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Biological protein precipitation: A green process for the extraction of cucumisin from melon (*Cucumis melo* L. *inodorus)* by-products

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ABSTRACT

Cucumisin (CUC) from industrial melon by-products was separated for the first time through biological precipitation using carrageenan (CRG). This approach could represent a cost-effective and environmentally friendly process for the industries, avoiding the use of expensive equipment and toxic salts or solvents, such as butanol and ethanol. In this study, biological precipitation of proteins from melon by-products using CRG was studied and compared with conventional precipitation with ammonium sulphate. Different methods were applied for the identification and characterization of isolated proteins, including SDS-PAGE gel, FPLC and proteolytic activity assays. The isolated CUC confirmed a molecular weight of 68 kDa and showed highly stable proteolytic (PA) and milk-clotting (MCA) activities in a wide range of $CaCl_2$ (20–60 mM), pH (5–7) and temperatures (30–85 °C). Melon peel extract demonstrated to possess significant PA (4.24 U/mg protein) and MCA (191.50 MCU/mg protein), but such values were increased by ammonium sulphate precipitation (1.60 and 2.06-folds, respectively), and specially a noticeable increment was observed by biological precipitation with 2.11 and 17.65-folds, respectively, demonstrating the capability to be an effective strategy to isolate and purify CUC, allowing a yield of 0.17 g CUC/100 g of by-products and keeping its biological properties.

1. Introduction

Globally, 1.3 billon tonnes of food by-products are generated mainly by the food producers and processing industries with an estimated economic loss of 990 billion dollars. In 2017, Europe generated 96 million metric tonnes of fruits and vegetables, corresponding to 8.5% of the global production and around 30% of such production has been rejected as by-products (Trigo J. P. et al., 2019; Campos D. A. et al., 2020). These by-products are poorly managed and discarded in landfills, causing environmental pollution and economic issues (Yousuf, Bonk, & Schmidt, 2016). In this context, melon fruit (*Cucumis melo* L.) is one of many examples related with fruit by-product generation, due to its high industrialization, producing great number of food products, such as juices, ready to eat salads, snacks, among other products. These industrial activities lead to an overproduction of melon by-products mainly seeds and peels, with around 8 to 20 million tonnes of melon by-products per year, which despite on their richness of nutritional and bio-functional compounds are still mismanaged or ignored (Rolim, Seabra, & de Macedo, 2019; Gómez-García, R. et al., 2020).

Currently, food by-products management has been recognized by the circular economy as one of the principal keys to reduce environmental and economic problems. Based on this fact, during the last decade biotechnological efforts have increased, focusing on the valorisation of food by-products, since they have been well-reported to keep high concentration of value-added biomolecules, such as proteolytic enzymes. In this regard, cucumisin (CUC) is an extracellular serine protease with molecular weight of 67 kDa, already purified from different Cucurbitaceae plants and characterized as plant subtilisin (Murayama et al., 2012). Serine proteases are one of the largest groups of proteolytic enzymes involved in several biological processes. Moreover, CUC is well-recognized as milk-clotting enzyme. Such enzymes are used in cheese making for promotion and regulation of milk coagulation properties (Ben Amira, A., Besbes, S., Attia, H., & Blecker, 2017). Based on its biological properties, many studies tested different techniques to isolate and extract this enzyme from melon juice. Traditional methods include precipitation with ammonium sulphate, ultrafiltration or gel and affinity

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chromatography, however such techniques have some disadvantages related with toxicity, high implementation costs and difficult to scale up (Galanakis, 2012). Biological precipitation using natural polyelectrolytes is a well-documented extractive method, which represent a non-toxic, low cost and efficient green-technological process for protein extraction which release proteins conserving its structure and biological activity (Woitovich Valetti, Brassesco, & Picó, 2016). Polyelectrolytes interact with proteins in solution arising soluble or non-soluble complex by the modification of pH or ionic strength, forming a semisolid (gel) mixture between proteins and polyelectrolyte, which then is precipitated by centrifugation (Campos D. A. et al., 2017). Carrageenan (CRG) is a natural polyelectrolyte obtained from certain species of red seaweed and is considered non-toxic and a water-soluble compound. This compound is well accepted in cosmetic, pharmaceutic and food industries. Recently, researchers from our group, successfully used CRG as precipitant agent to isolate and extract bromelain enzyme from pineapple by-products (Campos D. A. et al., 2019). Hence, the present research work tested this polyelectrolyte with CUC, since there are no studies regarding the molecular mechanism of complex formation between both compounds, and it is a good opportunity to develop a green process for precipitation of this enzyme.

2. Materials and methods

2.1. Chemicals

Azocasein, iota (ι)-carrageenan and all the other reagents of analytical grade were purchase from Sigma–Aldrich (St. Louis, Missouri, USA). Bradford Bio-Rad assay from Biorad (Hercules, CA, USA). Skim milk was obtained from NILAC™ (NIZO, Ede, The Netherlands).

2.2. Raw material - melon peels

Melon fruits (*Cucumis melo* L. *inodorus*) were harvested (ripening stage was not controlled) from the Alentejo region in Portugal and processed by Nuvi Fruits S. A. Company Torres Vedras (Portugal), during the season of summer in August–September 2018. Fresh melon peels were generated as by-products and then transported to our facilities (Centro de Biotecnologia e Química Fina - CBQF) at −20 °C. The peels were kept at −20 °C until their processing and pretreatment.

2.3. Melon peels juice (MPJ) preparation

Melon peels were processed as described previously by Gómez-García et al. (2021) and split in two fractions, fresh solid fraction (SF) and liquid fraction (LF), employing a commercial juice machine (HR1869/8, 900 W, Philips). The SF was manually pressed to recover the liquid excess, such liquid was mixed with the LF, which was subsequently centrifuged (11469 *g*, 15 min, 4 °C), then the clarified supernatant was called as melon peels juice (MPJ). The MPJ was collected and kept at −20 °C until its analysis.

2.4. Protein precipitation with ammonium sulphate

Traditional salt precipitation with ammonium sulphate was applied to precipitate melon protein fraction from MPJ. The precipitation was carried out by saturation of 200 mL of MPJ with ammonium sulphate (60% w/v). MPJ was kept in constant stirring when the salt was added carefully, after total addition of salt, the mixture was kept in agitation during 1 h. Later, the mixture was subjected to centrifugation (11469 *g*, 15 min, 4 °C) and the pellet obtained was solubilized in sodium acetate buffer (0.1 M, pH 4.6) and dialyzed overnight against the same buffer (buffer was changed three times) using 14 kDa MWCO membrane. The clear mixture was freeze-dried and stored until its analysis. This fraction was called as MPJ-(NH_4) (melon peels juice precipitated with ammonium sulphate).

2.5. SDS-PAGE electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using discontinuous gel, 12% (w/v) polyacrylamide for resolution gel and 6% (w/v) polyacrylamide for a stacking gel. Samples were analyzed employing a vertical system with running conditions for resolving gel established at 25 mA of intensity during 120 min. A standard mixture of proteins with a known molecular weight (precision plus protein dual color) obtained from Bio-Rad Laboratories (Hercules, CA, USA) was used for protein size estimation. Proteins were stained with Coomassie brilliant blue (0.25 %w/v). Protein patterns were than visualized after washing the gel with a distaining solution of 2.5:1 (v/v) acetic acid and methanol from Frilabo (Maia, Portugal), until protein bands became clearly visible in the colorless gel matrix.

2.6. Analysis by size exclusion chromatography (FPLC)

Protein profile was analyzed by gel filtration chromatography using the following conditions: the column was operated at a flow of 0.5 mL/min with phosphate buffer (0.025 M, pH 7) containing NaCl (0.15 M) and NaN_3 (0.02% w/v). Protein standards with known molecular weights (Thyroglobulin, 669 kDa; Ferritin, 440 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa; Ribonuclease A, 13.7 kDa; Aprotinin, 6.5 kDa) were used to elaborate a calibration curve. AKTA pure 25 L system, from GE Healthcare Life Sciences (Freiburg, Germany), was used with a configuration of two pumps with pressure control for column protection, a gel filtration column prepacked with Superdex® 200 10/300 GL connected in series with a column Superdex Peptide 10/300 GL (GE Healthcare Life Sciences, Freiburg, Germany) and an UV multiwavelength detection monitor U9-L, at a fixed wavelength of 280 nm. The software used to evaluate samples was UNICORN 7.0.

2.7. Turbidimetric titration curves vs pH

Turbidity of samples was measured at 420 nm. MPJ (10 mL) was mixture with phosphate buffer (8 mL, 0.1 M, pH 6) and CRG (at 0.001% (w/v) dissolved in ultrapure water). Medium pH modifications from 5.3 to 2 were performed by adding aliquots of HCl (0.1 M) and 5.3 to 12 with NaOH (0.1 M), leaving the system to equilibrate during 90 s before measuring the turbidity. These titration curves were made in order to estimate the pH range where the polyelectrolyte-protein complex was soluble or non-soluble (Valetti, Boeris, & Picó, 2013).

2.8. Turbidimetric titration curves with CRG

Formation of the CUC-CRG non-soluble complex was evaluated by the increment of turbidimetry in the solution. Briefly, 10 mL of MPJ were adjusted at pH 3 and titrated by adding successive aliquots (30 μL) of CRG in solution (1 mg/mL) and the absorbance was recorded at 420 nm in a spectrophotometer (UV mini 1240, Shimadzu, Tokyo, Japan) at room temperature. To avoid changes in pH value during titration, both MPJ and CRG solutions were adjusted to pH 3, corresponding to the highest absorbance obtained by the complexation of CRG with proteins (section 2.5). The total CRG concentration on the mixture was plotted against the absorbance. The complex formation was followed in the absence and presence of different ionic strengths, adding NaCl to the medium.

2.9. Protein content determination

Total protein content (TPC) was determined by the Bradford (1976) assay with a slight modification. Briefly, 0.05 mL of each sample was combined with 0.950 mL of Bradford regent at room temperature, the reaction was vortex mixed and then kept in darkness during 20 min. The

absorbance was measured at 595 nm and the protein concentration was quantified with a calibration curve using bovine serum albumin (BSA) as standard. Results were expressed as mg BSA/mL. All the measurement was made in triplicated.

2.10. Proteolytic activity determination

Proteolytic activity (PA) was determined as described by Biondi, Maria, Paiva, Lúcia, and Vieira (2003), using azocasein as substrate with slight modifications. Azocasein (1% w/v) was dissolved in Tris-HCl 0.2 M buffer in different ranges of pH (5, 6.5, 8, 9). In triplicate, the reaction was carried out with 0.1 mL of substrate mixed with 0.060 mL of sample at 37 °C in darkness during 1 h. Later, reaction was stopped by adding 0.480 mL of trichloroacetic acid (TCA) (10% w/v) and the precipitated protein was removed by centrifugation (16469 *g*, 5 min, 4 °C). A volume of 0.320 mL from the clear supernatant was mixed with 0.560 mL of sodium hydroxide (1 M) and the absorbance was measured at 440 nm. A blank was created by mixing TCA to the substrate prior to the sample addition. One unit (U) of enzyme activity was defined as the amount of enzyme able to hydrolyze azocasein, resulting in an increase of 0.001 units of absorbance per minute. The proteolytic recovery yield was defined according to equation (1):

$$Yield = \frac{PA_f}{PA_i} \quad (1)$$

Where PA_f is the proteolytic activity after the precipitation and PA_i is the initial proteolytic activity of MPJ. The purification factor (PF) was defined as (Equation (2)):

$$PF = \frac{SPA_f}{SPA_i} \quad (2)$$

Where the SPA_f is the specific proteolytic activity after precipitation and the SPA_i is the specific proteolytic activity in the MPJ. The SPA was defined as (equation (3)):

$$SPA = \frac{PA}{TPC} \quad (3)$$

Where the TPC is the total protein content.

2.11. Milk-clotting activity

Milk-Clotting Activity (MCA) was determined by triplicated following the procedure described by Arima, Yu, and Iwasaki (1970). Low-heat skim milk powder was reconstituted (10% w/v) in 10 mM aqueous $CaCl_2$ (pH 6.5). Enzyme extract was added a volume rate of 0.1 mL per mL of a pre-incubated milk (10 min, 37 °C). Test tubes containing the reaction were gently rotated by hand (at short time intervals) and time taken for solid clot formation was recorded. One Milk-Clotting Unit (MCU) was defined as the amount of protein that clots 10 mL of reconstituted milk at 37 °C within 40 min (2400 s). The following formula (4) was employed:

$$MCA = \frac{MCU}{mL} = \frac{(2400)^*(V)}{(t)^*(v)} \quad (4)$$

Where "V" is the volume of milk (mL), "v" the volume of enzyme (mL), "t" the clotting time taken in seconds. The MCA/PA is the ratio between specific milk-clotting activity (SMCA) and specific proteolytic activity (SPA). The effect of different concentrations of $CaCl_2$ on MCA was determined using a milk solution with varying concentration of $CaCl_2$ (0–60 mM). Additionally, MCA was tested by using different temperatures ranging from 30 to 85 °C and pH ranges from 5 to 7.

3. Results and discussions

3.1. SDS–PAGE analysis and FPLC profile

The protein profile by SDS-PAGE (Fig. 1a) and FPLC (Fig. 1b) of MPJ confirmed the presence of native proteins and homogeneity. SDS-PAGE shows 5 main bands of proteins. One protein is identified with an estimated molecular weight (MW) of 68 kDa, which was in agreement with the well-characterized cucumisin identified in *Cucumis melo* (Sotokawauchi et al., 2016). Also with the range of cucumisin-like serine proteases from different plant sources (60–80 kDa), including *Ficus religiosa* (Sharma, Kumari, & Jagannadham, 2012), *Cucumis rigonus* Roxburghi (Asif-Ullah, Kim, & Yu, 2006) and *Euphorbia supina* (Arima Kazumari at al., 2000). Moreover, proteins with MW of 54 kDa protease and ca. 17 kDa polypeptide were identified in the MPJ. Both bands may be originated from the autolysis products of original cucumisin (Nakagawa et al., 2010). Also, two other bands were identified with 25 and 27 kDa of MW which have been reported as proteins without relevant proteolytic activity and correspondent to fragments or contaminants of the native enzyme (Gagaoua, M et al., 2017). Additionally, through precipitation with ammonium sulphate was possible to concentrate and identify a protein with molecular size around 60 kDa (Fig. 1c), indicating that melon peel by-products keep considerable amount of proteins with potential biological activity.

3.2. Solubility phase diagram of CRG and MPJ proteins

Proteins precipitation from melon by-products was investigated through complex formation with commercial CRG as function of pH and ionic strength. The complex formation showed to be strongly influenced by the modification of pH medium. When the pH decreased below the initial pH 5.3 in the MPJ, the absorbance increased, showing a high turbidity at pH 3 (Fig. 2a). This behavior could be attributed due to the presence of proteins, such as CUC with an isoelectric pH of 8.7, which is positively charged in acid pH (Gagaoua, M., et al., 2017). This protein interacts directly with the negative electric charges of CRG sulphonic groups, allowing the complex formation (CUC-CRG), which is a semi-solid (gel) mixture. The turbidity decreased at higher values than pH 5.3 because the positive electric charge of CUC also decreased progressively, making difficult the interaction with CRG and therefore minor complex formation. Fig. 2b presents the variation of the absorbance at 420 nm when MJP was present at pH 3 and tested against different ionic strength. The absorbance values increased until constant value (plateau) representing a higher extent of complex formation. It was possible to calculate the minimal amount of CRG to precipitate melon peel proteins, which corresponds to the case in which most proteins have been precipitated as an insoluble complex. The stoichiometric ratios of proteins/CRG were 3 and 1.65 mg of proteins/mg CRG, were two CRG concentrations were predicted by the maximum absorbances given. These values correspond to the minimal concentrations needed to precipitate CUC, 0.0033% (C1) and 0.006% (C2) (w/v) respectively, allowing the partial precipitation of CUC from melon by-products. On the other hand, the salt added to the medium helps to break the non-soluble complex, releasing the proteins from CRG by increasing the solubility of the complex through the electrostatic interaction between proteins and Na^+ and Cl^-. This helps to improve the repulsive electrostatic forces between proteins and CRG, resulting in a salting effect as observed in the reduction of turbidity, being almost null at 500 mM NaCl (Fig. 2b). The interaction between CUC positively charged and CRG negatively charged appears to be natural coulombic attraction. These results obtained by this methodology showed that very low concentration of CRG was needed to precipitate proteins, compared with different reports employing time consuming processes or toxic salts, demonstrating great potentiality for its application as green extractive technology.

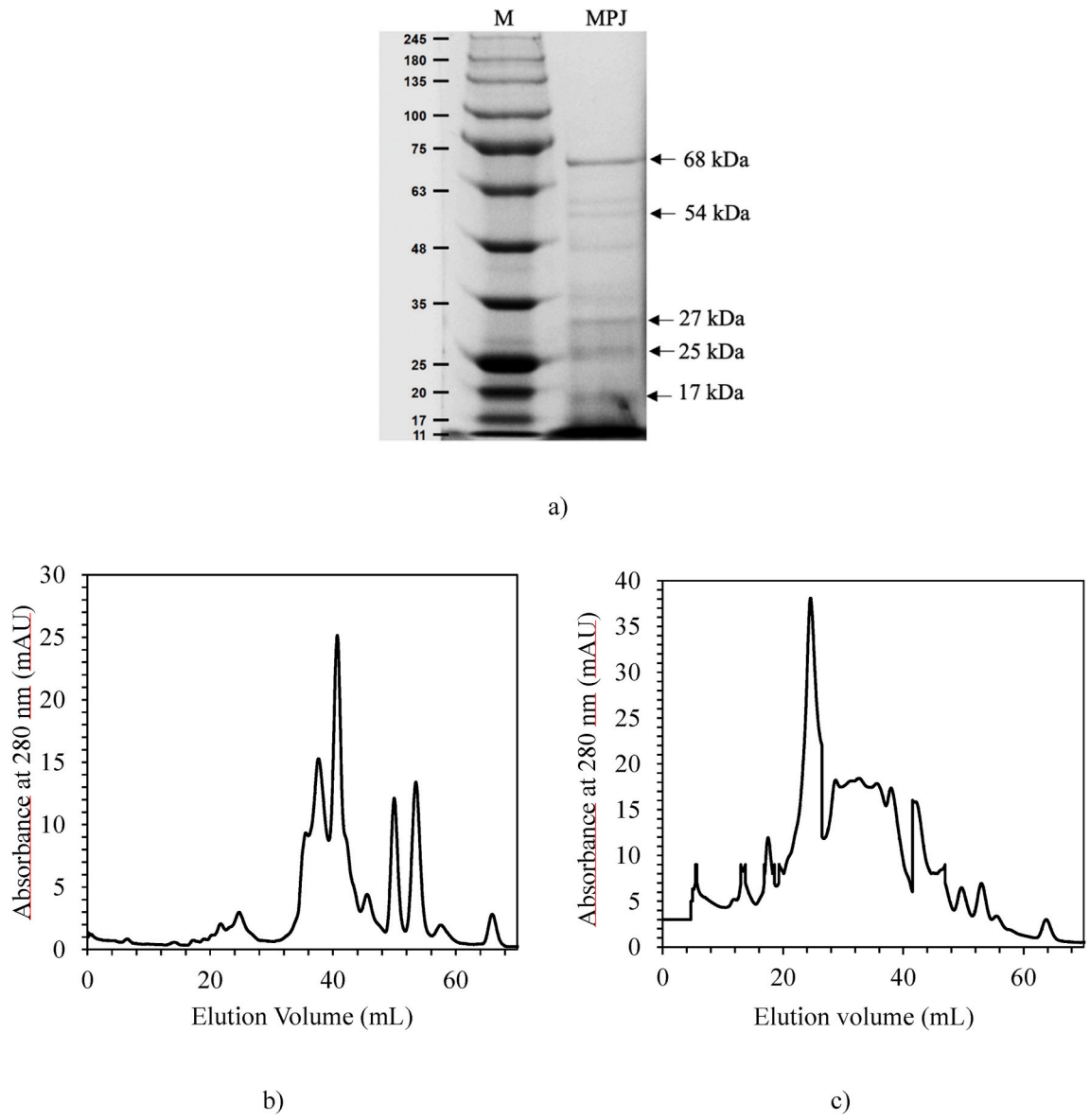

Fig. 1. a) Electrophoresis gel by SDS-PAGE from *Cucumis melo* L. *inodorus* peels and protein molecular size profile by FPLC of b) melon peel juice (MPJ) and c) proteins precipitated with ammonium sulphate MPJ-(NH_4). M: molecular weight standards.

3.3. Effect of CRG on MPJ proteins

The MPJ was subjected to biological precipitation using the two predicted CRG concentrations (C1 and C2). The non-soluble complex (COM) was separated from the supernatant (SUP) by centrifugation and re-dissolved in 0.5 Tris-HCl buffer (adding, 0.5 M NaCl at pH 8.2), then the COM was again centrifuged to eliminate the excess of CRG. The COM and SUP both filtered through 0.45 μm (Orange Scientific, Braine-l'Alleud, Belgium) were analyzed by FPLC to observe the protein behaviour in contact with the CRG. Fig. 3a presents the protein profile at 0.003% (w/v) and clearly shows that the CRG was able to precipitate proteins between 60 and 25 kDa (peaks at 22 and 32 mL). This behaviour is probably due to the functional groups responsible for the anionic character of the polyelectrolyte, which consists of sulphated galactans, the sulphate moieties make it a strong anionic molecule that can attract positively charged proteins (Fabian, Huynh, & Ju, 2010). Several studies reported the use of polyelectrolytes at low concentration as the best concentration to precipitate proteins instead using high concentration, since the increment of polyelectrolyte concentration in the medium could lead to an interaction between the same molecules, avoiding the non-soluble complex formation (Lombardi, Valetti, Picó, & Boeris, 2013). Such effect can be observed at 0.006% (w/v) where most of the high MW proteins remain in SUP (peaks at 22 and 32 mL) and shows one peak in complex at 24 mL, corresponding to 60 kDa protein (Fig. 3b). Previous reports have demonstrated that high concentration of polyelectrolyte influence and induce a modification of the secondary and tertiary protein structure, which could lead a loss of enzyme activity (Woitovich Valetti, Lombardi, Boeris, & Picó, 2012). Despite these effects, using low concentration of CRG (C1: 0.003% w/v) seems an appropriated concentration to be employed in protein extraction from melon by-products.

3.4. Complex formation phase diagrams of CRG and MPJ proteins

Proteins precipitation from MPJ was studied at pH 3 increasing the concentration of CRG. Non-soluble complex was handled as the same conditions described above. The TPC, PA and MCA were determined in complex (COM) and supernatant (SUP). Fig. 4a shows that the concentration of non-precipitated protein in the supernatant decreased as the same time as the complex was formed, demonstrating the capacity of

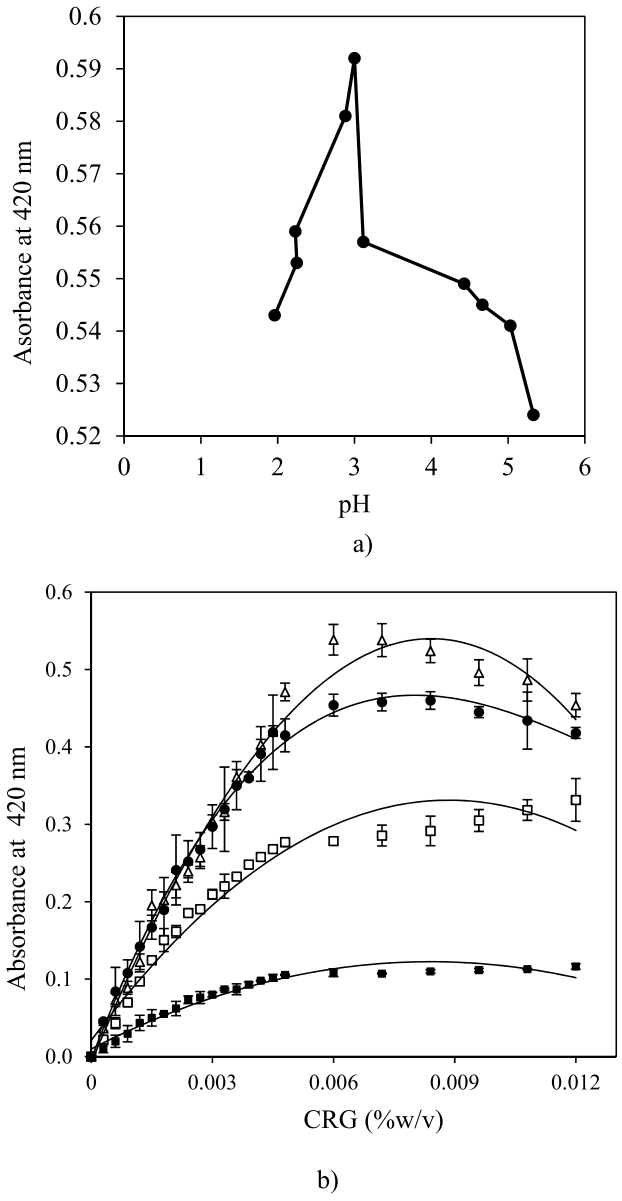

Fig. 2. a) Turbidimetric evaluation of CUC-CRG non-soluble complex and b) Titration of CUC from melon peels juice with increasing concentration of CRG at different constant concentration of NaCl (Δ 0, • 200, □ 400 and ■500 mM). All determinations were carried out in triplicated and mean value ± standard deviation.

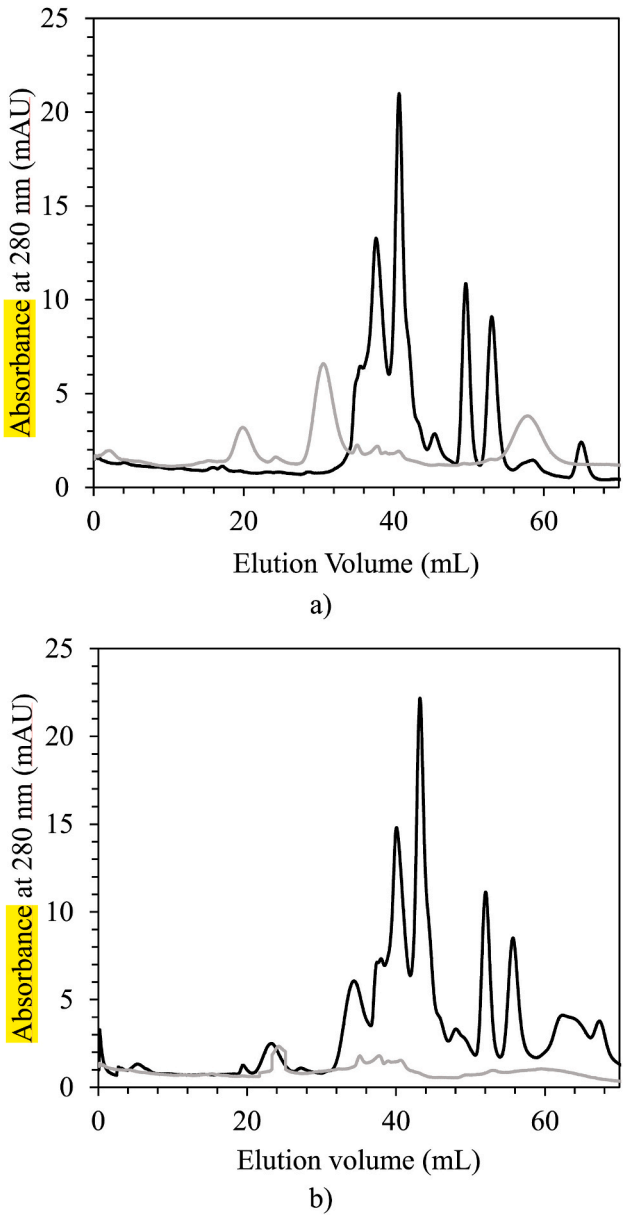

Fig. 3. Size exclusion chromatograms from FPLC of MPJ precipitated with CRG at a) 0.003% and b) 0.006% (w/v). COM: complex (gray line); SUP: supernatant (black line).

CRG to precipitate proteins from MPJ. Moreover, the PA reached a yield of 90% in the COM, while in the SUP was around 35% with total CRG concentration of 0.015% w/v (Fig. 4b). On the other hand, COM presented stable clotting activity when low concentration of CRG (C1: 0.003% w/v) was used, while at higher concentrations than 0.004% (w/v) (data not shown) no clotting activity was detected. Therefore, CRG was removed by centrifugation and the recovery value of the MCA was around 60% in the COM, while for the SUP the enzymatic activity decreased until 12% at higher CRG concentrations (Fig. 4c). This effect was similarly reported by different authors, describing a negative effect by excessive concentration of polyelectrolyte in the system, which could promote unwanted reactions or possible protein inactivation (Boeris, Spelzini, Farruggia, & Picó, 2009). Overall, the behaviors observed for TPC, PA and MCA confirmed that the equilibrium was displaced to a large extent to the insoluble complex formation. These effects could be attributed by the mechanism of complexation with proteins, which has been well-demonstrated that occurs in two steps: first, the formation of a primary link between protein-polyelectrolyte, which is soluble; and second the interaction of soluble complex molecules among them to form bigger and non-soluble composite (Valetti et al., 2013). Moreover, it is important to mention that this separation technique is highly recommended to be firstly tested on pure/standard enzymes to understand the complexation behavior, since undesirable interactions may occur with other macromolecules with similar isoelectric charge as the target protein on the homogenous samples, thus, avoid such negative interactions are the main challenge to overcome in the recovery of the target enzymes in complex mixtures. However, pure CUC has presented several limitations associated with its availability on the market and its extraction, making difficult its obtainment. Therefore, the present work highlights a selective and efficient separation of proteins from melon peels through precipitation with CRG.

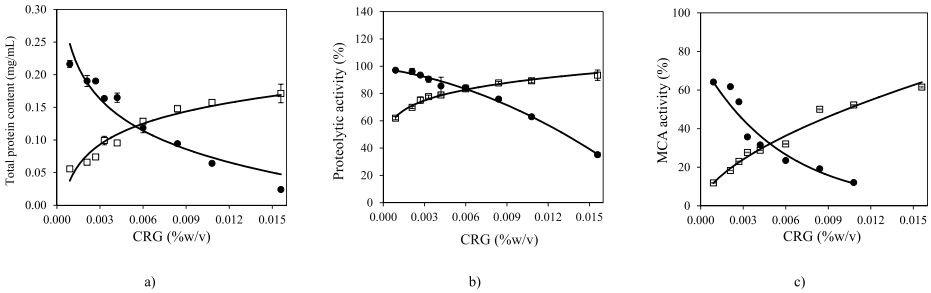

Fig. 4. a) Total protein content, b) recovery of proteolytic activity and c) milk-clotting activity measured in the complex (□ COM) and in the supernatant (• SUP) at different initial concentration of CRG. Proteolytic activity was measured using azocasein 1% w/v; MCA; milk-clotting activity was measured at constant temperature (37 °C) and pH (6.5). All determinations were carried out in triplicated and mean value ± standard deviation.

3.5. Enzymatic activities: PA and MCA

Based on the results obtained through the biological precipitation process in section 3.3 and 3.4, the lower concentration (C1) of CRG was selected to continue with the enzymatic evaluations, due to such C1 was able to preserve both enzymatic activities (PA and MCA). Fig. 5 shows the results of PA obtained from the evaluation using azocasein at different pH values. The specific activity was favored, preserved and increased progressively increasing pH. According to the previous reports of subtilisin/cucumisin serine proteases, they were more active in alkaline ranges of pH (8–9) than in acid ranges (Gagaoua, M et al., 2019). However, the best conditions (Table 1) were obtained from the complex at pH 6.5 with 74.86 U/mg protein of PA and 405 MCU/mg protein of MCA. These were 17.65 and 2.11-folds higher than the maximum initial PA (4.24 U/mg protein) and MAC (192.50 MCU/mg protein) of MPJ, respectively. Although, through traditional precipitation the results of PA (6.78 U/mg protein) and MCA (389.33 MCU/mg protein) were similar to those obtained in MPJ, biological precipitation showed better results regarding time saving, less volumes usage and the null use of toxic chemicals, as well as better purification factors. To the best of our knowledge, this was the first study related with the extraction of CUC from melon peel by-products applying polyelectrolytes. Hence, these results indicated that melon by-products still keep attractive concentration of proteins, which can be effective extracted by green precipitation keeping their biological activity.

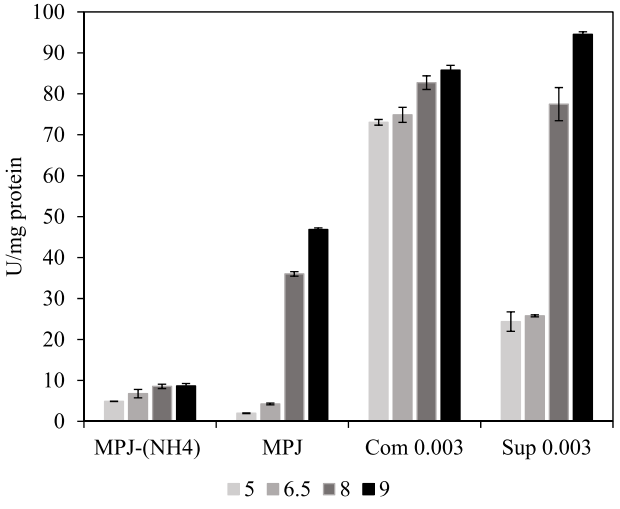

Fig. 5. Specific proteolytic activity of melon samples at different range of pH using azocasein (1% w/v) as substrate. MPJ: melon peels juice; MPJ-(NH4): melon peels juice precipitated with ammonium sulphate; COM 0.003: complex at 0.003% w/v of CRG. All determinations were carried out in triplicated and mean value ± standard deviation.

Table 1
Protein purification from melon peels.

	MPJ	MPJ-(NH4)	COM 0.003
Total protein content (mg/mL)	0.202 ± 0.0	1.96 ± 0.1	0.089 ± 0.0
PA (U/mL)	0.87 ± 0.0	13.30 ± 0.4	6.66 ± 0.4
Specific PA (U/mg protein)	4.24 ± 0.2	6.78 ± 1.0	74.86 ± 1.8
Fold purification of PA		1.60 ± 0.6	17.65 ± 1.3
MCA (MCU/mL)	38.63 ± 0.6	764.29 ± 86.6	36.10 ± 0.1
Specific MCA (MCU/mg protein)	191.50 ± 1.0	389.33 ± 33.7	405.28 ± 3.3
Fold purification of MCA		2.03 ± 0.9	2.11 ± 0.3

MPJ: melon peels juice; MPJ-(NH4): melon peels juice precipitated with ammonium sulphate; COM 0.003: complex at 0.003% w/v of CRG. PA: proteolytic activity; MCA: milk-clotting activity; MCU: milk-clotting units. The values are means of three independent measurements.

3.6. Effect of $CaCl_2$, temperature and pH on MCA

The effects of $CaCl_2$, pH and temperature on MCA were studied using the MPJ and COM at 0.003% (w/v). The results show that milk-clotting time is dependent on these parameters (Fig. 6). Commonly, $CaCl_2$ is added to milk to improve the milk coagulation in cheese manufacture. Thus, the effect of $CaCl_2$ on milk-clotting time is given in Fig. 6a where in both cases the clotting time decreased, which means that clotting activity increased by increasing the concentration of salt, where COM presented the highest MCA (1300 MCU/mg protein) at 60 mM (Fig. 6b). As observed, clotting time of COM decreased by increasing $CaCl_2$ concentration until a plateau is reached between 30 and 60 mM of salt. Thus, the enzymatic activity was progressively increased, and this behavior could be attributed to the native structures of serine proteases, which are stabilized by calcium ions. According to Hedayati & Ani, 2015, the calcium ions do not interfere in the binding and catalytic sites of proteins, providing additional electrostatic binding with anionic bonds of the enzyme. On the other hand, by increasing pH values from 5 to 7 (Fig. 6c and d), the clotting time increased because of the reduction of the enzymatic activity, in which MPJ had higher MCA (880 MCU/mg protein) than COM (490 MCU/mg protein) at pH 5 while under this value and above pH 7 were not showed coagulation activity. The effect at lower pH could be attributed by the disorganization of caseins micelles structural properties, since caseins are near to their isoelectric point (pH 4.6). This decreases the net charge, and therefore, decreases the electrostatic repulsion between charged groups, leading to milk clots agglomeration and enzymatic action (Ranadheera et al., 2019). Previous reports on MCA from plant proteases *(Wrightia tinctorial, Ficus carica, Colatropis procera)* also exhibited activity over a wide range of pH (Rajagopalan & Sukumaran, 2018). Fig. 4e shows the progression of milk-clotting time between 30 and 85 °C. This parameter showed that the clotting time decreased as function of increased temperature. 1 mL of milk was coagulated with 0.1 mL of MPJ or COM within 5–55 min with

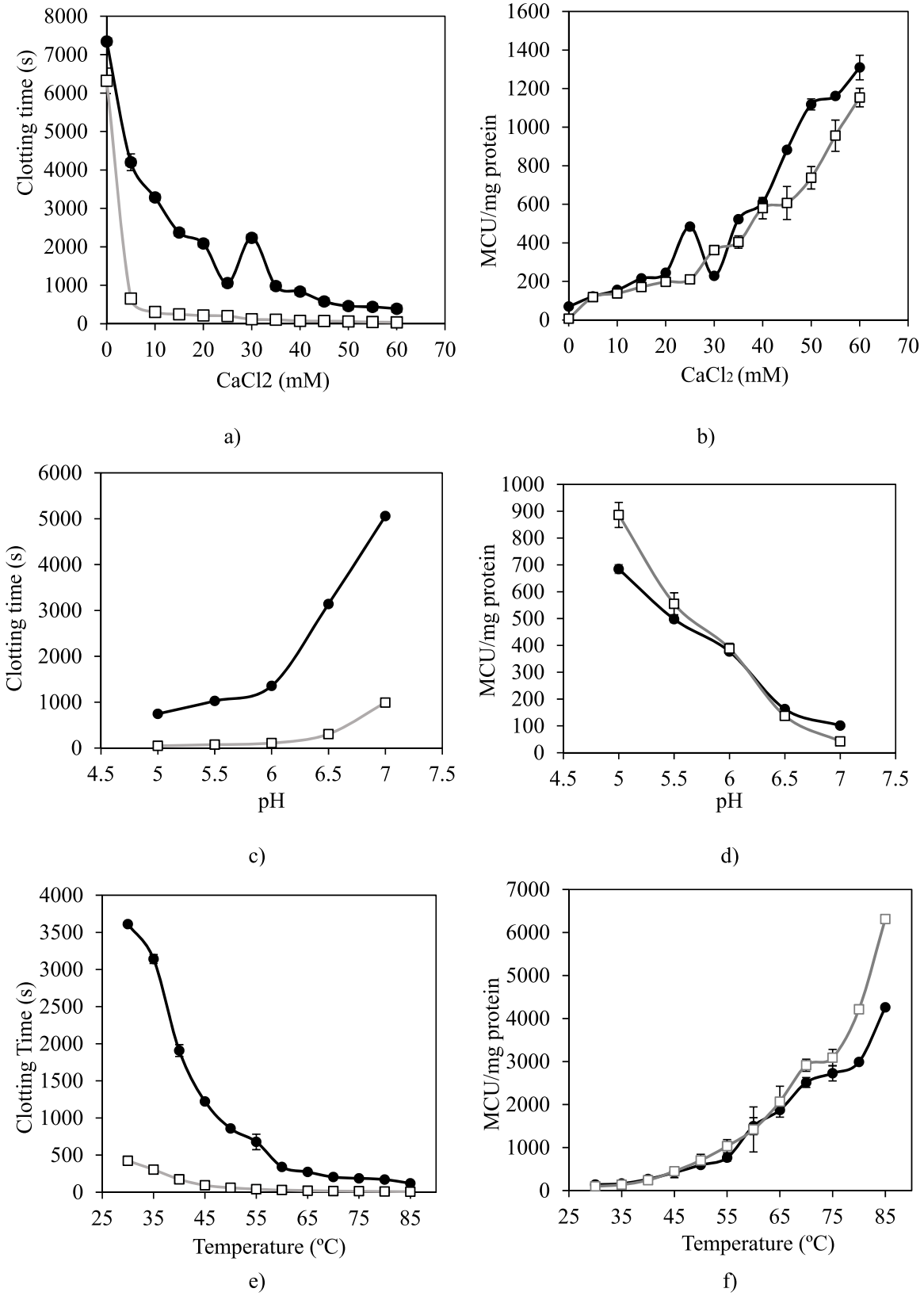

Fig. 6. Effect of $CaCl_2$, pH and temperature on milk-cotting acivity of the COM 0.003 (•) and MPJ (□). a) and b) Effect of $CaCl_2$ concentration, c) and d) pH and e) and f) temperature. Data are average of three independient experiments ± standard deviation of determined clotting activity.

an acceptable coagulation time (less than 90 min). The ample range of temperatures showed the high thermal stability of both MPJ and COM, producing the best MCA values at 85 °C with titles of 6300 and 4200 MCU/mg protein, respectively. These results are in concordance with the reported for milk-clotting proteases from *Citrus aurantium* L., *Wrightia tinctoria* and *Morinda citrifolia* L. at temperatures of 35–80 °C (de Farias et al., 2020; Mazorra-Manzano et al., 2013; Rajagopalan & Sukumaran, 2018). To the best of our knowledge, these findings were obtained for the first time from melon by-products, suggesting that MPJ still maintained bioactive proteins with relevant enzymatic activity, which can be effectively extracted by the natural chemical interaction with CRG without any previous purification step, leading a reduction of process time and cost when compare with traditional extractive methods.

3.7. Melon by-products as sources of proteolytic enzymes

The industrial enzymes market, especially those used in food industry, is expected to increase their global demand of over $3.6 billion by 2024, and proteases are one of the most important groups of industrial enzymes, with approximately 60% of the enzyme market (Ibrahim et al., 2019; Mangiagalli, Brocca, Orlando, & Lotti, 2020). Hence, different practical strategies for future melon by-products applications could be developed as vegetable rennet for cheese production: this approach could be achieved taking into account the current increasing demand of alternatives against animal rennet, since different issues have been arrived related with its production, less consumers acceptance and high cost. In order to evaluate melon peels extracts as vegetable rennet, one important parameter that must be considered is the ratio of milk-clotting activity to proteolytic activity (MCA/PA). It is well-known that excessive proteolysis negatively affect coagulation force, as well as some, sensorial characteristics of the final products (Gomes et al., 2019). In turn, depending on the temperature MPJ showed an MCA/PA ratio of 995.34 ± 111.6 and 1485.84 ± 107.2 at temperatures of 80 and 85 °C, respectively. These values were similar to the commercial rennet such as CoalhoPar® rennet (1730.4), Halamix® rennet (1077.6) and some plant extracts from *Cryptostegia grandiflora* (1154.8) and *Morinda citrifolia* L. (1124.31) (Freitas et al., 2016; de Farias et al., 2020), showing the possible application of MPJ as a vegetable rennet. Other possible application could be the production of bioactive peptides from whey protein or different protein sources by enzymatic hydrolysis. Bioactive peptides have a wide spectrum of bioactivities, such as, antioxidant, antimicrobial, inti-inflammatory activity and enzyme inhibition, which could be used in cosmetic and pharmacological industries (Aguilar-Toalá, Hernández-Mendoza, González-Córdova, Vallejo-Cordoba, & Liceaga, 2019). Several researchers employed *Cynara cardunculus* and *Maclura pomifera* proteases to produce bioactive peptides from bovine whey, such peptides exhibited antioxidant and ACE-inhibitory activity (Corrons, Bertucci, Liggieri, López, & Bruno, 2012; Tavares et al., 2011). Therefore, melon by-products coupled with biological precipitation can deliver different proteolytic fractions to be tested for their ability to produce value-added molecules. These evaluations should be conducted in future research studies to improve the application of melon by-products as sources of bioactive proteins.

4. Conclusions

The use of melon by-products as new feedstock for proteins recovery by biological precipitation could decrease the environmental impact as well as minimize the costs associated to the traditional extractive processes. This research demonstrated that melon peels have proteolytic (4.24 U/mg protein) and milk-clotting (6300 MCU/mg protein) activities with an MCA/PA ratio of 1485 at 85 °C. Besides, biological precipitation with CRG allows to improve the biological activity of the proteins recovered from melon peels. A yield of 0.17 g of CUC/100 g of melon by-products can be obtained with the best conditions of precipitation at pH 3 with low concentration of CRG (0.003% w/v), improving 17.65 and 2.11-folds the PA (74.86 U/mg protein) and MCA (405 MCU/mg protein), respectively. Furthermore, these findings support the importance to valorise melon by-products to avoid economic and environmental issues through their reincorporation into the industrial chains as rich sources of value-added proteins.

CRediT authorship contribution statement

Ricardo Gómez-García: Writing - original draft, Visualization. **Débora A. Campos:** Data curation, Investigation, Resources. **Cristóbal N. Aguilar:** Supervision, Validation. **Ana R. Madureira:** Resources, Supervision, Validation. **Manuela Pintado:** Conceptualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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