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Development of immobilized-pepsin microreactors coupled to nano liquid chromatography and tandem mass spectrometry for the quantitative analysis of human butyrylcholinesterase

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Abstract

Human butyrylcholinesterase is a serine hydrolase that reacts with organophosphorus compounds (OP) to form stable adducts. These adducts are valuable biomarkers for OP exposure and can be analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after a preliminary digestion step in solution. However, this digestion step is time-consuming and cannot be directly coupled with LC-MS set ups. Therefore, the aim of this work was to develop pepsin-based immobilized enzyme microreactors (IMERs) for the rapid digestion of human butyrylcholinesterase (HuBuChE). Various IMERs were synthesized by grafting different amounts of pepsin on a CNBr-sepharose gel and the grafting yield was measured by a bicinchoninic acid assay (BCA). A sensitive nanoLC-MS/MS method was developed to evaluate the digestion yields of HuBuChE on IMERs which was made possible by a synthetic peptide which was used as a calibrant. The digestion was optimized by studying the impact of different parameters such as the digestion time, the temperature and the amount of pepsin grafted on IMER. This optimization allowed HuBuChE to be digested with-in 20 min

without pretreatment and with digestion yields up to 20%. The repeatability of the IMER synthesis and HuBuChE digestion was highlighted with the characterization of 3 similar IMERs. Finally, the digestion yields of HuBuChE were higher with IMERs when compared to a typical in solution digestion.

Keywords

HuBuChE; nanoLC-MS/MS; Immobilized Enzyme Reactor; pepsin digestion

1. Introduction

Human butyrylcholinesterase (HuBuChE) is a serine esterase glycoprotein present in plasma and is made up of four 85 kDa subunits [1,2]. HuBuChE catalyzes the hydrolysis of esters including acetylcholine, aspirin, cocaine or heroin [3]. It can also be used as a biomarker for exposure to organophosphorus compounds (OPs) such as sarin [4–7], soman [5,6,8], tabun [6,8,9], or VX [5,10]. These nerve agents covalently bind to acetylcholinesterase (AChE) and HuBuChE and irreversibly inhibit their activity. Inhibition of AChE activity induces the accumulation of acetylcholine within nerve synapses, leading to paralysis and respiratory dysfunction which can result in death [11,12]. Because of their high degree of toxicity, these nerve agents can be used as chemical warfare agents [4]. Thus, the retrospective detection of OP exposure after a chemical weapons attack is of the utmost importance. It provides a reliable diagnosis leading to appropriate medical countermeasures, as well as confirming the alleged use of these chemical warfare agents which are prohibited under the terms of the Chemical Weapons Convention [13]. HuBuChE is usually the preferred biomarker of OP exposure over AChE because it can form adducts with a broad range of OPs, is 10 times more abundant than AChE in the plasma (40-80 nM) and can persist in the blood longer than AChE [14]. The binding of OP nerve agents on the 198 serine residue of the HuBuChE active site allows retrospective detection of HuBuChE-OP adducts in blood for as long as 16 days when urinary metabolites are no longer detectable [15].

Protein determination is commonly performed by liquid chromatography coupled to mass spectrometry analysis (LC/MS). However, ionization of intact proteins produces multiply charged ions envelopes, leading to a very complex mass spectrum. In order to avoid such problems, the detection of HuBuChE-OP adducts is usually performed after a preliminary digestion step. This digestion produces small peptide fragments that are then analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

The digestion of HuBuChE-OP is typically achieved in solution with trypsin [16], chymotrypsin [6] or pepsin [17,18], commonly over 2 hours. Generally, to decrease lengthy digestion time of proteins, the enzyme/protein ratio can be increased. However, as the enzyme is free in solution, autoprotolysis can occur thus generating peptides that can interfere during the mass spectrometry analysis. To overcome this drawback, the proteolytic enzyme can be immobilized on a functionalized solid support [19]. The resulting immobilized enzyme reactors (IMERs) allow for an efficient digestion and low digestion time while minimizing sample contamination during handling and avoiding autoprotolysis. In addition, as the stability of the enzyme is enhanced, immobilized enzyme reactors can be reused thus decreasing the cost of the digestion step [19]. IMERs have been synthesized on various formats for the digestion of proteins including pre-columns [20–25], membranes [26,27], capillaries [28–30], pipette tips [31], disks [32,33] and using various supports such as silica particles [34], silica monolith [34,35], agarose [36–39], graphene [40], or synthetic polymers such as monolithic polymethacrylate derivatives [31,41–43] and polystyrene-divinylbenzene particles (Poroszyme) [21–23,30, 44,45].

Performances of the IMERs depend on many factors including the technique used for enzyme immobilization (adsorption, encapsulation or covalent linkage), digestion conditions and the nature of the functionalized immobilization support [46]. The ideal support should be biologically inert, easily activated, homogeneous with high surface area and should favor a good accessibility of the substrate [47]. It should also present good mechanical and chemical stability over a wide range of pH, pressure, temperature and solvent conditions [48,49]. Finally, this support should be hydrophilic to avoid non-specific hydrophobic interactions with proteins or peptides. A study comparing the performance of IMERs made with 3 different precolumn-format supports [36], highlighted the importance of the immobilization support for the efficient digestion of cytochrome C on a trypsin-based IMER. Between CNBr-activated Sepharose (Seph-CNBr), NHS-activated Sepharose and glutaraldehyde-activated silica, Seph-CNBr appeared to be the best immobilization support in terms of capacity and digestion yield [36].

Due to the high specificity of its cleavages, trypsin has been frequently used for the digestion of HuBuChE [16,17]. Yet, HuBuChE digestions with trypsin require pretreatments (carboxymethylation and deglycosylation) and induce large peptide-OP adducts (29 amino acids) that can excessively fragment in the mass spectrometer ion source and result in an insensitive detection. In the case of HuBuChE digestion by pepsin, pretreatment of the enzyme is not necessary and the digestion leads to smaller peptides including the nonapeptide FGESAGAAS on which OPs bind [17]. Moreover, digestion by pepsin can be directly coupled

to a preliminary selective extraction step by immobilized antibodies (immunoextraction) in order to remove other interfering proteins from real plasma thus improving the selectivity and the sensitivity of the whole analytical method. Additionally, the acid conditions used for pepsin digestion (pH 2.2) has been shown to be compatible with the elution of proteins such as cytochrome C from antibodies [50].

Recently, Carol-Visser et al. developed an on-line set-up that consists of a direct coupling of a sarin-intoxicated HuBuChE digestion using a commercial pepsin-IMER precolumn (Poroszyme® 30 x 2.1 mm i.d.) with LC-MS/MS analysis [7]. Efficiency of the digestion was determined by comparing the intensities of the HuBuChE specific peptide obtained after digestion on IMER to those obtained after a digestion in solution. In this study, intensity of adduct nonapeptide derived from on-line pepsin digestion was about 70% of that obtained after in-solution digestion. However, no information was provided on Poroszyme® column digestion yields or on the grafting yields of pepsin in the reactor. Apart from a study on the development of trypsin-based microreactors related to cytochrome C digestion [36], quantification of digestion yields on IMERs have not been evaluated. In addition, rather than using commercial IMERs, it appears interesting to control the synthesis of IMERs to be able to choose an appropriate grafting support and control the amount of immobilized enzymes.

Although many developments were made on trypsin microreactors [51], few studies deal with the development of pepsin-based IMERs. Moreover, none have been specifically developed for HuBuChE digestion despite the ability of pepsin to digest HuBuChE as already mentioned above. Thus, the aim of this study was the development and characterization of laboratory-made pepsin-based IMERs for the digestion of HuBuChE. Different supports were prepared by grafting different amounts of pepsin on cyanogen bromide (CNBr)-activated Sepharose and were packed in precolumns (20 x 2 mm i.d.). Digestions on pepsin-based IMERs were optimized with the evaluation of several key parameters such as the digestion time, temperature and the amount of pepsin grafted on the IMERs. This evaluation was based on the quantification of a specific nonapeptide that was quantified in the digest by nanoLC-MS/MS. The repeatability of the digestion and IMER synthesis were assessed with the preparation of 3 similar microreactors. Finally, digestion yields obtained on IMERs were compared to those obtained by digestion in solution.

2. Experimental

2.1. Chemicals

Pepsin from porcine gastric mucosa, sodium hydrogen phosphate (Na_2HPO_4), sodium azide (NaN_3), sodium chloride (NaCl) and cyanogen bromide-activated Sepharose 4B were purchased from Sigma Aldrich (Sigma-Aldrich, Saint-Quentin Fallavier, France). Potassium dihydrogen phosphate (KH_2PO_4), di-sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4(2\text{H}_2\text{O})$), sodium acetate ($\text{CH}_3\text{CO}_2\text{Na}$), acetic acid (CH_3COOH), formic acid (HCOOH), glycine, hydrochloric acid and acetonitrile (ACN) were purchased from VWR (Fontenay-sous-Bois, France). Sodium hydroxide was purchased from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was ordered from Carlo Erba (Val de Reuil, France). High purity water was obtained from a Milli-Q purification system (Millipore, Saint Quentin en Yvelines, France). Two different sources of HuBuChE, obtained by the purification of human serum, were used: HuBuChE₁ (480 μg of lyophilized HuBuChE, 50 U/mg, B4186) was purchased from Sigma Aldrich and HuBuChE₂ (480 μg of lyophilized HuBuChE with 0.02 mol.L⁻¹ ammonium bicarbonate, 160 U/mg, MBS173030) was purchased from MyBioSource (San Diego, California, USA). Synthetic nonapeptide (FGESAGAAS) was obtained from Proteogenix (Schiltigheim, France). BCA protein assay reagents were obtained from Thermo Fisher Scientific.

The phosphate buffer saline solution (PBS, pH 7.4) consisted of 0.01 mol.L⁻¹ of both Na_2HPO_4 and KH_2PO_4 and 0.15 mol.L⁻¹ of NaCl . The PBS-azide solution is a solution of PBS with 0.1% (w/w) of NaN_3 . HuBuChE solutions (480 mg.L⁻¹) were prepared in PBS-azide. Pepsin solutions were prepared in saline sodium acetate solution ($\text{CH}_3\text{CO}_2\text{Na}$, 0.1 mol.L⁻¹, pH 5.8, NaCl 0.5 mol.L⁻¹).

2.2. Apparatus and HPLC-MS analysis

The digestion was performed using a device composed of two six-port switching valves (**Fig. S.1**), one connected with the injection loop (5 μL) and a preconcentration pump (isocratic pump LC-10AS, Shimadzu, Champs sur Marne, France) and the other to an IMER precolumn set in an oven (Crococil oven, CIL). Because sepharose is not pressure resistant, the backpressure generated by the system was not over 4 bars.

The nanoLC-MS/MS analytical system is composed of a nano-pump (NCP-3200RS Nano Pump, Dionex, controlled by Chromeleon 6.8 SR11), a 250 nL injection loop, an EASY-Spray PepMap[®] analytical column (C18, 15 cm x 75 μm i.d, 3 μm , 100 Å) and a triple quadrupole

TSQ Quantum Access MaxTM (Thermo Scientific) equipped with an EASY-SprayTM source (Thermo Scientific). The mobile phase contained a mixture of water and acetonitrile acidified with 0.1% formic acid. The separation of peptides was achieved by increasing the amount of acidified acetonitrile from 2% to 35% from 0 min to 20 min at 300 nL.min⁻¹. The synthetic nonapeptide was used as a calibrant (MW: 795.2 g.mol⁻¹) and peptides resulting from HuBuChE digestion were analyzed by nanoLC-MS and nanoLC-MS/MS. Ionization was performed in positive mode with the following source conditions: source voltage was set at 1900 V, capillary temperature at 200°C, tube lens offset at 175 V and skimmer offset at 10 V. The scan time was set to 100 ms and a m/z range [450-2000] was used for scan experiments. In MRM analysis, the collision-induced dissociation (CID) experiments were carried out with a fragmentation voltage of 30 eV. The transitions m/z 796.2→691.0; 796.2→673.0 and 796.2→602.1 corresponding respectively to the b₈, b_{8-H₂O} and b_{7-H₂O} fragments of the nonapeptide were followed to quantify the amount of nonapeptide obtained after digestion (**Fig. 1**).

2.3. Preparation of pepsin-based IMER

For pepsin immobilization, different grafting solutions were prepared by diluting various amounts of pepsin ranging from 1 to 8 mg.mL⁻¹ in 0.1 mol.L⁻¹ of sodium acetate solution. Pepsin-based IMERs were prepared according to a protocol similar to the one developed in our laboratory for trypsin immobilization [36]. Briefly, 1.7 mL of grafting solution at different concentrations of pepsin (1, 4, and 8 mg.mL⁻¹ corresponding to 1.7, 6.8 or 13.6 mg of pepsin) was added to 35 mg of CNBr activated sepharose that had been swollen in 1 mL of 10⁻³ mol.L⁻¹ HCl solution. The IMER was washed twice with 1 mL of saline sodium acetate solution. The sorbent was incubated for 16 h at 4°C and 30 mg of sorbent was packed in a 20 x 2 mm i.d precolumn. The remaining uncoupled sites were blocked by percolating a glycine solution at 0.2 mol.L⁻¹, (pH 5) through the precolumn for 2 h at room temperature with a flow of 0.4 mL.min⁻¹. The gel was washed 3 times to remove unbounded enzymes by alternating between sodium acetate (pH 5.8 + 0.5 mol.L⁻¹ NaCl) and acid solutions (HCl, pH 3 + 0.5 mol.L⁻¹ NaCl) for 10 min each at 0.4 mL.min⁻¹. Finally, IMERs were stored at 4°C in a formic acid solution (pH 3). The IMERs grafted with 1.7 mg, 6.8 mg and 13.6 mg of pepsin were respectively named IMER 1.7, IMER 6.8 and IMER 13.6. The repeatability of IMER synthesis was evaluated by making 3 IMERs grafted with 6.8 mg of pepsin. These resulting IMERs were called IMER 6.8B, IMER 6.8C and IMER 6.8D.

2.4. Quantification of pepsin grafted on IMER

A bicinchoninic acid assay (BCA) was developed to calculate the amount of pepsin immobilized on sepharose. Two calibration curves (6 levels of concentration) were made by adding 10 μL of pepsin at a concentration ranging from 20 $\mu\text{g}\cdot\text{mL}^{-1}$ to 8 $\text{mg}\cdot\text{mL}^{-1}$ (depending on the concentration of pepsin used in the coupling solution). Briefly, 10 μL of supernatant was added in 3 microwells of a 96 microplate. Then, 100 μL of working reagent (mix of bicinchoninic acid and Cu^{2+}) was added in each microwell. The microplate was incubated at 37°C for 30 min with stirring at 300 rpm. Absorbance was measured at 562 nm with a spectrophotometer (SpectraMax M2, Molecular Devices, St Gregoire, France). Finally, grafting yields were calculated by comparing the amount of pepsin remaining in the supernatant to the initial amount of pepsin in coupling solution.

2.5 HuBuChE digestion procedures

2.5.1. In-solution digestion

Pepsin is a proteolytic enzyme active at an acidic pH (1-3) [52]. Thus, HuBuChE (2.4 μg) was digested in 100 μL of formic acid solution (pH 2.2) by adding pepsin in an enzyme/substrate molar ratio of 10. The digestion was carried out for 2 hours at 37°C as previously described by Jiang et al. [18,53]. Then, 250 nL of the digest solution was analyzed by nanoLC-MS/MS. Digestion yields were evaluated by comparing the quantity of nonapeptide resulting from pepsin digestion determined with the nonapeptide calibrations curves, to the theoretical quantity of nonapeptide corresponding to a 100% digestion yield.

2.5.2. IMER digestions

Briefly, 2.4 μg of HuBuChE solution was loaded into a 5 μL injection loop and transferred to the IMER with a formic acid solution (pH 2.2) at 50 $\mu\text{L}\cdot\text{min}^{-1}$. After the digestion made by a “stop flow”, peptides were recovered with 100 μL of formic acid (50 $\mu\text{L}\cdot\text{min}^{-1}$) and analyzed by nanoLC-MS/MS. Evaluation of digestion yields on each IMER was identical to the method used in Section 2.5.1 for in solution digestion. Different parameters such as the temperature, the digestion time and the volume of formic acid used to transfer peptides were investigated to determine the optimal digestion conditions leading to the highest digestion yield of HuBuChE on the IMERs.

3. Results and discussion

3.1. Analysis of HuBuChE peptides by nanoLC-MS/MS

The aim of this study was to develop a rapid and efficient HuBuChE digestion utilizing an IMER prior to the analysis of the nonapeptide. Thus, a separation method based on the use of nanoLC-MS/MS was developed to analyze the nonapeptide and evaluate the HuBuChE digestion yields on each IMER. Nano-chromatography enables a sensitive method while enabling lower costs of development than conventional LC/MS systems by injecting smaller amounts of a standard solution into the analytical system (a few nanoliters instead of microliters). To optimize the nanoLC-MS parameters and to determine the retention time of the nonapeptide on the 75 μm i.d.-column, a synthetic nonapeptide (108 $\text{ng}\cdot\text{mL}^{-1}$) was injected into the nanoLC-MS system and detected in scan mode. An extracted ion chromatogram was performed and the nonapeptide ($[\text{M}+\text{H}]^+$, $m/z = 796.2$) was observed at a retention time of 10.6 min (**Fig. 1A**). Then, a fragmentation spectrum was carried out to compare its b-fragments to the theoretically expected b-fragments [54,55]. The three most intense b-fragments (b_8 , $b_{8-\text{H}_2\text{O}}$ and $b_{7-\text{H}_2\text{O}}$) corresponding respectively to the transitions m/z 796.2 \rightarrow 691.0; 796.2 \rightarrow 673.0 and 796.2 \rightarrow 602.1 were chosen for MRM analysis to further identify unambiguously and quantify the amount of nonapeptide resulting from HuBuChE digestion. For this purpose, calibration curves were established for each transition by injecting five different concentrations of the synthetic nonapeptide (6.75, 13.5, 27, 54 and 108 $\text{ng}\cdot\text{mL}^{-1}$) into the nanoLC-MS/MS system. The injections were repeated 3 times for each concentration level. The variation of the LC retention time was estimated at 2% while an 11% R.S.D. was obtained for LC peak area. To consider inter-day variations of the nanoLC/MS² signal, nonapeptide calibration curves were repeated before each digestion procedure. The linear regression equations and coefficients values of the regression curves obtained for the transitions m/z 796.2 \rightarrow 691.0; 796.2 \rightarrow 673.0 and 796.2 \rightarrow 602.1 for this concentration range were respectively equal to $y = 16590 x + 7159.7$, $R^2 = 0.9959$; $y = 16105 x + 6282.8$, $R^2 = 0.9979$; $y = 15796 x + 6035.9$, $R^2 = 0.996$ (**Fig. S.2**). For the transition m/z 796.2 \rightarrow 602.1, the limit of detection (LOD, S/N = 3) and quantification (LOQ, S/N = 10) of the nonapeptide were 0.15 $\text{ng}\cdot\text{mL}^{-1}$ (38 fg injected) and 0.5 $\text{ng}\cdot\text{mL}^{-1}$ (125 fg) respectively. For both transitions m/z 796.2 \rightarrow 691.0 and 796.2 \rightarrow 673.0, the LOD and LOQ values were estimated to 0.16 $\text{ng}\cdot\text{mL}^{-1}$ (LOD) and 0.5 $\text{ng}\cdot\text{mL}^{-1}$ (LOQ). These LOD values are 7 to 20 times below the limit of detection reported by Sporty et al. (3 $\text{ng}\cdot\text{mL}^{-1}$) [15], Pantazides

et al. (1 ng.mL^{-1}) [14] and Carter et al. (0.96 ng.mL^{-1}) [56]. Those values were obtained by injecting $3 \mu\text{L}$ [56] to $10 \mu\text{L}$ [14] of nonapeptide in LC-MS/MS while nanoLC-MS/MS allows a reduction in the injected volume by a factor 12 to 40 (250 nL). Thus, these LOD values demonstrate that the nanoLC-MS/MS method leads to a sensitive analysis while reducing the cost by injecting smaller amounts of standards. To confirm that the nonapeptide resulting from HuBuChE digestion can be detected by nanoLC-MS, HuBuChE₁ ($480 \mu\text{g}$ of lyophilized HuBuChE, 50 U/mg , diluted in 1 mL of PBS azide solution), was digested in solution as described in Section 2.5.1 and analyzed by nanoLC/MS in scan mode. An extracted ion chromatogram was performed, confirming the presence of the nonapeptide in the HuBuChE digest at 10.6 min (**Fig. 1B**). The corresponding fragmentation spectrum displayed nonapeptide b-fragments similar to those obtained from the fragmentation of the synthetic nonapeptide (**Fig. 1A and 1B**). Thus, a sensitive method was developed to detect the target nonapeptide resulting from a HuBuChE digestion and could thus be applied to the detection of HuBuChE peptides from an IMER digestion.

3.2. Development of an optimized HuBuChE digestion on IMER

3.2.1 Choice of the support

As previously described, the choice of the immobilization support is a key parameter controlling the performance of IMERs. Because pepsin is active at a low pH (1-3), a support with good chemical stability over this pH range was essential for the preparation of pepsin-based IMERs. Sepharose gel is a beaded-form of agarose, a polysaccharide gel. It has been frequently used for protein immobilization including enzymes, and is stable over a large pH range (2-14). Moreover, this hydrophilic support avoids non-specific interactions that could retain peptides in the IMER and would result in a lack of peptides for nanoLC-MS/MS. It also presents good chemical stability, allowing the reusability of the sorbent. From several sepharose phases (2B, 4B, 6B), Sepharose 4B was chosen for its 700 \AA pore size suitable for the digestion of HuBuChE (140 \AA). Indeed, as described by Gustavsson and Larsson [46], for a protein of “normal” size (60 \AA) the pore diameter should be between three and five times the diameters of the protein to avoid restricted rates of diffusion. A pore size between 300 and 700 \AA is usually a good compromise to provide fair diffusion rates and a large surface area. Therefore, three IMERs were prepared by grafting 3 different amounts of pepsin on a CNBr-sepharose gel (4B) for the quantification and the optimization of HuBuChE digestion parameters.

3.2.2. Estimation of pepsin grafting yields on IMER

Various amounts of pepsin were introduced in the grafting solution (1.7 mg, 6.8 mg or 13.6 mg) to be immobilized on the CNBr-activated sepharose support (35 mg). The amount of effectively grafted pepsin was evaluated by the BCA assay as described in section 2.4. **Fig. 2** presents the amount of immobilized pepsin versus the amount of pepsin introduced in the grafting solution. As expected, an increase in the amount of pepsin in the grafting solution leads to a higher quantity of pepsin grafted on sepharose. A linear response was observed between the amounts of pepsin introduced and the pepsin grafted on IMER for amounts of pepsin ranging from 1.7 to 13.6 mg. The highest grafting density of pepsin was evaluated to 88 mg per gram of sepharose. The amount of immobilized pepsin did not reach a plateau, meaning that the capacity of the sepharose was certainly not reached using 1.7 mL of pepsin concentrated at 8 mg.mL^{-1} . However, it was not possible to estimate this capacity because the limit of solubility of pepsin in formic acid is close to this concentration (10 mg.mL^{-1}). The mean grafting yield of pepsin on CNBr-sepharose was evaluated to be 22%, a result close to those previously described for trypsin-IMERs made with CNBr sepharose (20%) [36]. As a central point, the grafting of 6.8 mg of pepsin on 35 mg of CNBr sepharose was repeated 3 times and showed good repeatability of the grafting yields (R.S.D 11%). Thus, these values highlighted the repeatability of the grafting process.

3.2.3. Optimization of the digestion procedure

According to the conditions of digestion that were applied by Cingoz *et al.* [36] for the digestion of cytochrome C on trypsin based IMER, the first digestion of HuBuChE was made at ambient temperature (27°C) by digesting $5 \mu\text{L}$ of HuBuChE₁ on IMER 1.7. This IMER was grafted with 1.7 mg of pepsin with the same concentration used for the grafting of trypsin-IMER made by Cingoz *et al.* (1 mg.mL^{-1}). The HuBuChE solution was transferred from a $5 \mu\text{L}$ loop to the IMER 1.7 with a $30 \mu\text{L}$ formic acid solution (pH 2.2) at $50 \mu\text{L.min}^{-1}$. After digestion by stop flow for 20 minutes, peptides were collected in two fractions of $100 \mu\text{L}$ of formic acid (pH 2.2) percolated at $50 \mu\text{L.min}^{-1}$. Considering the volume of the IMER ($37 \mu\text{L}$) a volume of $100 \mu\text{L}$ ensured the total recovery of the peptides produced by HuBuChE digestion. Each $100 \mu\text{L}$ fraction was analyzed by nanoLC-MS/MS. No peptide was observed in the second fraction, indicating that $100 \mu\text{L}$ of formic acid was enough to recover the totality of peptides

produced by HuBuChE digestion. The amount of nonapeptides recovered in this fraction has allowed evaluating a digestion yield of $14 \pm 2\%$ thanks to the nonapeptide calibration curves. This first result demonstrates the ability of the IMER to digest HuBuChE₁ without pretreatment despite the strong glycosylation of HuBuChE. However, to enhance the digestion yields, several parameters were evaluated and optimized.

3.2.3.1. Optimization of the transfer volume

Before optimization of the transfer volume, the first digestion was performed with a 30 μL transfer volume. This volume was estimated taking into account the volume of the loop (5 μL), the volume of connections tubes between the loop and the IMER (4.4 μL) and the volume of IMER (37 μL). The latter volume was evaluated by multiplying the volume of the 20 x 2 mm i.d. precolumn (62.8 μL) by a porosity value of 0.6. It was presumed from these values that the transfer volume should be at least superior to 9.4 μL and should not exceed 46.4 μL to digest the totality of HuBuChE in the solution. The volume of formic acid required to transfer HuBuChE from the loop to the IMER was evaluated with the digestion of 5 μL of HuBuChE₁ on IMER 1.7 using transfer volumes from 20 to 60 μL (20, 30, 40, 50 and 60 μL). HuBuChE₁ was again digested for 20 min (stop flow) at ambient temperature (27°C) and recovered in 100 μL of formic acid (pH 2.2). Each digest was injected 3 times into the nanoLC-MS/MS system. As shown in **Fig. 3**, a transfer volume of 20 μL was not sufficient to push the totality of the HuBuChE solution to the IMER (yield of 6%). As expected, volumes above 50 μL resulted in a significant decrease of the digestion yield (from 12 to 7%) because HuBuChE solution exceeded the void volume of the IMER. Transfer volumes of 30 and 40 μL gave the best results (yields of respectively $14 \pm 2\%$ and $14 \pm 3\%$). A transfer volume of 30 μL , showing more repeatable digestion yields, was used for future HuBuChE digestions on IMERs.

3.2.3.2. Optimization of the temperature

Temperature is a key factor controlling the enzyme activity. Thus, optimization of the digestion temperature was evaluated by carrying out 4 digestions of HuBuChE₁ at 22, 27, 32 and 37°C on IMER 6.8. As IMER 6.8 contains an amount of pepsin that is approximately the average of the two extreme pepsin quantities grafted on IMERs, this IMER was chosen for the optimization of the temperature. The IMER was set in an oven at the given temperature and 5 μL of HuBuChE solution was transferred to the IMER by 30 μL of formic acid and digested

for 20 min. The resulting digest solutions were recovered in 100 μL of formic acid and the digestion yields were evaluated by nanoLC-MS/MS. Considering that in solution pepsin-digestion of HuBuChE generally occurs at 37°C, it was expected that this temperature will lead to the highest digestion yield. Nevertheless, the digestion yields were constant for the lowest tested temperatures and started to decrease from 32°C to drastically decrease at 37°C. Indeed, it was not possible to perform a digestion at 37°C because of the clogging of the IMER which leads to a high pressure when applying the 50 $\mu\text{L}\cdot\text{min}^{-1}$ formic acid flow. This phenomenon can be explained by the inherent properties of the CNBr sepharose support having a gelling point of 37°C. A temperature of 27°C was then chosen for further experiments. The experiment was not reproduced on the others IMERs to avoid their deterioration.

3.2.3.3. Optimization of the digestion time

To enhance the efficiency of the digestion, the contact time between the proteolytic enzyme and the protein can be increased by reducing or stopping the flow. It was reported that an efficient digestion of cytochrome C could occur on trypsin based-IMERs with the use of a 20 min stop-flow [36]. Stop-flow digestions varying from 5 to 60 min were also performed by Carol *et al.* for the digestion of myoglobin on a trypsin-Poroszyme® column and showed no significant variation of peptides area when applying different digestion times [45]. After the deterioration of IMER 6.8 during the temperature optimization step, it was impossible to perform further digestions on this IMER. Then, to evaluate the best residence time of HuBuChE in the laboratory-made IMERs, five digestions were made on a newly IMER prepared with 6.8 mg of pepsin. HuBuChE₁ was transferred to the IMER 6.8B with 30 μL of a formic acid solution at pH = 2.2 and digested at 27°C for 5, 10, 20, 30 and 60 min. The resulting digest solutions were recovered in 100 μL of formic acid (pH 2.2) and injected into the nanoLC-MS/MS system. As illustrated in **Fig. 4**, it turned out that a residence time of 5 min was sufficient to digest HuBuChE. Nevertheless, the most efficient and repeatable digestions were obtained for a 20 min digestion time with a digestion yield of $13 \pm 1\%$. Unexpectedly, the digestion yields decrease from 30 min to 60 min. This may be due to the occurrence of non-specific digestion of the nonapeptide by pepsin, leading to further cleavage and smaller peptides than those expected. In conclusion, the optimal conditions for HuBuChE digestions on IMERs were defined as a transfer volume of 30 μL , a temperature of 27°C and a digestion time of 20 minutes.

3.3. Repeatability of IMERs digestion

As the optimized digestion parameters were fixed, the reproducibility of IMERs digestions were evaluated with the comparison of digestion yields obtained using three different IMERs prepared by the grafting of 6.8 mg of pepsin on 35 mg CNBr-activated sepharose. The evaluation of pepsin grafting yields on these IMERs was already discussed in section 3.2.2 and showed a good repeatability of the grafting process. Three digestions were carried out the same day on each IMER with a new solution of HuBuChE called HuBuChE₂ (480 µg of lyophilized HuBuChE diluted in 1mL of PBS pH 7.4, 160 U/mg). As shown in **Fig. 5**, a digestion yield of $20 \pm 3\%$ was obtained for the digestion of HuBuChE₂ on IMER 6.8 whereas digestion yield on this IMER was previously evaluated to $13 \pm 1\%$ for the digestion of HuBuChE₁ (section 3.2.3.3). This variation could be explained by the different origins of HuBuChE solutions. Indeed, as described Section 2.1, HuBuChE₁ was purchased from Sigma Aldrich with a specific activity of 50 U/mg, whereas HuBuChE₂ was purchased from Mybiosource with a specific activity of 160 U/mg. In addition, the purification and lyophilization steps used by each supplier are not communicated and could differ. Thus, it seemed crucial to use the same HuBuChE solution to compare the digestion yields of HuBuChE on each IMER and to check the intermediate precision of IMER synthesis. As illustrated **Fig. 5**, it was difficult to estimate the repeatability of the synthesis by simply comparing the mean digestion yields and standard deviations obtained using the 3 IMERs. Consequently, a Cochran test was first used and showed no significant variance between IMERs, which enabled us to perform an ANOVA test ($\alpha = 5\%$). The conclusion of this test demonstrated that there is no significant variation between the 3 IMERs and the R.S.D. value of the intermediate precision was evaluated at 16%. The RSD values related to digestion yields were compared to those related to the nanoLC/MS² analysis of a digest to evaluate the contribution of the digestion step in the variability of the whole analytical procedure. For this, three digestions of HuBuChE₂ were carried out on IMER 6.8 during 20 min at 27°C and each resulting digest was injected one time in nanoLC/MS² while one of these digests was injected 3 times. The resulting R.S.D. values were 13% and 10% respectively. The similarity of these values indicates that the main variations were due to the nanoLC/MS² analysis. Therefore, it was concluded that a repeatable method was developed for the synthesis and the digestion of HuBuChE on IMERs.

3.4. Effect of the amount of pepsin on digestion yields

As depicted sections 3.2.3.1 and 3.2.3.3, digestions of HuBuChE₁ on IMER 1.7 and IMER 6.8 respectively prepared with 1.7 mg and 6.8 mg gave similar digestion yields ($14 \pm 2\%$ and $13 \pm 1\%$). Nevertheless, it seemed interesting to evaluate if a higher amount of pepsin (> 6.8 mg) could lead to higher digestion yields. Consequently, 3 digestions were carried out on IMER 13.6. As illustrated **Fig. 2** and section 3.2.2., IMER 13.6 contained a higher quantity of immobilized pepsin than IMER 1.7 and IMER 6.8. Considering this information, it could be expected that IMER 13.6 would lead to higher digestion yields of HuBuChE than IMER 6.8 when applying the same conditions of digestion. Hence, digestion yields on IMER 6.8 and IMER 13.6 were compared by digesting 5 μ L of HuBuChE₂ ($n = 3$) according to the optimal digestion conditions (Section 3.2.3.3). As shown in **Fig. 5**, similar digestion yields were obtained for IMER 6.8 and IMER 13.6 (20%), showing that 6.8 mg of pepsin was enough for an efficient digestion of HuBuChE₂ in 20 minutes. It was assumed that beyond a certain threshold, the pepsin density was too high, leading to a low accessibility of the active site of the protease. To confirm the benefit of IMER-digestions over digestion in solution, 5 μ L of HuBuChE₂ were digested during 20 minutes at 37°C with a ratio E/S of 10 as commonly used in the literature for HuBuChE digestion in solution [18,53] and described section 2.5.1. A digestion yield of 11% was obtained. It can be concluded that the yields obtained for HuBuChE₂ digestion on IMERs in 20 min is 2 times higher than the yield obtained for a 2 hours digestion in solution. Indeed, while E/S of 10 was used for digestion in solution, by immobilizing pepsin we can report E/S > 4000 for IMER digestion. Thus, the use of IMERs leads to faster and more efficient digestions of HuBuChE than in solution digestions. As a comparison, Carol-Visser *et al.* estimated that the yield of adduct nonapeptide from a 5 minutes on-line pepsin digestion of HuBuChE on poroszyme pepsin cartridge was approximately 70% of those obtained after 2 hours digestion in solution [7]. Therefore, the optimization of the HuBuChE digestion parameters on IMERs enabled us to obtain better results than those described for the use of commercial pepsin-IMER. In conclusion, we have demonstrated the potential of laboratory made pepsin-IMERs for fast and efficient HuBuChE digestion compared to in-solution digestion and commercial IMERs.

Conclusions

A highly sensitive nanoLC-MS/MS method was developed for the analysis and quantification of the HuBuChE nonapeptide which is used as a biomarker of nerve agent intoxication. The sensitivity of the analytical system was demonstrated by the analysis of a

synthetic nonapeptide and of a HuBuChE digest. Its limit of quantification was estimated to a few attomoles. HuBuChE digestions were carried out on reusable IMERs and without pretreatment despite the strong glycosylation of HuBuChE. The yields of HuBuChE digested on IMERs were quantified using the synthetic nonapeptide as calibrant and digestion yields up to 20% were obtained after 20 minutes. The repeatability of IMERs synthesis was evaluated with the development of 3 similar IMERs that showed similar grafting and digestion yields. Various parameters such as the transfer volume, the temperature and the digestion time were optimized. Finally, HuBuChE digestion yields on IMERs were compared to those in solution and proved their superiority in term of digestion yields and digestion times.

Our future work will be focused on the on-line coupling of IMER to LC-MS/MS analysis. At last, an antibody-based support will be developed to extract HuBuChE from plasma, allowing the on-line analysis of HuBuChE.

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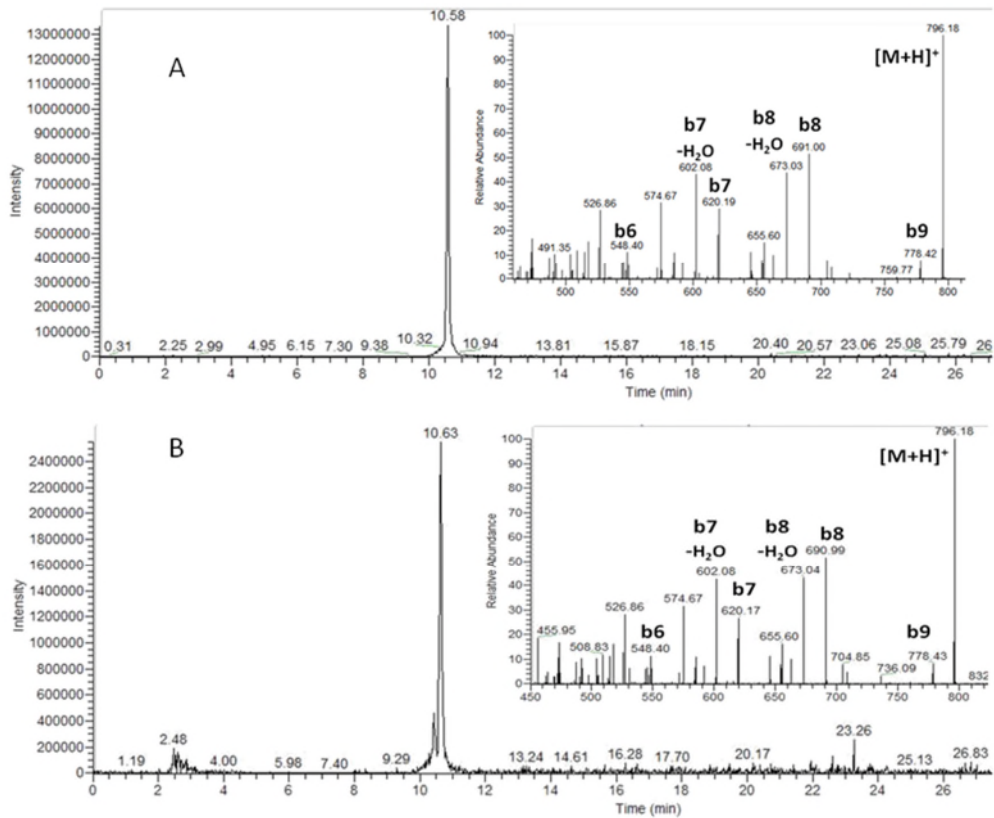


Fig. 1. Extracted ion chromatograms and fragmentation spectra of the synthetic nonapeptide FGESAGAAS (m/z 796) (A) and of the nonapeptide resulting from the digestion of HuBuChE in solution (B).

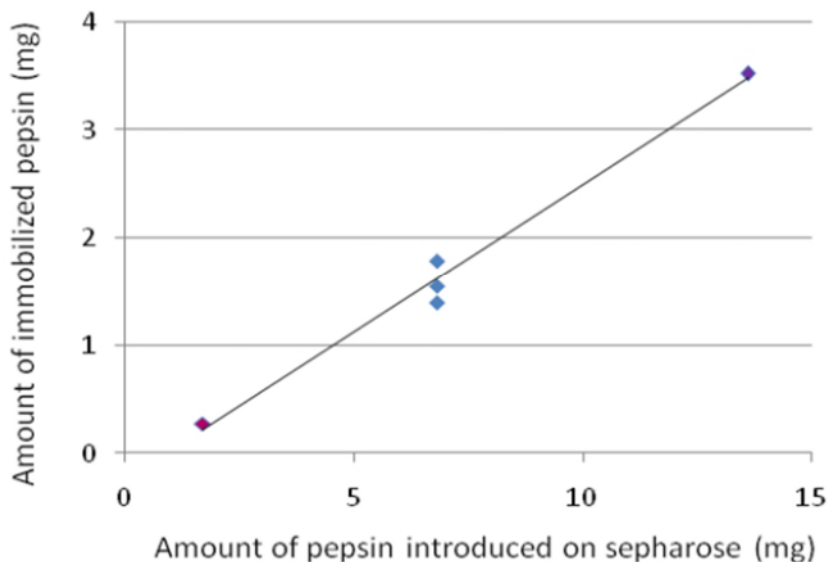


Fig. 2. Amount of pepsin immobilized on 35mg of CNBr-activated sepharose grafted by 3 different amounts of pepsin in solution (1.7, 6.8 and 13.6 mg in 1.7 mL of coupling solution).

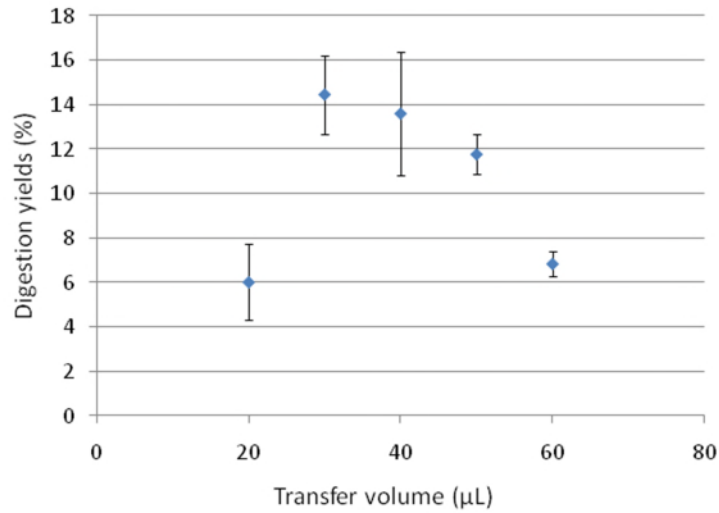


Fig. 3. Influence of the transfer volume on the digestion yields of 7.1 pmol of HuBuChE₁ digested during 20 minutes at 27°C on IMER 1.7 prepared with 1.7 mg of pepsin (3 injections).

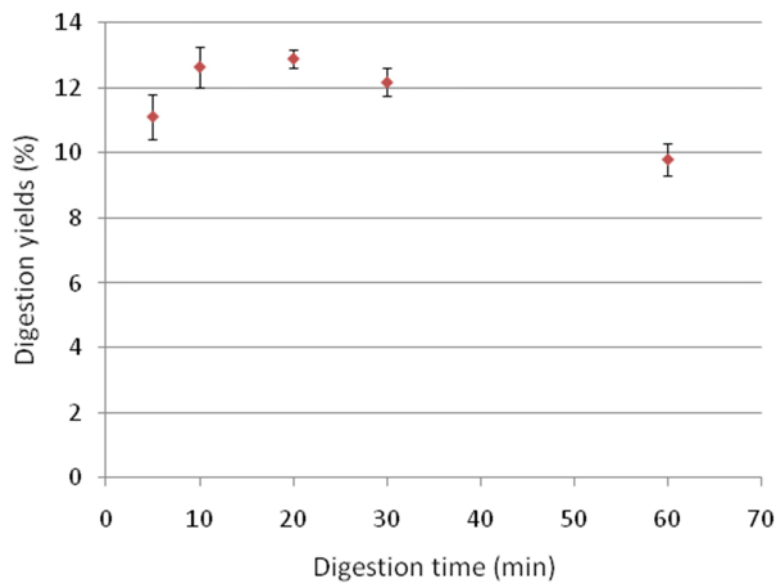


Fig. 4. Influence of the digestion time on the digestion yields of 7.1 pmol of HuBuChE₁ digested at 27°C on IMER 6.8B prepared with 6.8 mg of pepsin with a transfer volume of 30 µL (3 digestions).

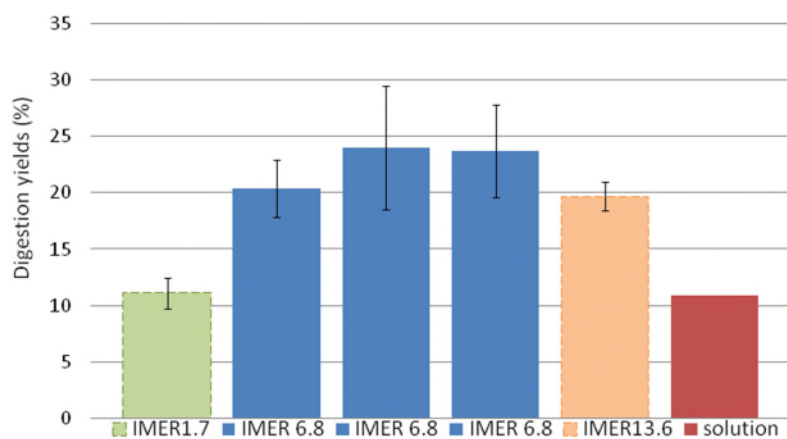


Fig. 5. Digestion yields obtained after the digestion of 7.1 pmol of HuBuChE₂ by pepsin in solution (E/S: 10) at 37°C during 2 h (1 digestion) and obtained after the digestion at 27°C of 7.1 pmol of HuBuChE₂ during 20 min on 3 IMERs prepared with 6.8 mg of pepsin and one IMER prepared with 13.6 mg of pepsin (3 digestions on each IMER).

Supplementary material

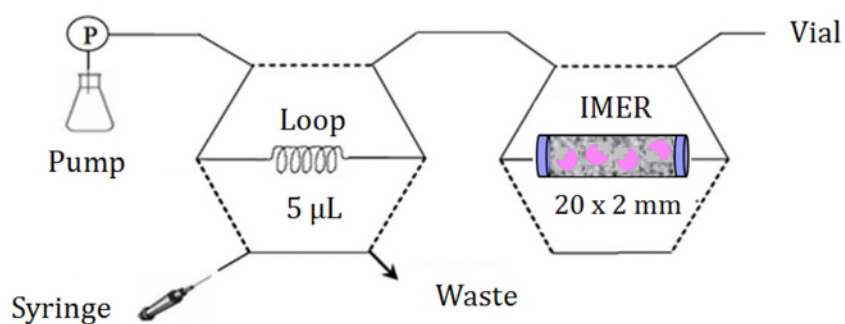


Fig. S1. Scheme of the assembly used to digest HuBuChE on IMER.

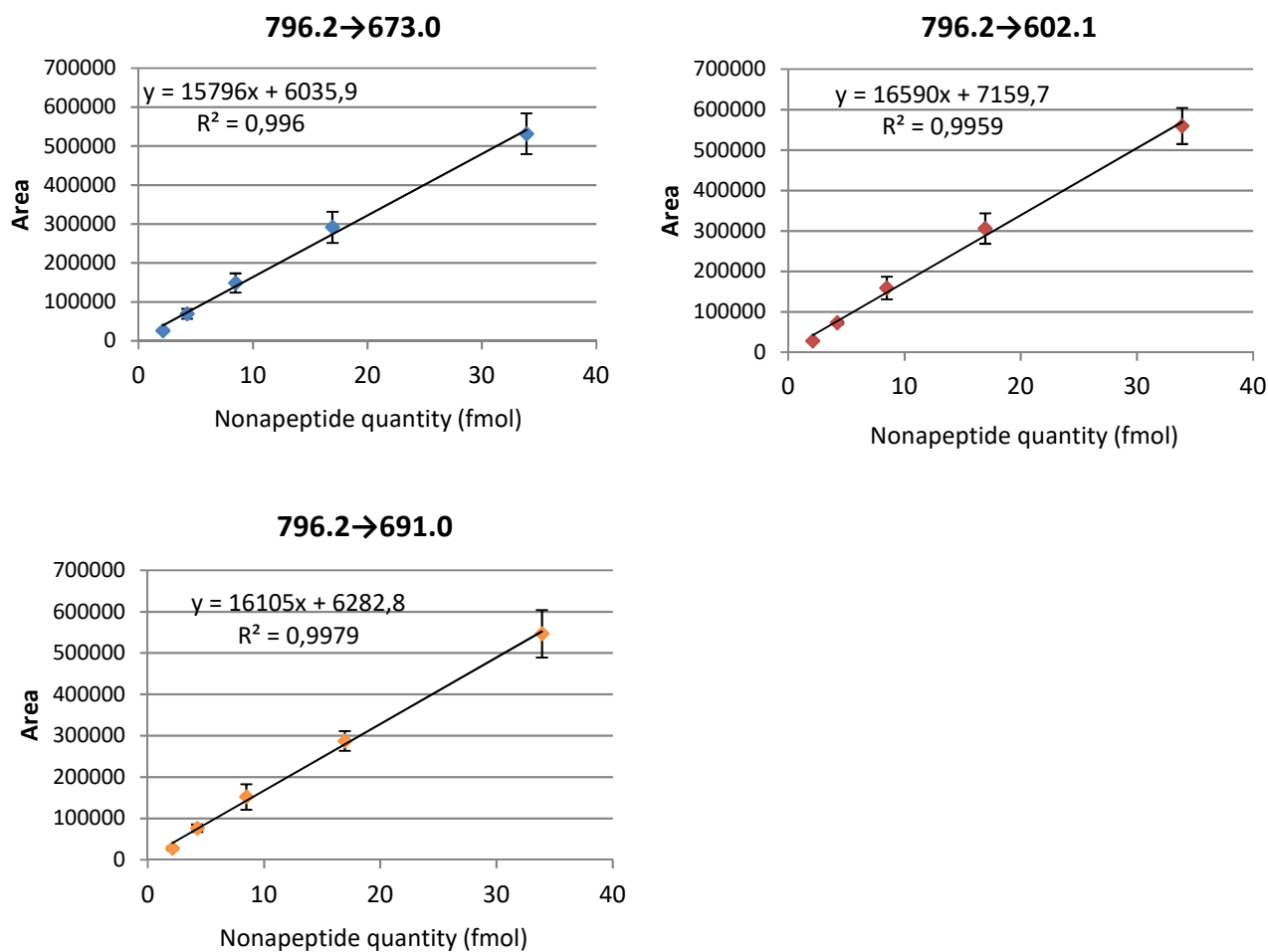


Fig. S2. Calibrations curves obtained after the injection of various amounts of synthetic nonapeptide varying from 2.12 fmol (6.75 ng.mL⁻¹) to 33.9 fmol (108 ng.mL⁻¹) for the transitions m/z 796.2→673.0; 796.2→602.1 and 796.2→691.0.

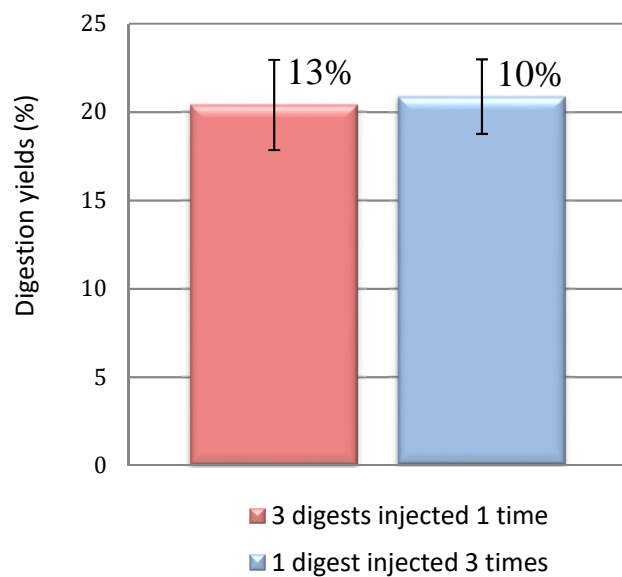


Fig. S.3. Digestion yields and RSD value corresponding to three digestions of HuBuChE₂ on IMER 6.8 (each digest being injected 1 time) compared to those resulting from three injections of one of these digests.