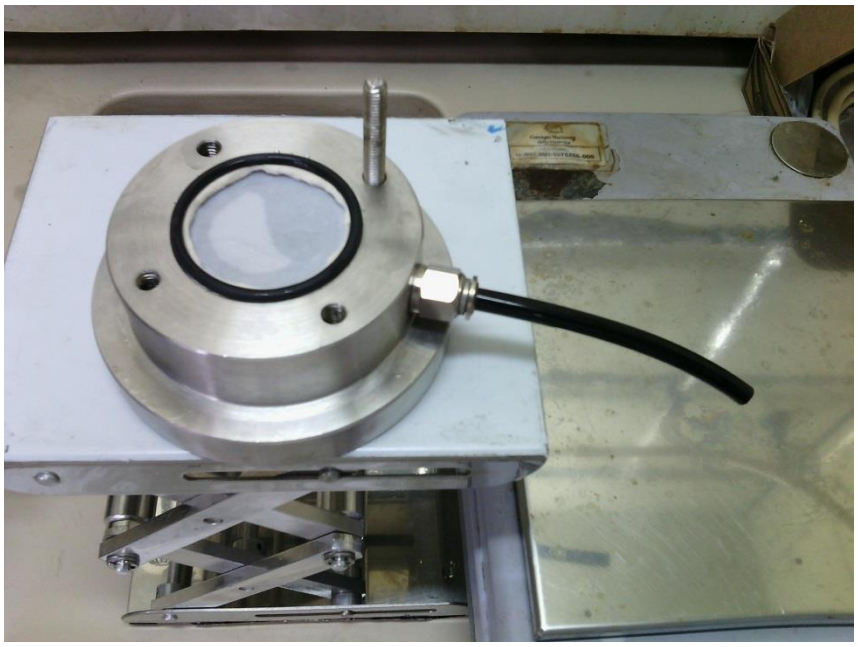


Figure 2. Particle size and shape characterisation for tibia BBHP

Figure 3. Assembly of the ultrafiltration system: 1) feed/retentate, 2) peristaltic pump, 3) manometer, 4) cross-flow membrane cell, 5) permeate



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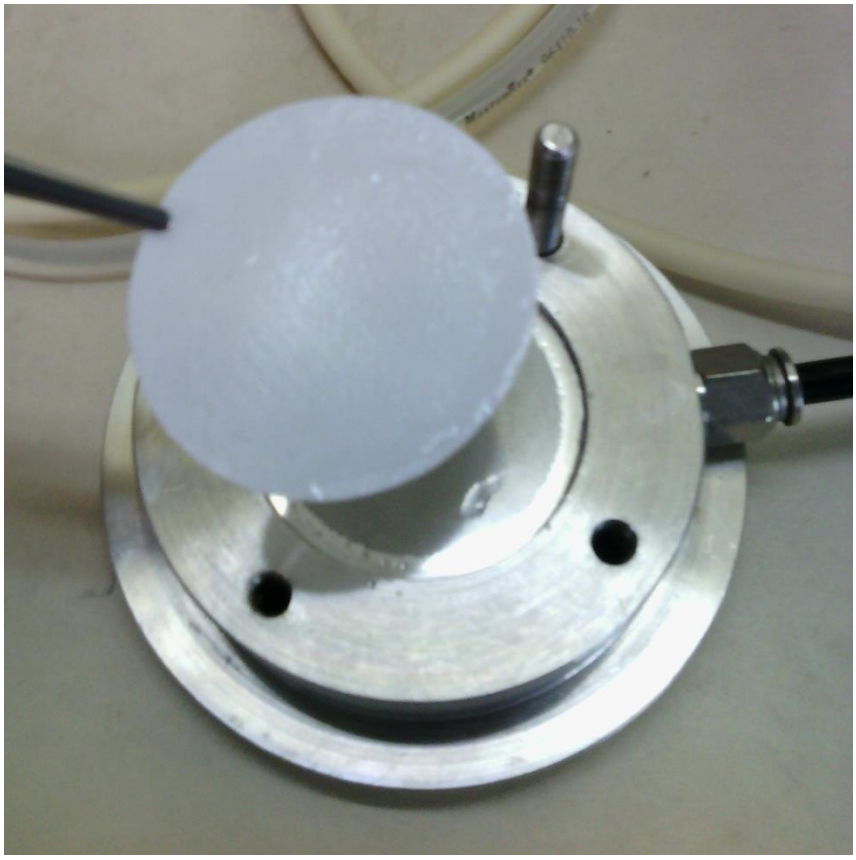

b

Figure 4. Inner of the cross-flow membrane cell (1) and membrane (b)

3. Results and Discussion

3.1 Collected fractions

Ultrafiltration process of BBHP allowed the recovery of 4 fractions, namely 100 kDa cut-off retentate and permeate, and 30 kDa cut-off permeate and retentate, for each sample. The fraction permeated through the 100 kDa cut-off (named P100) was filtered through the 30 kDa cut-off.

The volume reduction factors (VRF) for each sample and each filtrating step are reported in Table 2, where VRF is the ratio between the weight of solution undergoing filtration and the weight of retentate. Separation process was stopped when pressure increased near 1 bar (limit of the pump).

Table 2. Volume reduction factor

Sample	Membrane cut-off (kDa)	VRF	Filtration time (h)
F.Y. BBHP	100	11.4	1.5
F.Y. P100	30	9.5	2.83
T.Y. BBHP	100	12.7	2,33
T.Y. P100	30	13.1	3

Particle size characterisation demonstrated a different distribution in BBPH from femur and tibia (Figure 1, 2 and 5), where tibia young hydrolysates were bigger and the sample is more concentrated. According to the polydispersity index (PI) found (>0.7) those starting samples (feed) contained one major population by volume but there is a broad range distribution within this population, suggesting the presence of multiple species. Particles dimension recoded are bigger than protein dimension (25 nm), meaning the occurrence of strong aggregation of molecules inside each sample.

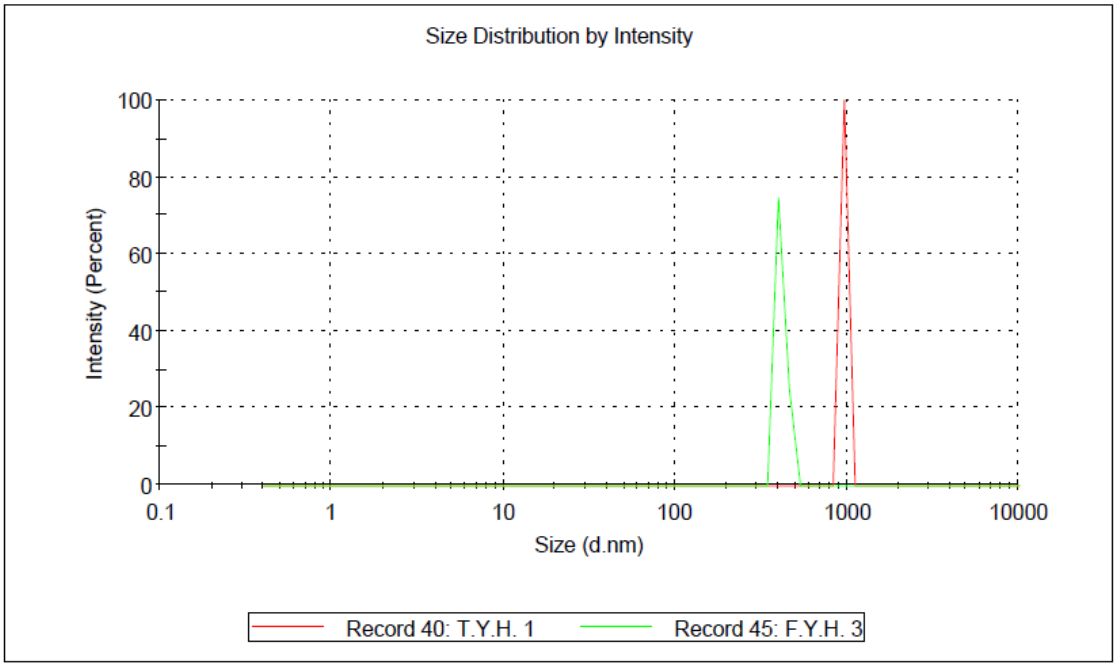

Figure 5. Overlay of size distribution in BBPH from femur and tibia before filtration.

3.1.1. Femur BBPH filtration

Filtration of femur BBPH through 100 kDa cut-off allowed the separation of smallest fractions as can be seen from Figure 6. Permeate showed low PI (0.19) meaning the occurrence of a short range distribution in particle size. On the contrary, the retentate is polydispersed (PI 0.67) suggesting the presence of multiple species.

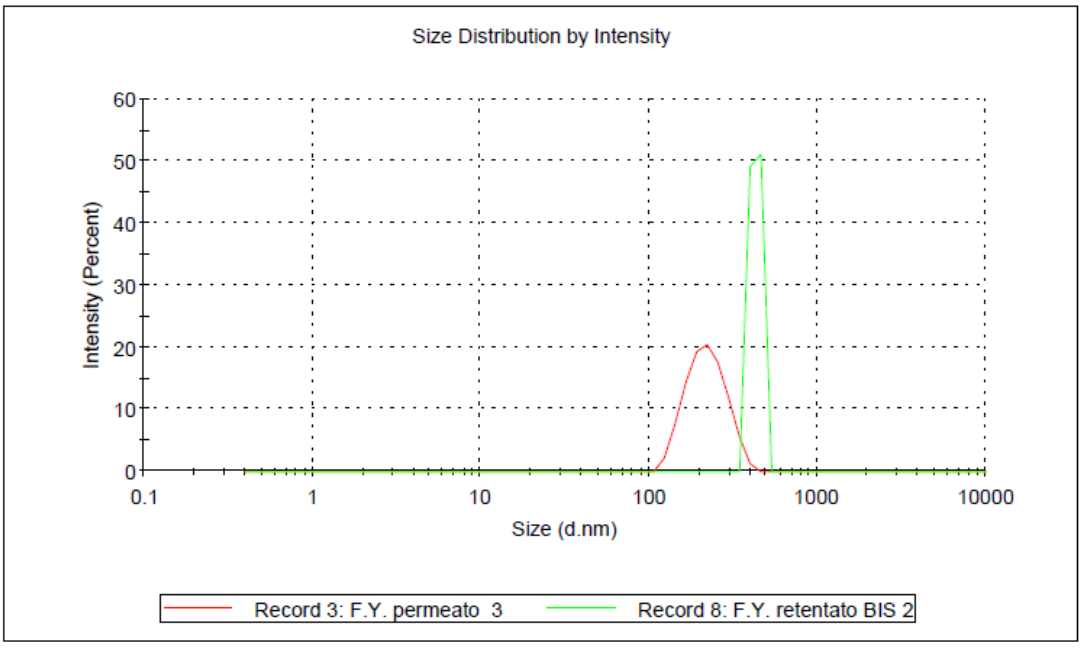

Figure 6. Overlay of 100 kDa permeate and retentate size distribution in femur BBHP.

Filtration of femur P100 through the 30 kDa cut-off membrane demonstrated that the presence of species having molecular weight under 30 kDa is scarce; the intensity is in fact very low (Figure 7). Permeate and retentate are both highly polydispersed (PI 0.92 for permeate and 0.67 for retentate). Also, in the permeate, there are strong aggregation phenomena.

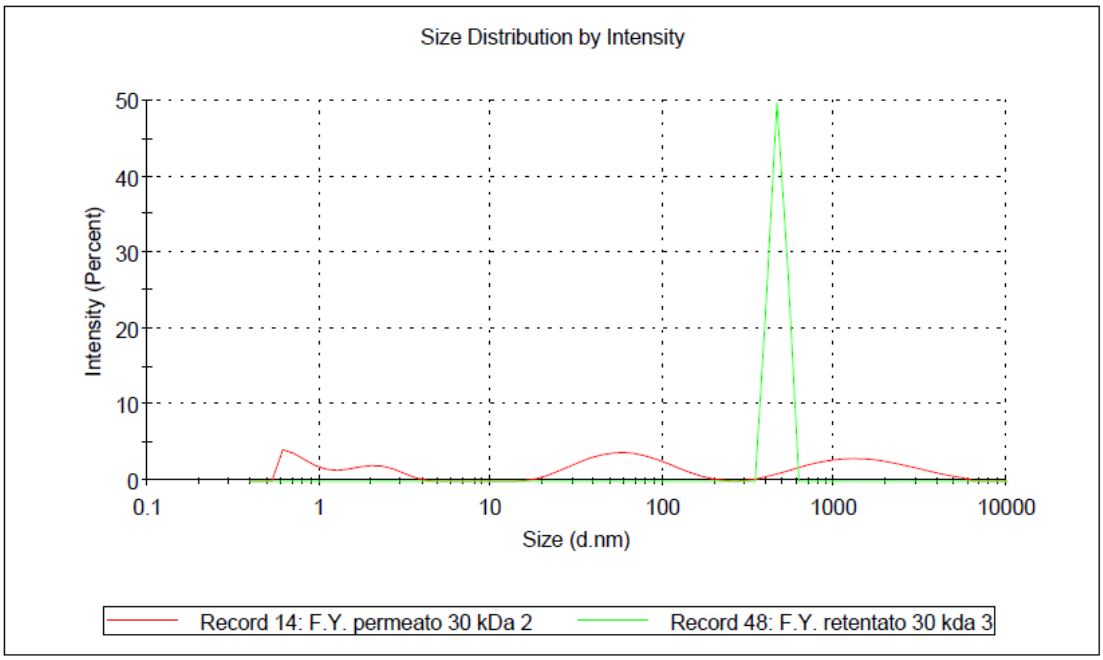

Figure 7. Overlay of 30 kDa permeate and retentate size distribution in femur BBHP.

3.1.2 Tibia BBPH filtration

Filtration of tibia BBPH through 100 kDa cut-off allowed the separation of smallest fractions as can be seen from Figure 8. Permeate and retentate showed to be less polydispersed than the femur analogues, where PI was 0.34 for permeate and 0.25 for retentate. From the intensity, it is also possible to notice than species with molecular weight under 100 kDa are less than in femur BBPH.

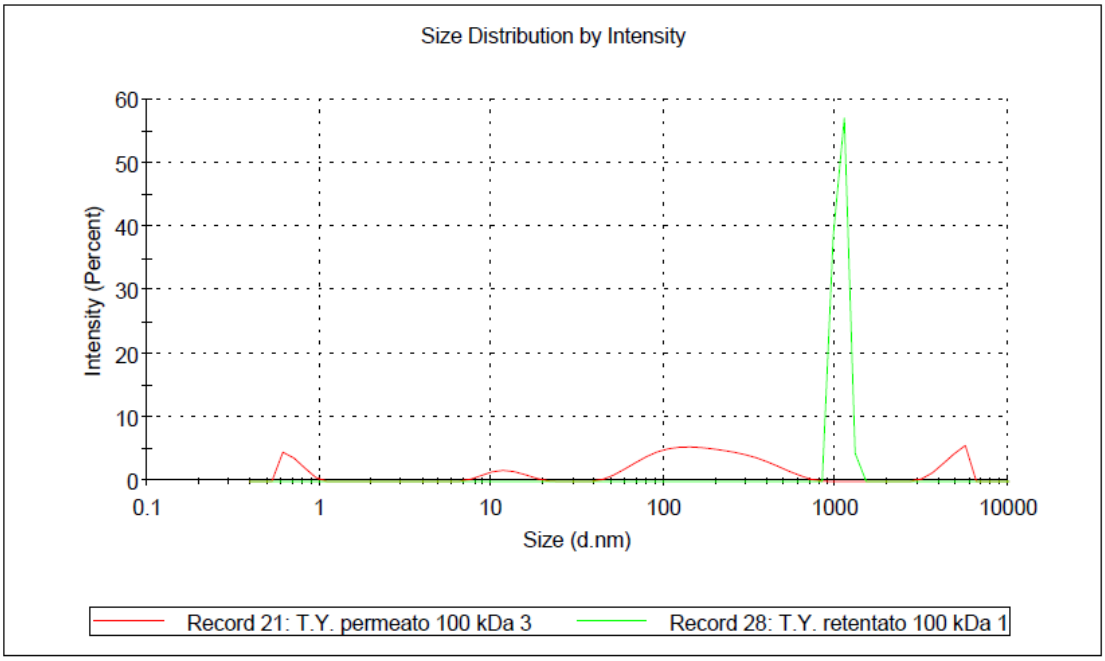

Figure 8. Overlay of 100 kDa permeate and retentate size distribution in tibia BBHP.

Filtration of tibia P100 through the 30 kDa cut-off membrane demonstrated that the presence of species having molecular weight under 30 kDa is scarce, as in the case of femur; the intensity is in fact very low (Figure 9). Permeate is less polydispersed than retentate (PI 0.36 against PI 1.00). Also, in the permeate, there are strong aggregation phenomena.

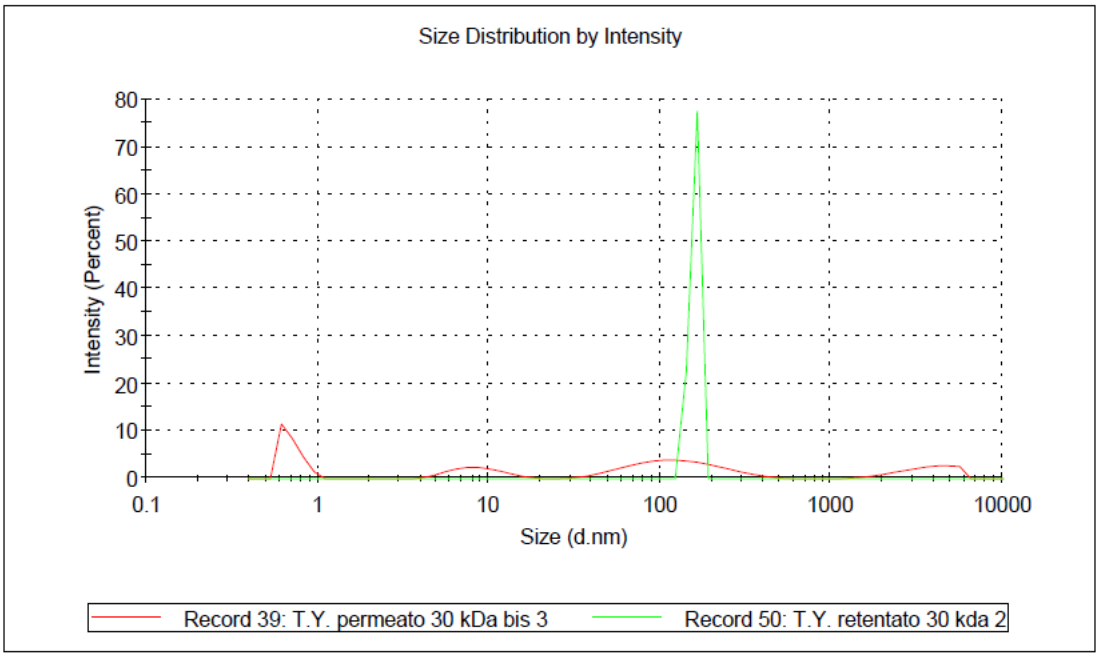

Figure 9. Overlay of 30 kDa permeate and retentate size distribution in tibia BBHP.

Making a comparison between Figure 8 and Figure 9, it is possible to conclude that the ultrafiltration process was effective allowing obtaining two retentate fractions with significant different particle size (see also Table 3).

3.1.3 Comparison between femur and tibia BBPH fractions

Molecular weight distribution in R100 (R=retentate) from tibia and young BBPH was different, as can be noticed from Figure 10. As expected from particle size analysis for BBPH (Figure 5), species in tibia R100 had bigger dimension and higher intensity.

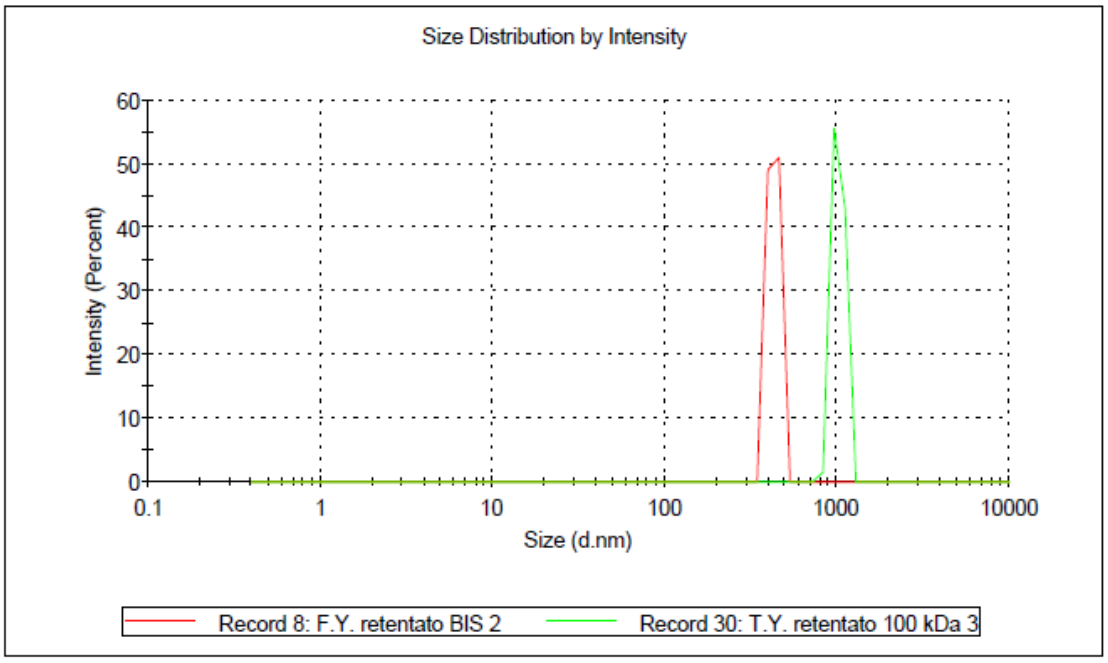

Figure 10. Overlay of femur and tibia BBPH 100 kDa retentate size distribution.

P100 femur and tibia permeates (Figure 11) also have different size distributions.

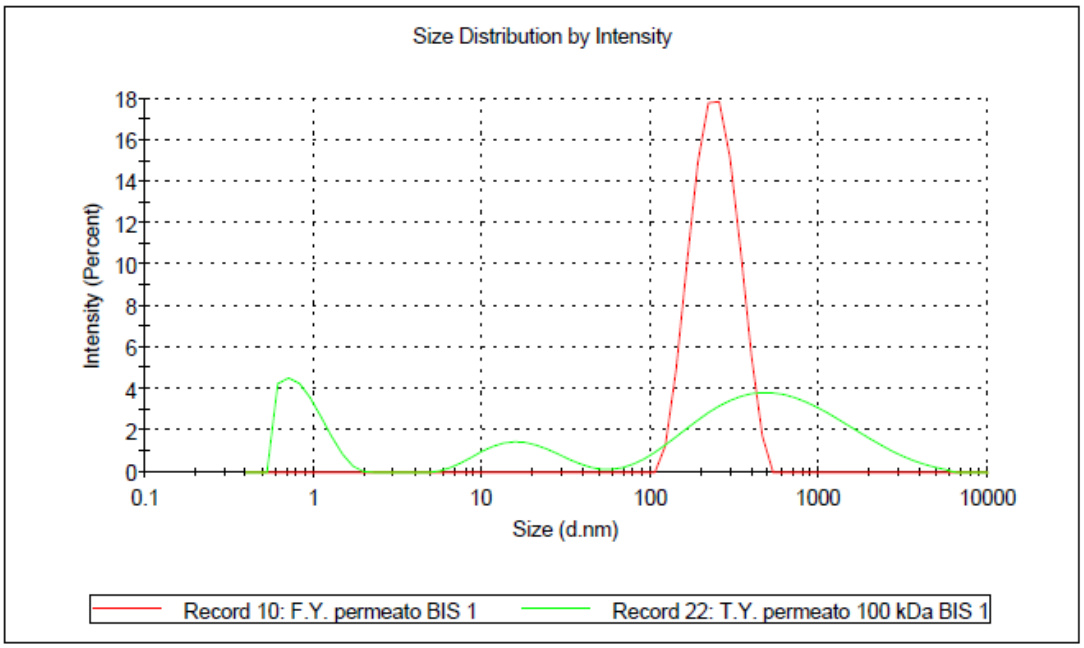

Figure 11. Overlay of femur and tibia BBPH 100 kDa permeate size distribution.

Filtration through 30 kDa cut-off membrane showed that particles size of femur R30 is higher that particle size in tibia R30 (Figure 12).

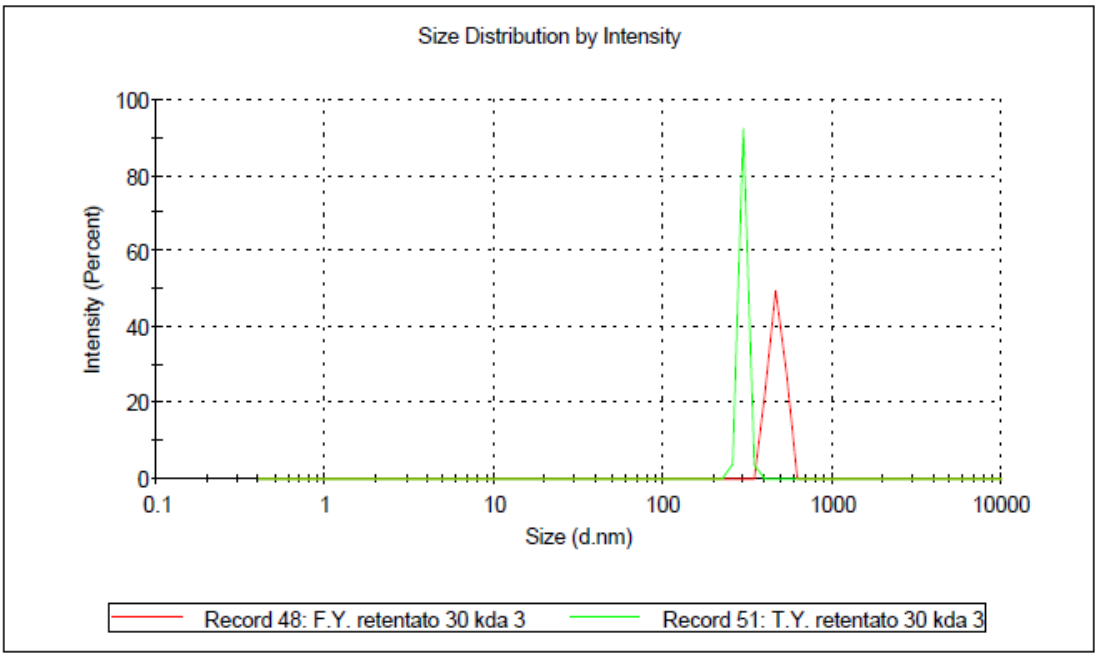

Figure 12. Overlay of femur and tibia BBPH 30 kDa retentate size distribution.

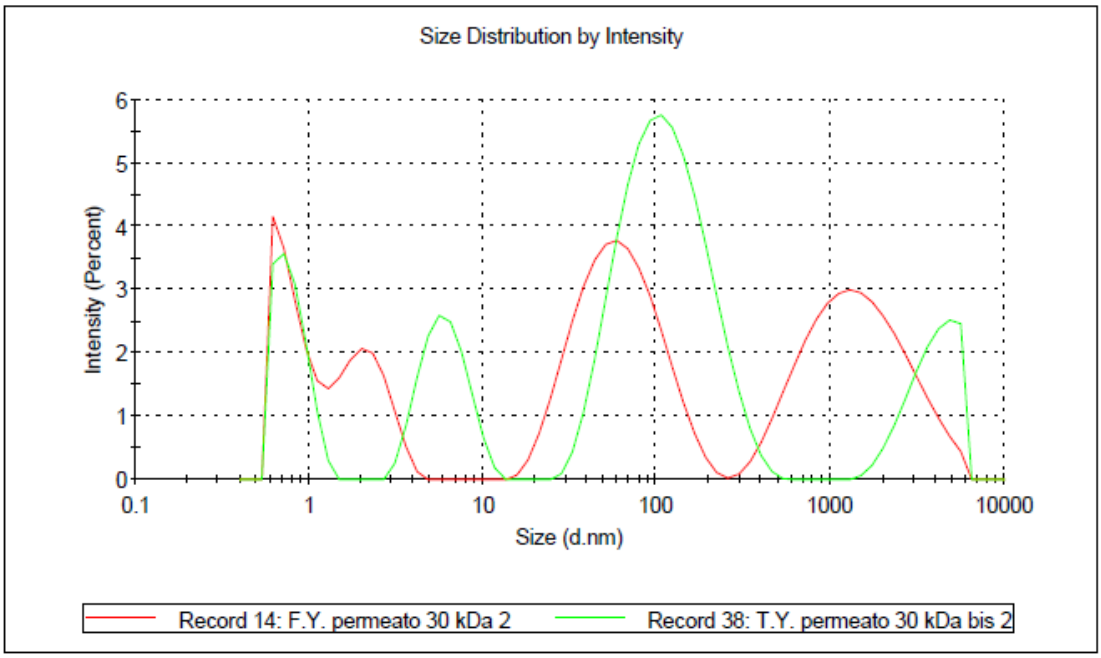

Figure 13. Overlay of femur and tibia BBPH 100 kDa permeate size distribution.

Table 3. Particle size dimension in permeate and retentate

Sample	Particle size (nm)	Sample	Particle size (nm)
Femur BBPH	412	Tibia BBPH	955
Femur R100	428	Tibia R100	1056
Femur P100	222	Tibia P100	0.7 -14 -150 - 5500
Femur R30	464	Tibia R30	159
Femur P30	0.83 - 2.2 -67-1500	Tibia P30	0.65 - 8 -110 - 5000

3.2 Membranes Fouling

Results showed reversible fouling in the case of regenerated cellulose membrane (100 kDa cut-off) and irreversible fouling in the case of SEPA *UltraFillic* membrane (30 kDa cut-off). As an example, the material retained on the 100 kDa cut-off membrane and totally removed after washing with ultrapure water is reported Figure 14. Permeability values to ultrapure water

after filtration of each sample, along with the permeability decrease are reported in Table 4.

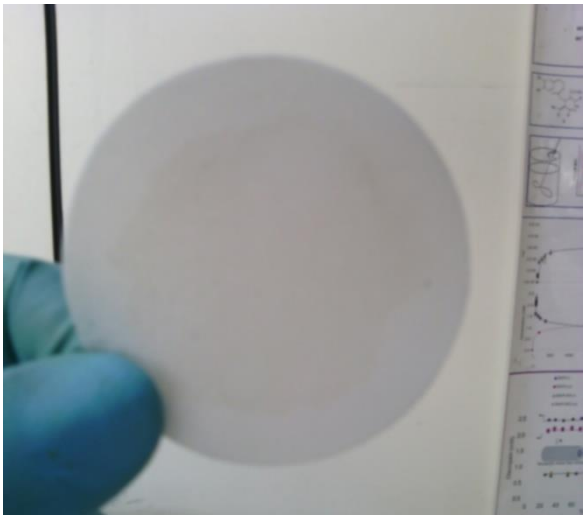

Figure 14. T.Y. BBHP 100 kDa retentate collected in the membrane

Table 4. Membranes permeability to ultrapure water after filtration of samples

Filtered Sample	Membrane	Permeability ($L/h \cdot m^2$)	Error (%)	Permeability decrease (%)
	100 kDa	665		
Dissolving solution	100 kDa	674	5	-
F.Y. BBHP	100 kDa	673	5	-
T.Y. BBPH	100 kDa	648	2.7	-
	30 kDa	195		
Dissolving solution	30 kDa	170	10	13
F.Y. P100	30 kDa	146	10	25
T.Y. P100	30 kDa	135	5	30

4. Conclusions and Future Work

The research work carried out allowed the recovery of fractions with different particle size distributions and degree of aggregation. The membranes used demonstrated to be effective. Other parameters should be evaluated in order to improve the separation process and mainly aiming at investigating the effect of aggregation phenomena. Thermal conditions, ionic strength and fluid dynamic should be studied for an optimisation of the separation process. Membrane fouling should be also studied to assess the nature of fouling itself, i.e. if chemical (irreversible) or physical (reversible by washing with specific chemicals).

Acknowledgements

I would like to kindly acknowledge Dr. Lidietta Giorno, Director of CNR-ITM, for having accepted my STM application, and Dr. Rosalinda Mazzei for all the support provided before and during my stay at the hosting laboratory.
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