

Article

Centrifugal Filtration Devices to Separate Peptides/Proteins as a Characterization Tool: Good Specificity but Poor Quantification

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Abstract

Centrifugal filtration devices are increasingly being used to separate (bio)molecules thanks to their molar masses. This is partly due to their ease of use, with sample preparation requiring no complicated protocol and often being able to be run as is. Nevertheless, several limitations have been highlighted in the literature, such as proteins' selectivity according to their molar masses, clogging problems and some post-separation quantification issues. We wanted to test the real potential of the centrifugal filtration devices as a characterization tool, by verifying their specificity using a library of peptides and proteins. The impact of the peptide charge on separation performance as well as the impact of biological material concentration and centrifugation rate were evaluated using three membranes with different cut-offs. A good specificity was confirmed: biological materials that have molar masses which are below the membrane cut-off can pass almost completely through the membrane, while those with molar masses above the membrane cut-off are almost fully retained. The findings proved more problematic when protein mixtures were analyzed: despite still having good specificity, biological materials with molar masses below the membrane cut-off only partially crossed the membranes, due to clogging effects, making quantification of the initial mixture incorrect. It is therefore essential to be very careful when working with centrifugal filtration devices, as their use requires precise and relevant controls, without necessarily being able to optimize the separation of mixtures. Thus, this remains far from an easy and ready-to-use system.

Keywords: centrifugal filtration device; peptide; protein; separation; quantification



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1. Introduction

Centrifugal filtration devices use filters with a membrane that allows certain molecules to pass through during centrifugation. A change in the size of the membrane pores enables the separation and thus the selectivity of molecules and biomolecules of different molar masses. Consequently, (bio)molecules with a molar mass above the membrane cut-off are retained by the membrane, while those with a lower molar mass are found in the filtrate. This technique was first used in the 1970s [1], but is still in use today in a variety of applications, such as the purification and concentration of DNA samples from crime scenes [2], proteins [3–7], proteome fractionation [3,8], the quantification of small molecules [9], etc. In the field of nanomedicine, centrifugal filtration devices are used, for

example, to separate nanoparticles from a free molecule prior to quantification steps [10–13], or to purify extracellular vesicles [14,15].

However, this technique has several limitations. Although it can be used to increase concentration in biological samples, particularly in the case of DNA traces, a loss of biological material is often observed [2,9,16–18]. Indeed, when purifying DNA with a molar mass above the cut-off of the membranes of centrifugal filtration devices (Amicon® filters – Merck, Darmstadt, Germany; and Microsep® filters – Pall Corporation, Port Washington, NY, USA), Norén et al. observed that around 35% and 77% of the DNA, respectively, passed through the membranes [17]. Moreover, Garvin et al. showed that this loss can be significant depending on the centrifugation rate and cut-off of the devices used [18]. A lack of membrane selectivity has also been noted by Johnsen et al. Indeed, they observed similar separation profiles when proteins were passed through filters with different cut-offs [9]. These limitations could also be found when separating free biological material from nano-objects and thus bias the quantification of adsorption or the encapsulation of it [19].

The aim of this study was therefore to evaluate the ability of centrifugal filtration devices to select peptide or protein from a simple solution or a mixture, in order to test the performance of this separation technique. Firstly, we wanted to check whether peptides or proteins' charge could be a limit to the separation. Three homopeptides—neutral, cationic and anionic—were synthesized and separated on the centrifugal filtration device. These homopeptides had very low molar masses compared with the cut-off of the membranes; thus, their molar masses were not a hindrance to separation and their charge was the only parameter that could influence separation. Then, the separation profiles of eight peptide/proteins with different molar masses were analyzed using membranes with different cut-offs. The influence of protein concentration and centrifugation rate was also studied. Finally, the separation capacities of proteins mixtures with different molar masses were evaluated.

2. Materials and Methods

2.1. Material

N-(9-Fluorenylmethoxycarbonyl)-arginine (Fmoc-Arg-OH), *N*-(9-Fluorenylmethoxycarbonyl)-alanine (Fmoc-Ala-OH), *N*-(9-Fluorenylmethoxycarbonyl)-glutamic acid (Fmoc-Glu-OH), formic acid (FA), porcine liver esterase (EST), β -galactosidase from *Aspergillus oryzae* (GAL), bovine serum albumin (BSA), pepsin (PEP), bovine pancreas α -chymotrypsin (CHY), lysozyme from chicken egg white (LYS), bovine pancreas insulin (INS), sodium hydroxide (NaOH) and sodium chloride (NaCl) were supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Dimethylformamide (DMF), methanol (MeOH), trifluoroacetic acid (TFA), phenol, thioanisole triisopropyl silane (TIS), acetonitrile (LC/MS grade) (ACN), water (LC/MS grade) and diethyl ether were provided by ThermoFisher Scientific (Waltham, MA, USA). Piperidine, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), diisopropylethylamine (DIEA) were supplied by Biotech GmbH (Burgwedel, Germany). Biotinylated NFL-TBS.40-63 peptide (NFL) was synthesized by PolyPeptide (Strasbourg, France). Deionized water was obtained from a Milli-Q plus_system (Millipore, Billerica, MA, USA).

2.2. Synthesis, Purification and Characterization of Polyarginine, Polyalanine and Polyglutamic Acid Peptides

Polyarginine (pArg₇), polyalanine (pAla₇) and polyglutamic acid (pGlu₇) were synthesized on a rink amid p-methylbenzhydrylamine (0.69 mmol/g) resin (Multisyntech GmbH, Witten, Germany) via a standard Fmoc solid phase peptide synthesis method, with

an automated microwave peptide synthesizer (CEM corporation, Matthews, NC, USA). First, the resin was immersed in DMF and gently stirred to swell the resin beads for 2 h at room temperature. The first step was Fmoc deprotection using piperidine/DMF 20% (v/v). Then, the resin was washed several times with DMF. Subsequently, the amino acid coupling reaction was achieved by adding a DMF-mixed solution of Fmoc-protecting amino acid (5 eq.), HBTU (4.5 eq.), and DIEA (10 eq.). These steps were performed using a microwave-assisted system; cycles of deprotection–washing–coupling–washing were repeated until the desired amino-acid sequence was obtained. Finally, the resin was washed with DMF and MeOH, three times, and dried under vacuum for 48 h. The peptide was cleaved from the resin by adding the appropriate cleavage cocktail based on the amino acid sequence. For pArg₇, reagent K (TFA/phenol/water/thioanisole/TIS 82.5/5/5/5/2.5 (v/v/v/v/v)) was used, while reagent B (TFA/phenol/water/TIS 88/5/5/2 (v/v/v/v)) was used for pAla₇ and pGlu₇. After adding the cleavage cocktail (5 mL for 0.5 g of resin), the resin was stirred at 37 °C for 1–4 h. The crude peptide was precipitated by adding the cleavage mixture dropwise to at least a 10-fold excess of cold diethyl ether. The resulting precipitate was centrifuged for 30 min at 4 °C at 2000 rpm. Afterward, the crude product was dissolved in water before freeze-drying.

The crude products were purified by semi-preparative reversed phase high-performance liquid chromatography (RP-HPLC) (Waters, Guyancourt, France). The purification was performed at room temperature using a XBridge BEH C18 Prep column (130 Å, 5 µm, 250 × 30 mm) (Waters). The mobile phase consisted of water (TFA 0.1% (v/v)) and ACN (TFA 0.1% (v/v)). The purification was conducted at a flow rate of 44 mL/min, with an injection volume of 2.5 mL and a detection wavelength at 214 nm, using a gradient: 0 min: 95% water; 0 → 12 min: linear gradient until 80% water; 12 → 45 min: linear gradient until 70% water; 45 → 47 min: linear gradient until 0% water; 47 → 57 min: 0% water; 57 → 59 min: linear gradient until 95% water; and 59 → 75 min: 95% water. Before purification, the crude product was dissolved in water (TFA 0.1% (v/v))/ACN (TFA 0.1% (v/v)) 95/5 (v/v) at a concentration of 4 mg/mL. The samples were vortexed, sonicated and filtered with a 0.22 µm Millex-LG filter (Merck, Darmstadt, Germany).

pArg₇, pAla₇ and pGlu₇ were characterized by a LC-MS/MS method, developed on an Alliance[®] 2695 system (Waters) with a Uptisphere C18 5ODB column (5 µm, 150 × 2.0 mm) (Interchim, Montluçon, France). The mobile phase consisted of water (FA 0.1% (v/v)) and ACN (FA 0.1% (v/v)). The purified peptides were dissolved in water (FA 0.1% (v/v))/ACN (FA 0.1% (v/v)) 95/5 (v/v) at a concentration of 1 mg/mL. The samples were vortexed, sonicated and filtered with a 0.22 µm Millex-LG filter before analyses. For pArg₇, the gradient was: 0 → 8 min: 50% water; 8 → 9 min: linear gradient until 0% water; 9 → 12 min: 0% water; 12 → 13 min: linear gradient until 50% water; and 13 → 15 min: 50% water. For pAla₇ and pGlu₇, the gradient was: 0 → 5 min: linear gradient from 95% to 0% water; 5 → 6 min: linear gradient until 95% water; and 6 → 12 min: 95% water. The total HPLC effluent was injected into a Quattro Micro[®] triple quadrupole mass spectrometer (Waters). Ionization was achieved using electrospray in positive ion mode in the *m/z* 200–1500 range (full scan acquisition) for pArg₇, negative mode in the *m/z* 20–600 range for pAla₇ and negative mode in the *m/z* 50–1000 range for pGlu₇. An option of cone ramp was used between 20 and 100 V to optimize the acquisition.

The purity was established by UPLC method, using an UPLC Acquity H-Class Bio (Waters). The column used for pArg₇ was an Acquity[®]UPLC BEH C18 (1.7 µm, 100 × 2.1 mm) (Waters). For pArg₇, the mobile phase consisted of water (TFA 0.1% (v/v)) and ACN (TFA 0.1% (v/v)). The purified pArg₇ was dissolved in water, vortexed and filtered with a 0.22 µm Millex-LG filter. The purity analysis was conducted at a flow rate of 0.2 mL/min, with an injection volume of 10 µL and a detection wavelength at 214 nm, using a gradient:

0 → 5 min: linear gradient from 95% to 78% water; 5 → 10 min: 78% water; 10 → 15 min: linear gradient until 0% water; 15 → 20 min: 0% water; 20 → 21 min: linear gradient until 95% water; and 21 → 25 min: 95% water. The column used for pAla₇ and pGlu₇ was an Acquity[®] UPLC BEH200 SEC (1.7 µm, 4.6 × 150 mm) (Waters). The purified pAla₇ was dissolved in water while pGlu₇ was dissolved in NaCl at 0.1 M, vortexed and filtered with a 0.22 µm Millex-LG filter. The purity analysis was conducted at a flow rate of 0.2 mL/min, with an injection volume of 10 µL and a detection wavelength at 214 nm, using an isocratic method: NaCl 0.1 M in water.

2.3. Assays for Separation Using Centrifugal Filtration Devices

pAla₇ (~0.5 kDa), pArg₇ (~1.1 kDa), NFL (~2.7 kDa), LYS (~14.6 kDa), CHY (~25 kDa), PEP (~34.6 kDa), BSA (~66.4 kDa), GAL (~105 kDa) and EST (~162 kDa) were dissolved in water (Final pH between 6.5 and 7.5). pGlu₇ (~0.9 kDa) was dissolved in NaCl 0.5 M (Final pH between 6.5 and 7.5) while INS (~5.7 kDa) was dissolved in NaOH 0.1 M (Final pH close to 1). The final concentrations for all the peptides and proteins' solutions were 400, 600, 800 and 1000 µg/mL. Proteins mixtures were also prepared in water. Concentration ratio of 400/400, 600/600, 800/800 and 1000/1000 (µg/mL/µg/mL) for the mixture LYS/BSA, and 200/200, 400/400, 1000/200 and 1000/400 (µg/mL/µg/mL) for the mixture LYS/EST. A volume of 500 µL of peptides, proteins, or mixtures were centrifuged through 30 K, 50 K or 100 K Amicon[®] Ultra filters (Merck, Darmstadt, Germany) for 30 min at 7000 or 14,000 × g. The supplier recommendations were respected to prepare the membranes before centrifugation. The filtrate volume was measured by pipetting. The peptide and protein concentrations in the filtrate were analyzed using the bicinchoninic acid (BCA) assay (Section 2.4) or by size exclusion chromatography (SEC) or UPLC (Section 2.5).

2.4. Peptides and Proteins' Titration Using BCA Assay

The concentration of peptides and proteins were measured using the BCA assay (Pierce[™] BCA Protein Assay Kit, ThermoFisher Scientific) before and after the filtration through the centrifugal filtration devices. Seven standards (25 to 175 µg/mL) and one blank (0 µg/mL) were prepared from all the peptides and proteins, in water or NaOH 0.1 M (only for INS). The supplier's recommendations were respected. The reagent was prepared by mixing 50 parts BCA reagent A and 1 part BCA reagent B. All standards and filtrates (25 µL) were added in triplicate to microplate wells (Nunc[™], ThermoFisher Scientific), followed by the addition of 200 µL reagent. The microplate was mixed for 30 s, covered, and incubated at 37 °C for 30 min. After cooling the plate, the absorbance was measured at 562 nm on a SpectraMax[®] M2 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). The peptides and proteins' concentrations in the various samples were determined using the various calibration curves (determination coefficients: R² = 0.9918–0.9999).

2.5. Peptides and Proteins' Titration Using SEC and UPLC

The concentrations of pGlu₇, pAla₇ and the mixtures LYS, BSA, and EST were measured before and after the separation through the centrifugal filtration devices using an UPLC Acquity H-Class Bio (Waters), with an Acquity UPLC Protein BEH200 SEC (200 Å, 1.7 µm, 4.6 mm × 150 mm) (Waters) column. The analyses were conducted at a flow rate of 0.3 mL/min, with an injection volume of 10 µL and a detection wavelength at 220 nm, using an isocratic method: NaCl 0.1 M in water. The peptide and protein concentrations were determined using calibration curves for the area under the curves with standard concentrations from 200 to 1000 µg/mL for pAla₇, BSA and LYS (R² = 0.9919–1.0000), from 100 to 1000 µg/mL for pGlu₇ (R² = 1.0000) and from 100 to 800 µg/mL for EST (R² = 0.9980–0.9996). The concentrations of pArg₇ were measured before and after the separation through the centrifugal filtration devices using the same method described in

previous section for purity characterization. The pArg₇ concentrations were determined using calibration curves for the area under the curves, with standard concentrations ranging from 100 to 1000 µg/mL ($R^2 = 1.0000$).

2.6. Statistical Analysis

All experiments were performed in triplicate, regardless of the nature of the biomolecule and its concentration, or the mixture composition. Statistical evaluation of all the data was performed using the parametric analyses, such as ANOVA 1F followed by either Tukey's post hoc test for pairwise comparisons, or Welsh's *t* test. The difference between the groups was accepted as significant with *p*-values lower than 0.05.

3. Results and Discussion

The principle of the separation of peptides or proteins by centrifugal filtration devices is based on their molar masses. However, the impact of the charge of biological molecules is not so clear in the literature, since this parameter is rarely studied. As a first step, we wanted to test the influence of the charge of biological molecules while minimizing a separation weakness due to excessive molar mass. Short homopeptides (sequence of seven amino acids) were synthesized: (i) the pGlu₇, an anionic peptide (920.83 g/mol-[M-H]⁻ = 919.37 g/mol-purity > 95%), (ii) the pAla₇, a neutral peptide (514.53 g/mol-[M-H]⁻ = 513.332 g/mol-purity > 95%) and (iii) the pArg₇, a cationic peptide (1109.73 g/mol-[M+H]⁺ = 1111.082 g/mol-purity > 95%). The proportion of peptides recovered in the filtrate was close to 100% after separation using centrifugal filtration devices with a membrane cut-off of 50 kDa (Figure 1). No difference was observed whatever the charge of the homopeptide—96 ± 3%, 99 ± 1% and 100 ± <1% for pGlu₇, pAla₇ and pArg₇, respectively—nor was there any difference in separation between homopeptide concentrations from 400 to 1000 µg/mL. These results clearly suggest that the charge of biological molecules has no influence on its passage through the centrifugal filtration devices.

The performance of centrifugal filtration devices with different cut-offs was tested when separating peptide and proteins of different molar masses. For this purpose, a peptide (NFL) and seven protein solutions (INS, LYS, CHY, PEP, BSA, GAL and EST) were used at a concentration of 1000 µg/mL—this was first examined through a centrifugal filtration device with a cut-off of 50 kDa. Overall, the separation was excellent, since the peptide and most of the proteins with a molar mass below the cut-off passed through the filter at over 90% (NFL, INS and LYS), while proteins with a molar mass above the cut-off were well retained (passing through the filter at less than 10% for BSA and EST) (Figure 2). As molar masses approach the cut-off, as in the case of CHY and PEP, but are still lower, more proteins are retained on the filter. Only about 80% for CHY (25 kDa difference between cut-off and molar mass) and about 70% for PEP (15 kDa difference between cut-off and molar mass) passed through the filter. An exception was observed for GAL, since about 20% of the protein was able to pass through the filter even though the protein has a molar mass greater than the cut-off—55 kDa—which is a difference greater than that for BSA. These differences cannot be explained by the clogging of the filters since the filtrate volumes after the centrifugation for all the peptide/proteins were very close to the one obtained with pure water (Figure 2 insert). The size distribution of the membrane below the 50 kDa cut-off as well as protein conformations different from the sphere could explain the partial protein retention for CHY and PEP, or the uncomplete retention for GAL. This observation has also been noted by Georgiou et al. [20]: a high-molar-mass protein was able to pass through the membrane of centrifugal filtration devices when it should not. Another hypothesis is that the pH of the solution influences the structure of GAL and thus its passage through the membrane. Yang et al. have shown that at pH levels above 5, GAL appears to break down

into fragments that are almost 10-fold smaller than the protein's initial size [21]. This may suggest that the proportion of GAL found in the filtrate corresponds to fragments of the protein and not to the whole protein.

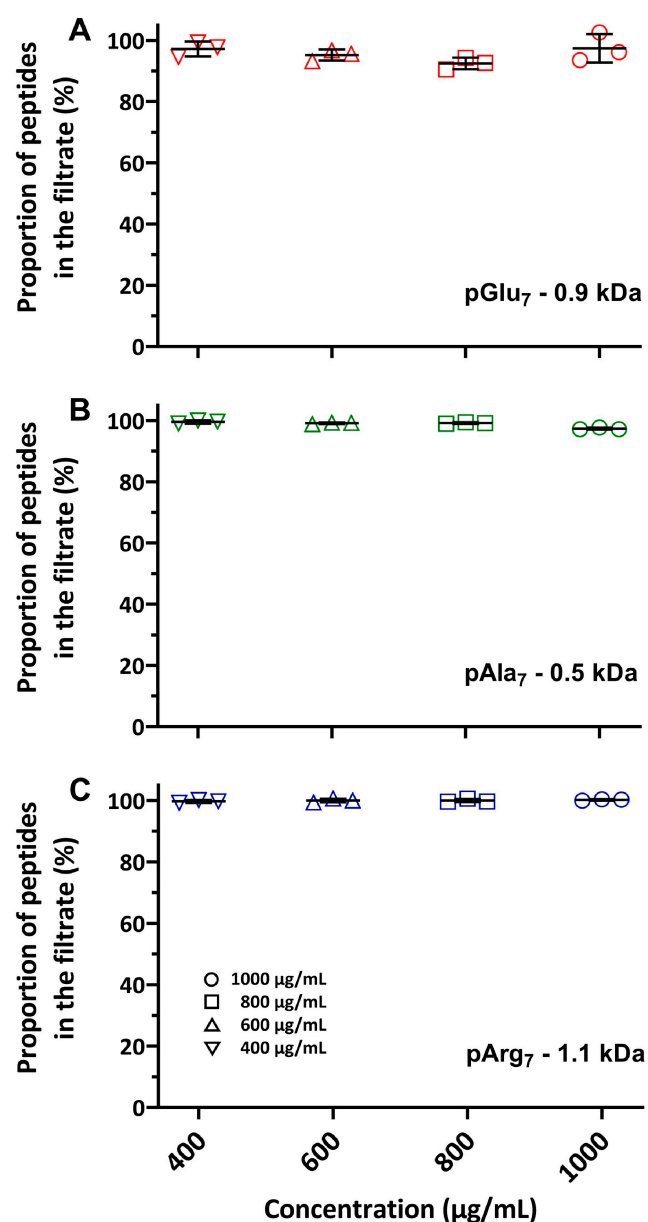


Figure 1. The charge of peptides does not modify their separation using centrifugal filtration devices. Solutions (500 μ L; 400, 600, 800 or 1000 μ g/mL) of pGlu₇ (A), pAla₇ (B) and pArg₇ (C) were deposited on centrifugal filtration devices (cut-off of 50 kDa) and centrifuged at 14,000 $\times$ g during 30 min. The proportion of peptides in the filtrate was determined using SEC or UPLC methods ($n = 3$; mean $\pm$ SD).

The same performance was tested using a centrifugal filtration device with a cut-off of 30 kDa. Separation abilities remained excellent, since the NFL with a molar mass below the cut-off passed through the membrane at over 90%, while PEP with a molar mass above the cut-off was well retained (passing through the membrane at about 1%, $p < 0.0001$ in comparison to the cut-off of 50 kDa) (Figure 3). Uncomplete separations were observed when the molar masses approached the cut-off. Only about 70% of INS (a difference of 25 kDa between the cut-off and the molar mass) and only 15% of LYS (a difference of 15 kDa between the cut-off and the molar mass) passed through the membrane, which is significantly less than with the 50 kDa cut-off ($p < 0.0001$ for both). Even though the

difference between the molar mass of CHY and the cut-off is very small, around 5 kDa, this protein still passed through the filter at over 60%, practically the same proportion as with the cut-off of 50 kDa. Membrane pore size distribution as well as protein conformations could also explain these differences and non-differences. However, clogging effects are not a hypothesis (Figure 3 insert).

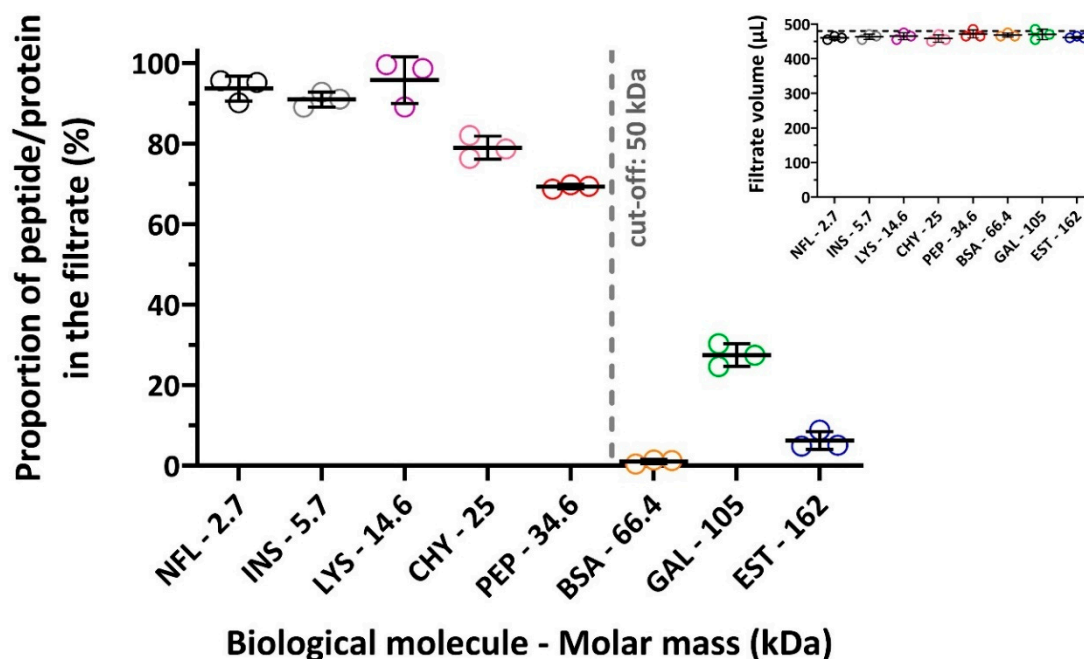


Figure 2. Separation of peptide or proteins with different molar masses confirms the performance of the centrifugal filtration device with a 50 kDa cut-off membrane. Solutions (500 μ L; 1000 μ g/mL) of biotinylated NFL-TBS.40-63 (NFL), bovine pancreas insulin (INS), lysozyme from chicken egg white (LYS), bovine pancreas α -chymotrypsin (CHY), pepsin (PEP), bovine serum albumin (BSA), β -galactosidase from *Aspergillus oryzae* (GAL) and porcine liver esterase (EST) were deposited on centrifugal filtration devices (cut-off of 50 kDa) and centrifuged at $14,000 \times g$ during 30 min. The proportion of peptide/proteins in the filtrate was determined using SEC or BCA assay ($n = 3$; mean $\pm$ SD). Insert: the volume of the filtrate after centrifugation was measured by pipetting ($n = 3$; mean $\pm$ SD). The dotted line corresponds to the filtrate volume of pure water after centrifugation.

The performance of the centrifugal filtration device with a cut-off of 100 kDa was finally tested. Separation remains excellent, since the peptide and most of the proteins with a molar mass below the cut-off passed through the filter at over 90% (NFL, INS, LYS (no change in comparison to a cut-off of 50 kDa, $p = 0.1189$) and CHY ($p = 0.0090$ in comparison to a cut-off of 50 kDa)), while proteins with a molar mass above the cut-off were well retained (passing through the filter at less than 5% for EST) (Figure 4).

No change was observed for GAL compared with the 50 kDa cut-off. Thirty percent still passed through the filter with a cut-off of 100 kDa, even though it is closer to its molar mass (5 kDa difference). This lack of selectivity was also observed by Johnsen et al. [9]. Indeed, the filtration of proteins on membranes with different cut-off values led to identical separation profiles. However, the hypothesis of GAL degradation as indicated previously could explain how the same proportions are found whatever the cut-off used. On the other hand, more PEP passed through the 100 kDa cut-off membranes than through the 50 kDa cut-off ones; is demonstrated a significant increase of about 10%, with $p = 0.0078$, since its molar mass was further away from the cut-off. Using the membrane with a cut-off of 100 kDa, the BSA with a molar mass of about 65 kDa was able to partially but not totally pass through the filter (about

25%), while it was totally retained by the membrane with a cut-off of 50 kDa ($p < 0.0001$). As for the other membranes, no clogging was observed (Figure 4 insert).

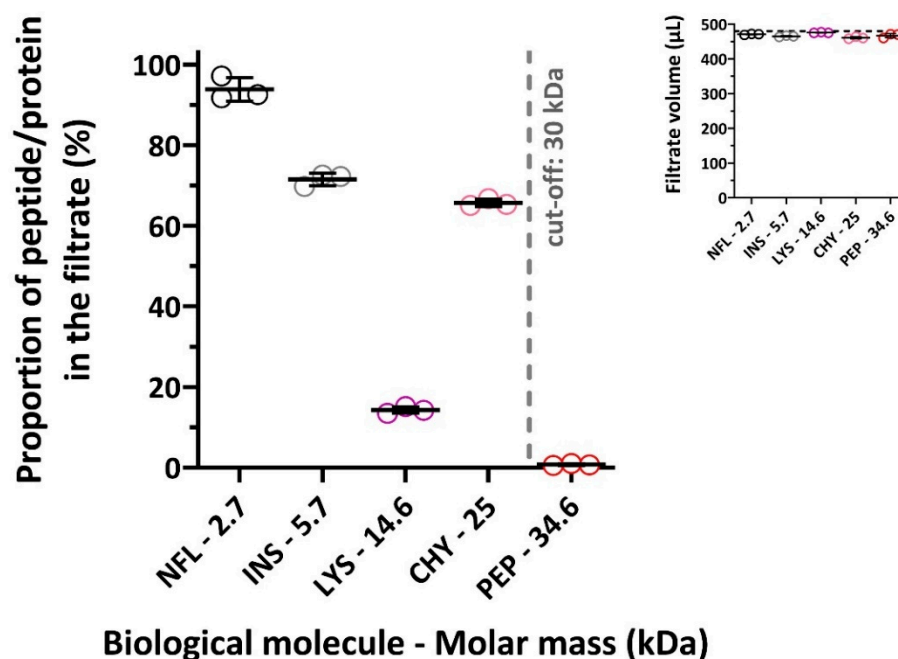


Figure 3. Separation of peptide or proteins with different molar masses confirms the performance of the centrifugal filtration system with 30 kDa membrane. Solutions (500 µL; 1000 µg/mL) of biotinylated NFL-TBS.40-63 (NFL), bovine pancreas insulin (INS), lysozyme from chicken egg white (LYS), bovine pancreas α -chymotrypsin (CHY) and pepsin (PEP) were deposited on centrifugal filtration devices (cut-off of 50 kDa) and centrifuged at $14,000\times g$ during 30 min. The proportion of peptide/proteins in the filtrate was determined using BCA assay ($n = 3$; mean $\pm$ SD). Insert: the volume of the filtrate after centrifugation was measured by pipetting ($n = 3$; mean $\pm$ SD). The dotted line corresponds to the filtrate volume of pure water after centrifugation.

The performance of centrifugal filtration devices was unchanged—it neither improved nor degraded—when peptide or protein concentrations were decreased from 800 to 400 µg/mL and when the centrifugation rate was reduced to $7000\times g$, for membranes with a cut-off of 50 kDa (Figure 5) or 100 kDa (Figure 6). The same proportions of peptide and proteins in the filtrate were found independently of concentration and centrifugation rate. These conclusions were also found in the work of Johnsen et al., where the dilution of human plasma prior to passage through centrifugal filtration devices with a cut-off of 100 kDa did not improve their selectivity [9].

To summarize, the molar mass of biological molecules is in fact the main factor controlling their separation from a centrifugal filtration device, enabling excellent selectivity to be achieved using this separation system. The performance is even more important the further the molar mass is from the membrane cut-off.

We previously proved the selectivity of the centrifugal filtration devices when a single peptide or protein is used, but is this always the case with protein blends? Two protein mixtures were tested—a mix of LYS and BSA and a mix of LYS and EST—at various concentrations from 200 to 1000 µg/mL. For both mixtures, LYS had a molar mass below the membrane cut-off, i.e., 50 kDa, while BSA and EST had a molar mass above the membrane cut-off. We specifically chose these proteins since LYS passes almost entirely through 50 kDa membranes, while BSA and EST are almost completely retained (Figure 6). Protein proportions in the filtrate were titrated by SEC using the mixture without separation as references. As a control, the proteins alone (unmixed) were separated using the centrifugal filtration

devices, their proportions in the filtrate were assessed, and compared to the protein proportions in the filtrate when the mixes were separated. After separation of the first mixture, the proportion of BSA in the filtrate was close to 0%, the same as after filtration of the protein alone. However, only 20–35% of LYS, depending on the concentrations tested, was found in the filtrate after separation of the mixtures, whereas this protein was capable of crossing the membrane by almost 100% when unmixed (Figure 7A) ($p = 0.000039, 0.0028, 0.0001$ and 0.0004 for LYS/BSA 400/400, 600/600, 800/800 and 1000/1000 ($\mu\text{g/mL}/\mu\text{g/mL}$), respectively, comparing mixed and unmixed proteins). This phenomenon was also visualized with the second mixture. EST was retained on the membrane (depending on the concentrations tested, EST proportions between 0 and 15% passed through the membrane), and full amounts of LYS were not found in the filtrate (proportions between 60% and 80% depending on the concentrations tested) (Figure 7B) ($p = 0.0178, 0.0006, 0.0026$ and 0.0046 for LYS/EST 200/200, 400/400, 1000/200 and 1000/400 ($\mu\text{g/mL}/\mu\text{g/mL}$), respectively, comparing mixed and unmixed proteins). These results suggest that centrifugal filtration devices can specifically separate a mixture of two biological molecules according to their molar masses. However, the proportions of proteins in the filtrate whose molar masses are below the membrane cut-off may be influenced by the presence of proteins whose molar mass is above the membrane cut-off, making quantification incorrect. No interaction between the proteins was observed when the mixtures without separation were analyzed, which means that BSA and EST prevent the total passage of LYS through the membrane. Even if no change in the filtrate volumes was observed after the separation of the mixtures, we can assume the clogging of the membrane pore by the protein with a high molar mass, thus preventing proteins that could cross the membrane from passing.

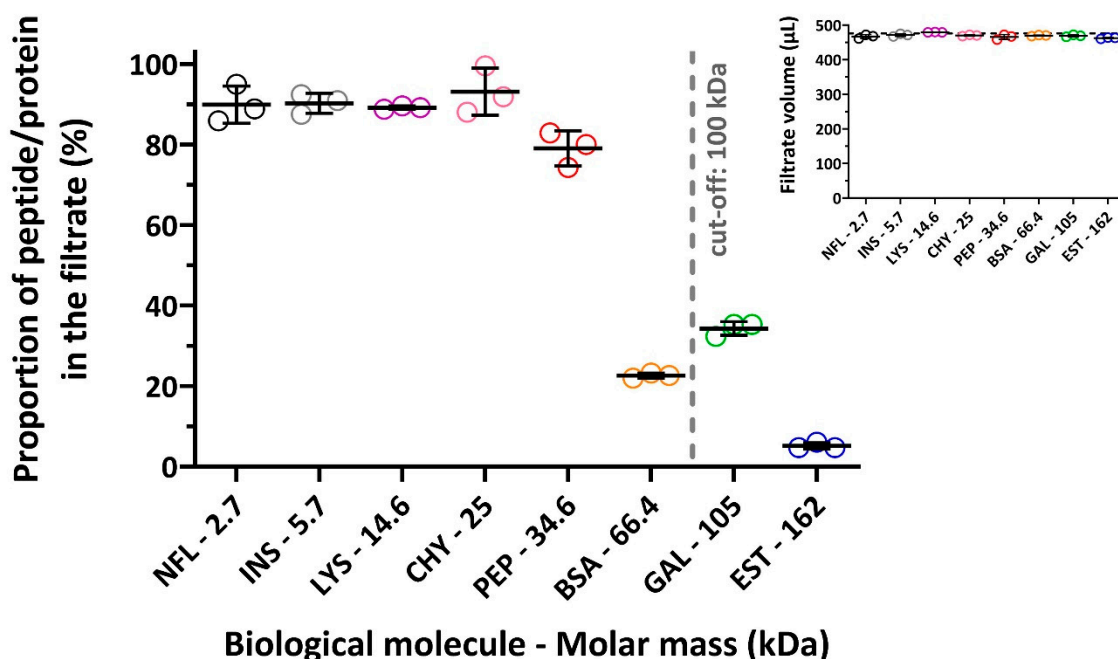


Figure 4. Separation of peptide or proteins with different molar masses confirms the performance of the centrifugal filtration system with 100 kDa membrane. Solutions (500 μL ; 1000 $\mu\text{g/mL}$) of biotinylated NFL-TBS.40-63 (NFL), bovine pancreas insulin (INS), lysozyme from chicken egg white (LYS), bovine pancreas α -chymotrypsin (CHY), pepsin (PEP), bovine serum albumin (BSA), β -galactosidase from *Aspergillus oryzae* (GAL) and porcine liver esterase (EST) were deposited on centrifugal filtration devices (cut-off of 50 kDa) and centrifuged at $14,000\times g$ during 30 min. The proportion of peptide/proteins in the filtrate was determined using SEC or BCA assay ($n = 3$; mean $\pm$ SD). Insert: the volume of the filtrate after centrifugation was measured by pipetting ($n = 3$; mean $\pm$ SD). The dotted line corresponds to the filtrate volume of pure water after centrifugation.

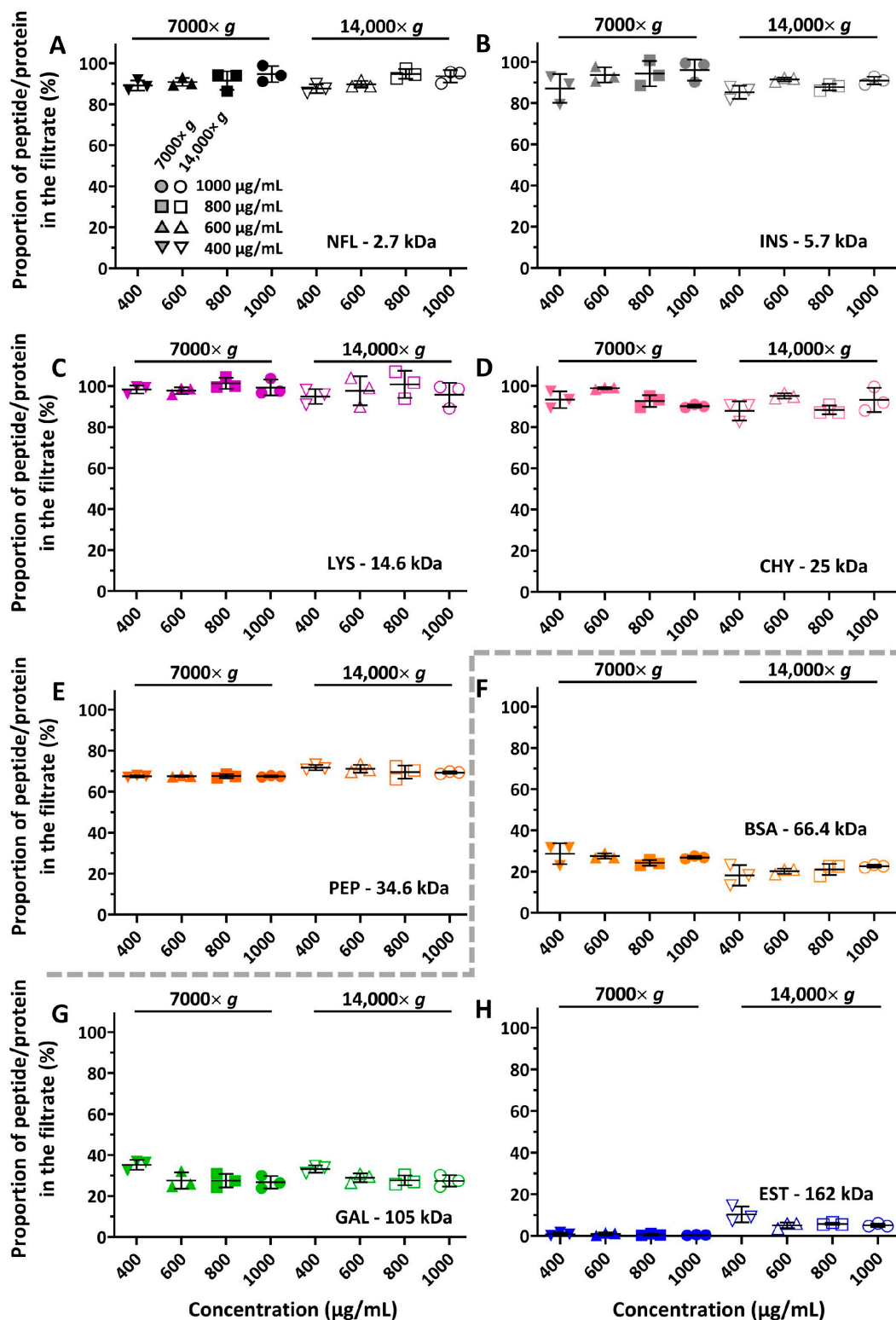


Figure 5. The centrifugation rate and the concentration of biological materials do not influence the separation of peptide or proteins with different molar masses, confirming the performance of the centrifugal filtration system with 50 Da membranes. Solutions (500 μ L; 400, 600, 800 or 1000 μ g/mL) of biotinylated NFL-TBS.40-63 (NFL) (A), bovine pancreas insulin (INS) (B), lysozyme from chicken egg white (LYS) (C), bovine pancreas α -chymotrypsin (CHY) (D), pepsin (PEP) (E), bovine serum albumin (BSA) (F), β -galactosidase from *Aspergillus oryzae* (GAL) (G) and porcine liver esterase (EST) (H) were deposited on centrifugal filtration devices (cut-off of 50 kDa) (grey dotted line) and centrifuged at 14,000 or 7000 $\times$ g during 30 min. The proportion of peptide/proteins in the filtrate was determined using SEC or BCA assay ($n = 3$ per concentration; mean $\pm$ SD).

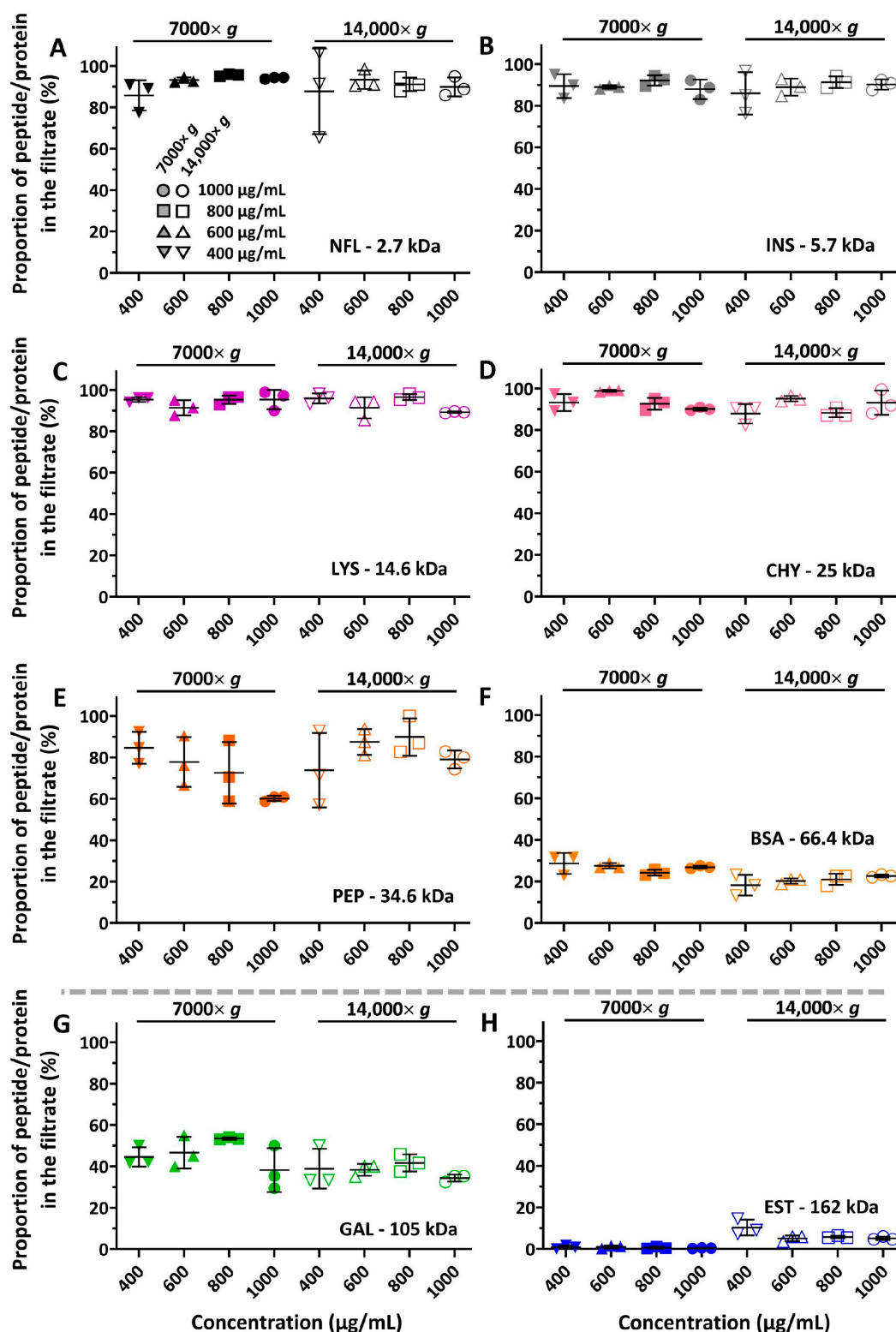


Figure 6. The centrifugation rate and the concentration of biological materials do not influence the separation of peptide or proteins with different molar masses, confirming the performance of the centrifugal filtration system with 100 kDa membranes. Solutions (500 μ L; 400, 600, 800 or 1000 μ g/mL) of biotinylated NFL-TBS.40-63 (NFL) (A), bovine pancreas insulin (INS) (B), lysozyme from chicken egg white (LYS) (C), bovine pancreas α -chymotrypsin (CHY) (D), pepsin (PEP) (E), bovine serum albumin (BSA) (F), β -galactosidase from *Aspergillus oryzae* (GAL) (G) and porcine liver esterase (EST) (H) were deposited on centrifugal filtration devices (cut-off of 100 kDa) (grey dotted line) and centrifuged at 14,000 or 7000 $\times$ g during 30 min. The proportion of peptide/proteins in the filtrate was determined using SEC or BCA assay ($n = 3$ per concentration; mean $\pm$ SD).

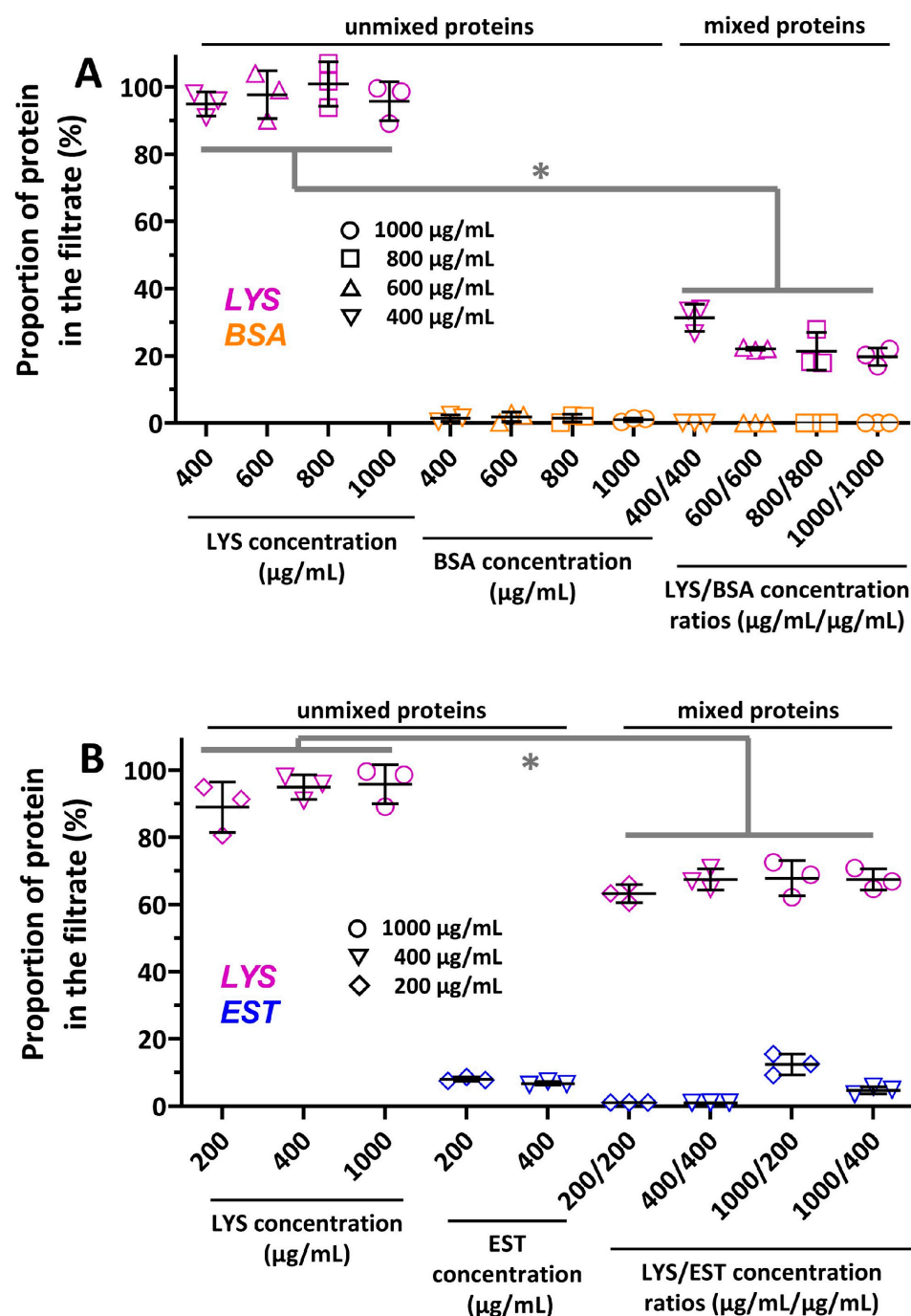


Figure 7. Separation of mixtures of proteins confirms the selectivity of the centrifugal filtration devices but with a poor quantification of the protein concentration after the separation. (A) Lysozyme from chicken egg white (LYS)/bovine serum albumin (BSA) mixtures in solution (500 μL ; 400/400, 600/600, 800/800 and 1000/1000 $\mu\text{g/mL}/\mu\text{g/mL}$) and unimixed protein at the same concentrations or (B) lysozyme from chicken egg white (LYS)/porcine liver esterase (EST) mixtures in solution (500 μL ; 200/200, 400/400, 1000/200 and 1000/400 $\mu\text{g/mL}/\mu\text{g/mL}$) and unimixed protein at the same concentrations were deposited on centrifugal filtration devices (cut-off of 50 kDa) and centrifuged at $14,000\times g$ during 30 min. The proportions of proteins in the filtrate for unimixed proteins and mixed proteins were determined using SEC method ($n = 3$; mean $\pm$ SD; * $p < 0.05$).

These conclusions corroborate other observations in which not all proteins able to pass through the membrane of the centrifugal filtration device were found in the filtrate after the separation of protein mixtures [9,20]. For example, Georgiou et al. centrifuged human plasma through another centrifugal filtration device (Centrex UF-0.5, Schleicher

& Schuell, Dassel, Germany) with a membrane cut-off of 30 kDa. Comparing proteins on a two-dimensional polyacrylamide gel before and after separation, they observed that proteins with a molar mass below the cut-off remained present in the retentate [20]. It is not only protein mixtures that can cause problems. Indeed, Gazaille et al. also showed the limits of the centrifugal filtration devices with nanoparticles and NFL peptide mixture. This tool had been used to quantify the peptide adsorbed at the surface of the nanoparticles, titrating the amount of non-adsorbed free peptide crossing the membrane. They established that the amount of free peptide was underestimated, due to nanoparticles clogging inhibiting the free peptide total crossing through the membrane during the centrifugation, leading to mis-quantification [19].

Is it possible to improve or optimize this separation and recover all of the proteins in the filtrate after using a centrifugal filtration device? One of the parameters that could have the most significant impact would certainly be the protein concentration of the mixture, if the samples can be diluted. Indeed, by reducing the concentration, one could imagine that the pores would no longer be totally clogged by the proteins retained in the retentate, thus allowing the proteins to pass completely through the membranes. The lower the concentration, the better the performance. On the other hand, it will then be necessary to quantify these low protein concentrations, certainly using methods requiring advanced techniques such as mass spectrometry, coupled or not with a chromatographic system, rather than the conventional methods used in our study (BCA or SEC and conventional UV detection). In any case, this latter technique will always be optimal as long as the protein mixture can be properly separated using the SEC column and quantified by adequate detector, without any other prior separation techniques [19].

4. Conclusions

This study demonstrated that centrifugal filtration devices have good selectivity for separating proteins of different molecular masses, irrespective of their concentrations (below 1000 µg/mL) and centrifugation rate. The charge of biological molecules does not seem to influence their retention; thus, molecular mass is the essential parameter for achieving a good separation. This selectivity is even more important the further the molar masses of the proteins are from the cut-off of the membranes used, especially for molar masses below the cut-off of the membranes. Nevertheless, the main limitation is quantification after the separation of a protein mixture. Indeed, when purifying protein mixtures, the proportions of proteins found in the filtrate do not correspond to the proportions found when separating proteins alone. A significant loss of proteins, trapped in the membrane due to the clogging of the proteins whose molar masses are above the membrane cut-off, has been observed. This lack of precision in quantification can be problematic, particularly in the case of encapsulation/adsorption of biological material in/on nano-systems. This is why we need to be very careful when using centrifugal filtration devices, a technique that is being increasingly used in the field of nanomedicine.

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Abbreviations

ACN	Acetonitrile
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CHY	Bovine pancreas α -chymotrypsin
DIEA	Diisopropylethylamine
DMF	Dimethylformamide
EST	Porcine liver esterase
FA	Formic acid
Fmoc-Arg-OH	N-(9-Fluorenylmethoxycarbonyl)-arginine
Fmoc-Ala-OH	N-(9-Fluorenylmethoxycarbonyl)-alanine
Fmoc-Glu-OH	N-(9-Fluorenylmethoxycarbonyl)-glutamic acid
GAL	β -galactosidase from <i>Aspergillus oryzae</i>
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
INS	Bovine pancreas insulin
LYS	Lysozyme from chicken egg white
MeOH	Methanol
NaCl	Sodium chloride
NFL	Biotinylated NFL-TBS.40-63 peptide
pArg ₇	Polyarginine
pAla ₇	Polyalanine
pGlu ₇	Polyglutamic acid
PEP	Pepsin
SEC	Size exclusion chromatography
TFA	Trifluoroacetic acid
TIS	Thioanisole triisopropyl silane
UPLC	Ultra-high-performance liquid chromatography

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