Modeling of the production of ACE inhibitory hydrolysates of Mediterranean horse mackerel (*Trachurus mediterraneus*) using protease mixtures

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ABSTRACT

Fish protein hydrolysates from Mediterranean horse mackerel were produced by a mixture of two commercial endoproteases (i.e. subtilisin and trypsin) under different levels of substrate concentration (2.5 g/L, 5 g/L, 7.5 g/L of protein), temperature (40ºC, 47.5ºC, 55ºC) and percentage of subtilisin in the enzyme mixture (0%, 25%, 50%, 75% and 100%). A crossed mixture process model was employed to predict the degree of hydrolysis (DH) and the ACE inhibitory activity of the final hydrolysates as a function of the experimental factors. Both models were optimized for a maximum DH and ACE inhibition. A maximum DH (17.1%) was predicted at 2.54 g/L of substrate concentration, 40ºC and an enzyme mixture comprising 38.3% of subtilisin and 61.7% of trypsin. Although its proteolytic activity is limited, the presence of trypsin in the enzyme mixture allowed obtaining higher degrees of hydrolysis at low temperatures, which is desirable to minimize thermal deactivation of the proteins. Similarly, a percentage of ACE inhibition above 48% was attained at 2.5 g/L of protein, 40ºC and a mixture 1:1 of both proteases. Higher values of ACE inhibition could be attained by increasing both the temperature and the amount of trypsin in the enzyme mixture (e.g. 50% ACE inhibition at 55ºC and 81.5 % of trypsin). Finally, those hydrolysates exhibiting the highest levels of ACE inhibition were subjected to simulated gastrointestinal digestion. These assays confirmed the resistance of active fractions against their degradation by digestive enzymes.

Keywords: ACE inhibitory activity; bioreactors; modeling; optimization; mixture of enzymes

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INTRODUCTION

According to the Magnuson-Stevens Fishery Conservation and Management Act (MSA), by-catch is defined as "fish which are harvested in a fishery, but which are not sold or kept for personal use, and includes economic and regulatory by-catch."[1] Bycatch comprises target species which are discarded due to legal, economic or personal considerations (i.e. minimal landing size, prohibitions on the retention of particular species, sexes or size ranges, fishing quota, low commercial value, highgrading practices). This definition also includes incidental catches (i.e. retained catches of non-targeted species) as well as "unobserved mortalities resulting from a direct encounter with fishing gear" [2]. International instruments, such as the FAO have highlighted the impact of bycatch on the sustainability of fishing. [3]

In compliance with the recent EU Fisheries Common Policy, by-catch is banned in European fisheries for most of pelagic fisheries (e.g. herring, sardine or Mediterranean horse mackerel) since the 1st of January of 2015. This measure will be extended to the rest of species in the following two years.[4] As a consequence, a supplementary amount of unwanted biomass will be no longer returned to the sea but brought ashore. This calls for the search of new solutions to handle and valorize these materials, involving the commercial promotion of non-target species, improvements in the fishing gears or up-grading strategies to obtain valuable products.[5]

In this context, enzymatic processing arises as a promising alternative to recover protein and lipid fractions from fish biomasses (e.g. by-catches, non edible fractions and other wastes from fish processing). The use of proteases ensures high protein recovery rates and allows converting the native proteins into fish protein hydrolysates (FPH). These do not only present improved functional properties,[6] but they also exhibit a number of biological activities such as antioxidant, antihypertensive or antimicrobial. [7–10] The main group of peptides displaying antihypertensive activity corresponds to the inhibitors of Angiotensin Converting Enzyme (ACE). Fish protein hydrolysates from marine origin have been widely reported to exhibit ACE-inhibitory activity, some of them belonging to target species in Mediterranean fisheries such as sardinelle *(Sardinella*

aurita) [11], cuttlefish *(Sepia officinalis)* [12], sardine *(Sardina pilchardus)* or Mediterranean horse mackerel *(Trachurus mediterraneus)* [13], among others. Apart from their intrinsic activity, bioactive peptides should resist gastrointestinal digestion before reaching the target organs and exerting their physiological effect. To this regard, *in vitro* digestion processes, which simulate the digestion process by employing gastrointestinal proteases in a reaction vessel, are effective to evaluate the bioavailabity of active peptides [14,15].

Enzymatic reactions entail a number of simultaneous phenomena (e.g. substrate solubilization, product or substrate inhibition, thermal enzyme deactivation) which cannot be accurately predicted by classical approaches (i.e. Michaelis-Menten mechanisms). Empirical models overcome these limitations since they are based on direct observation of experimental data, without considering the underlying mechanism [16] . These techniques require a small number of experimental data, arranged according to an experimental design, and have been successfully employed to model or optimize the yield of enzymatic reactions [17–19]. For instance, previous works on fish protein hydrolysis have predicted the optimum conditions for maximal degree of hydrolysis[19,20] which has a positive impact on many functional properties (e.g. protein solubility, water or lipid binding capacities). Other range of optimization problems target the maximization of the levels of some biological activities (e.g. ACE inhibition, DPPH scavenging, antimicrobial activity) in the final hydrolysate. [21–23]

This work studied the enzymatic hydrolysis of Mediterranean horse mackerel (*Trachurus mediterraneus*) by a variable mixture of two commercial endoproteases (subtilisin and trypsin). A design of experiments was performed to investigate the influence of the enzyme mixture and operating conditions (i.e. reaction temperature and enzyme-substrate ratio) on both the final degree of hydrolysis (DH) and the *in vitro* ACE inhibitory activity of the final hydrolysates (ACEI). Two predictive models were constructed for the final DH and ACEI, by means of a crossed mixture-process approach. These equations allowed the optimization for maximum DH and ACEI. Subsequently, those hydrolysates presenting the highest levels of ACEI were subjected to simulated gastrointestinal digestion to evaluate the loss of bioactivity of the hydrolysate after digestion.

MATERIALS AND METHODS

Proximate composition of the raw material

Mediterranean horse mackerel (*Trachurus mediterraneus*), was chosen as model species for this study. According to previous studies, this species is highly discarded in the Alboran Sea (i.e. portion of the Mediterranean Sea lying between northern Morocco and southern Spain)[24,25]. The raw material was provided by the fishing harbor of Motril (Spain) and kept in ice during transportation.

The raw material was partially dewatered prior to hydrolysis. To this end, 3 kg of fresh fish were preheated at 40ºC for 30 min in a water bath and then pressed stepwise at 120 bar by means of a hydraulic press (model ESP-K, Sanahuja, Spain). After completion of three pressing cycles the dewatered press cakes were recovered and analyzed for their proximate composition.

The moisture and ash content of press cakes was determined according to the official methods recognized by the A.O.A.C [26]. Total Nitrogen, determined by the Kjeldahl method, was reported to the content of crude protein by a conversion factor of 6.25 [27].

Production of the fish protein hydrolysates (FPH)

The hydrolysis experiments were conducted with two serine endoproteases: subtilisin (EC 3.4.21.62) and trypsin (EC 3.4.21.4), purchased from Novozymes (Denmark) as Alcalase 2.4L and PTN 6.0S, respectively. A sample of grinded press cake, containing the desired amount of crude protein, was suspended in 200 mL of demineralized water. This suspension was transferred to a jacketed reactor of capacity 250 mL, where it was adjusted at pH 8 and the desired temperature. An enzyme mixture of subtilisin and trypsin was employed as catalyst, whose composition was varied according to the experiment design. The amount of enzyme mixture was adjusted at 1% w/w of the protein content in the reactor. After addition of the enzymes, the hydrolysis reaction was allowed for 4 h. The degree of hydrolysis (DH) was monitored in the course of the reaction by the pH-stat method [27], employing an automatic titrator (718 Stat Titrino, Metrohm, Switzerland) and NaOH 0.5

N as titration agent. The degree of hydrolysis can be related to the amount of base consumed throughout the reaction to maintain the pH at 8, according to the Eq. 1:

$$DH = \frac{V_b \cdot N_b}{m_P} \cdot \frac{1}{\alpha} \cdot \frac{1}{h_{tot}} \qquad (1)$$

where DH stands for the degree of hydrolysis; V_b (mL) is the amount of base consumed; N_b (eq/L) is the normality of the base; m_P (g) is the mass of protein in the substrate; α is the average degree of dissociation of the α-NH_2 amino groups and h_{tot} (meq /g) was assumed to be 8.6 milliequivalents of peptide bonds per gram of protein, as commonly accepted for fish materials [27,28].

After completing 4 h, the hydrolysis was stopped by heating the reaction mixture at 100ºC for 15 min. These conditions ensure complete enzyme deactivation. Samples were then stored at -20ºC until freeze drying in a Labconco freeze drying system (Kansas City, MO, USA)

Experimental design

A crossed mixture-process design was proposed in this work, comprising two mixture variables: the percentage of subtilisin and trypsin in the enzyme preparation (X_1, X_2, %), and two process variables: the concentration of substrate (i.e. protein) in the reaction vessel (S, g/L) and the reaction temperature (T, ºC). The percentage of subtilisin was tested at five experimental levels: 0% (pure trypsin), 25%, 50%, 75% and 100 % (pure subtilisin). The substrate concentration was varied at three levels: 2.5 g/L, 5 g/L and 7.5 g/L. Protein concentrations above 7.5 g/L could hinder the correct stirring of the reactor vessel. On the contrary, producing hydrolysates with protein concentration below 2.5 g/L requires a high energy consumption for purification and stabilization. Three levels of reaction temperature were assayed: 40ºC, 47.5ºC and 55ºC. The lower bound corresponds to the optimal temperature of trypsin (around 37ºC), while the maximal activity of subtilisin is reported to be within the interval 50ºC - 60ºC [27]. The combination of these levels led to 45 experimental runs. As response variables, the final DH (at 4 h of reaction) and the ACE inhibition of the powdered hydrolysate were determined for each experiment. These results are summarized in Table 1.

Crossed mixture-process models for DH and ACEI

The designed experiment described above allowed obtaining mathematical models relating two response variables, the degree of hydrolysis (DH) and the ACE inhibitory activity (ACEI), to the three experimental factors assayed: enzyme composition (X_1, X_2, %), substrate concentration (S, g/L) and temperature (T, ºC). The mathematical models proposed were constructed by combination of a binary mixture model, which relates the responses with the composition of the enzyme preparation (i.e. percentage of subtilisin and trypsin) and a factorial design involving two process variables (i.e. substrate concentration and reaction temperature). Each of the responses could be related to the enzyme components by means of mixture model of second order [29], as shown in Eq. 3:

$$Y(X_1, X_2) = \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_{12} \cdot X_1 \cdot X_2 \qquad (3)$$

where Y denotes any of the response variables DH or ACEI, X_1 is the percentage of subtilisin in the enzyme mixture and X_2 is the percentage of trypsin in the enzyme mixture.

As for the process variables, a second order factorial model was proposed as follows:

$$Y(S,T) = \alpha_0 + \alpha_1 \cdot S + \alpha_2 \cdot T + \alpha_{12} \cdot S \cdot T + \alpha_{11} \cdot S^2 + \alpha_{22} \cdot T^2 \qquad (4)$$

Finally, a crossed mixture process model, containing 18 terms, was obtained by multiplication of both sub-models, as expressed by Eq. 5:

$$Y(X_1, X_2, S, T) = (\beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_{12} \cdot X_1 \cdot X_2) \times (\alpha_0 + \alpha_1 \cdot S + \alpha_2 \cdot T + \alpha_{12} \cdot S \cdot T + \alpha_{11} \cdot S^2 + \alpha_{22} \cdot T^2) \qquad (5)$$

Every term of the complete model was estimated by non linear regression of the experimental data. The significance of each term was then judged statistically by computing the associated probability (p-value) at a confidence level of 95%. This means that those terms whose p-value was below 0.05 were statistically significant on the output variable. The non significant terms can be sequentially removed from the regression model by backward selection. This approach starts with the complete model and eliminates the term with the highest p-value. This process is repeated with the new

model until obtaining a reduced model where all the terms are statistically significant.[30] The goodness of the reduced model was assessed by the coefficient of determination R^2, as well as the mean absolute error (i.e. average value of residuals) and the standard error of estimate (standard deviation of the residuals).

Determination of the ACE inhibitory activity of the FPH

The ACE inhibitory activity of the freeze dried hydrolysates was determined in vitro by the assay proposed by Shalaby et al.[31] This method is based on the hydrolysis of the synthetic tripeptide N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG, Sigma-Aldrich, St. Louis, USA) by the enzyme ACE (EC 3.4.15.1, Sigma-Aldrich, St. Louis, USA), which can be followed spectrophotometrically. The assays were conducted in a 96-well microplate at 37ºC, where the absorbance at 340 nm was monitored during 30 min by means of a Multiskan FC microplate photometer (Thermo Scientific, Finland). The hydrolysis of the substrate FAPGG causes a linear decrease of absorbance with time, whose slope is commonly related to the enzyme activity. Therefore, the percentage inhibition of the ACE activity by the hydrolysate can be expressed as:

$$\%\ \text{ACE Inhibition} = \left(1 - \frac{\rho_i}{\rho_0}\right) \cdot 100 \qquad (2)$$

where ρ_i and ρ_0 are the slopes of the absorbance curves in the presence and in the absence of inhibitor (hydrolysate), respectively. The slopes were calculated in the interval between 10 to 25 min, where the best linearity was observed.

In vitro digestion of the FPH

A modification of the method proposed by Garrett et al.[32] was employed to evaluate the effect of digestive enzymes. The reaction was carried out at 37 ºC with a continuous shaking (300 rpm) in a temperature-controlled shaker (Heidolph, Germany). Firstly, the lyophilized samples were dissolved in distilled water 5% (w/w) and the pH was set to 2 with 1M HCl. Subsequently, pepsin (EC 3.4.23.1, Merck, Darmstadt, Germany) was added at enzyme-substrate ratio of 4% (w/w). After 1h of reaction, a solution of 0.9 M $NaHCO_3$ was added to raise the pH until 5.3. Then, a mixture of

porcine pancreas enzymes (Pancreatin from Sigma-Aldrich, USA) was added and the pH was set to 7.5 with 1M NaOH. The digestion was maintained for 2 more hours and finally, the enzymes were thermally deactivated (100 ºC for 15 min). The samples were freeze-dried and stored until analysis.

Size exclusion Chromatography (SEC)

The hydrolysates with higher *in vitro* ACE inhibition and their digest were analyzed by fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) employing a Superdex Peptide 10/300GL column (GE Healthcare, Uppsala, Sweden). Aliquots of 100 μL (5 mg protein/mL) were eluted at 0.5 mL/min with mobile phase composed of 70:30 water/acetonitrile and 0.1% TFA. The absorbance was measured at 280 nm. The column was calibrated with the following standards: glycine (75 Da), alanine (89 Da), Phe-Gly-Gly (279 Da), $(Gly)_6$ (360 Da), vitamin B_{12} (1355 Da), insulin (5733 Da), aprotinin (6511 Da) and ribonuclease (13700 Da).

Optimization of DH and ACEI by evolutionary algorithm

The empiric models presented above allowed the optimization of the operating conditions (X_1, X_2, S and T) for a maximum degree of hydrolysis and ACE inhibitory activity. Among the optimization strategies currently available, an evolutionary algorithm was chosen for this work [33]. This algorithm is implemented in the Solver tool of the Microsoft Excel software. The optimization procedure starts with the generation of an initial random population of 100 individuals (i.e. combinations of S, T and t within their range of application) whose quality is evaluated by means of a fitness function (i.e. maximization of DH or ACEI). The best candidates are then combined to create a new population, employing a range of procedures inspired in biological evolution (i.e. elite, crossover and mutation). This procedure was repeated iteratively until completing 60 s of computation time.

RESULTS AND DISCUSSION

Curves of hydrolysis

The time evolution of the degree of hydrolysis followed the general pattern described for enzymatic reactions [27,34]. In general, hydrolysis curves presented a high reaction rate at the beginning and then

decreased progressively until attaining a steady state. This trend is explained by the exhaustion of peptide bonds available in the protein, combined to other phenomena such as thermal enzyme inactivation or product inhibition, among others.[35] As for the interactions between enzyme and temperature, the Figure 1 presents two examples of hydrolysis curves, illustrating the single use of subtilisin (Fig. 1a) or trypsin (Fig. 1b) at protein concentration of 5 g/L and increasing reaction temperature (experiments # 6, 10, 21, 25, 36 and 40 in Table 1). Subtilisin cleaves a wide range of peptide bonds, preferably those involving aromatic and methionine residues.[36] This enzyme exhibits high resistance against thermal degradation, presenting maximal proteolitic activity in the interval 50-60ºC [27,35]. Indeed, increasing temperatures favored the reaction rate, so the highest final value of DH was attained at 55ºC (DH = 14.73%). The hydrolysis curve at 55ºC was the only one attaining a steady state from 160 min on, suggesting possible loss of enzyme activity. In contrast, as shown in Fig. 1b, trypsin presented its highest proteolytic activity at 40ºC (final DH = 10.7%) while the curves at 47.5ºC and 55ºC flattened after 30 min, attaining a steady value of DH = 7.4 %. This enzyme presents a narrow selectivity, since it cleaves exclusively peptide bonds with participation of lysine or arginine residues. [37] Furthermore, trypsin is highly susceptible to thermal deactivation, combined to loss of activity by autolysis. Zhang et al.[38] reported maximal proteolytic activity of trypsin at 37-40ºC, which decreased in 50% at 47ºC. This is reflected by the curves at 47.5ºC and 55ºC, which present rapid loss of enzyme activity by thermal inactivation.

Modelization of the degree of hydrolysis

The experimental values of DH at the end of the hydrolysis (4 h) are summarized in Table 1. It can be observed that the final DH varied from 7.34% (single trypsin at 47.5ºC and substrate concentration 5 g/L) to 16.48% (mixture subtilisin-trypsin 1:1 at 40ºC and substrate concentration 2.5 g/L). These experimental data were fitted to the crossed mixture-process model proposed in Eq. 5, obtaining the set of 18 regression coefficients by non linear regression. The statistical significance of each term on the calculated DH was evaluated by the probability value (p-value) at a level of confidence of 95%. Those terms with non-significant impact on the full model (i.e. those

with p-value > 0.05) were removed by backward elimination, obtaining a reduced model with 8 terms, as expressed by Eq. 6:

$$DH = 0.1289 \cdot X_1 + 0.4655 \cdot X_2 - 0.0882 \cdot X_2 \cdot S - 5.25 \cdot 10^{-3} \cdot X_2 \cdot T + 8.01 \cdot 10^{-4} \cdot X_2 \cdot S \cdot T + 4.66 \cdot 10^{-3} \cdot X_2 \cdot S^2 + 7.02 \cdot 10^{-5} \cdot X_1 \cdot X_2 \cdot T - 1.20 \cdot 10^{-6} \cdot X_1 \cdot X_2 \cdot T^2 \quad (6)$$

The goodness of fit of the reduced model was confirmed by the value of the coefficient of determination (R^2 =0.9913, R^2 adjusted to the degrees of freedom = 0.9813) and the distribution of the residuals (i.e. difference between observed and calculated DH), which presented an average value of 1.2803 ± 0.9590 %.

The predicted model allowed the generation of the contour plots presented in Figure 2, where the final degree of hydrolysis calculated by Eq. 6 was plotted against the temperature and the percentage of subtilisin in the enzyme mixture at the three levels of substrate concentration assayed (i.e. 2.5, 5 and 7.5 g/L). The highest values of final DH were attained at 2.5 g/L of substrate in the reaction mixture (Fig. 2a). The optimization procedure calculated a maximum value of DH (15.94%), attained at 2.5 g/L, 40ºC and 41.2% of subtilisin in the enzyme mixture. This value is the absolute maximum for DH inside the experimental range of temperature, enzyme composition and substrate assayed. The current results are in line with a previous work by Morales-Medina et al.[20], where DH was modeled by an artificial neuronal network comprising 10 neurons in the hidden layer. In that case, the model which also included the time as a variable, predicted an absolute optimum for DH (17.1%) at 2.54 g/L of substrate concentration, 40ºC and 38.3% of subtilisin in the enzyme mixture

Overall, the final values of DH for 5 g/L (Fig. 2b) were significantly inferior, ranging between 8% and 13.5%. Similarly, the final values of DH were slightly improved in the case of 7.5 g/L (10-13.7%, Fig. 2c). These results are in agreement with the mathematical model, where several interactions between trypsin and substrate concentration are significant on the response variable. Indeed, the interaction $X_2 \cdot S$ affected negatively the final DH, while $X_2 \cdot S \cdot T$ and $X_2 \cdot S^2$ did positively. No interaction between subtilisin and substrate concentration was significant on the

response variable. The inhibitory effect of substrate on the proteolysis has been reported for fish protein hydrolysates,[35,39] and could explain the optimum DH at substrate concentration 2.5 g/L. Effectively, increasing levels of substrate slowed the reaction rate, leading to lower values of final DH. However, this negative modulation vanished at substrate concentrations above 5 g/L. Furthermore, high levels of substrate above 6.5 - 7 g/L exerted a positive effect on the final DH, especially in combination with low temperatures (40ºC – 47.5ºC). To this regard, Valencia et al.[35] reported that increasing levels of substrate (salmon muscle) protected Alcalase against thermal denaturation. In this case, this effect was more remarkable for trypsin and vanished when reaction temperatures above 50ºC were employed.

The mathematical model predicts a synergic effect between subtilisin and trypsin on the DH, modulated by the reaction temperature, as confirmed by the significant interactions $X_1 \cdot X_2 \cdot T$ and $X_1 \cdot X_2 \cdot T^2$. Overall, the combination of subtilin and trypsin improved the final DH, in comparison with their single use. This synergy has been reported in previous studies on other fish species such as sardine, mackerel or Mediterranean horse mackerel [13]. As shown in the contour plots, the optimal values of DH correspond to combinations of both enzymes. As the reaction temperature increased, so did the percentage of subtilisin required for maximal DH. For instance, as shown in Fig. 2a, the maximal DH reachable at 40ºC required 41.2% of subtilisin in the enzyme preparation, while this percentage rose to 66.7% (50ºC) and 100% at 55ºC. As mentioned above, this is attributed to the different thermal stability of subtilisin and trypsin.

Modelization of the ACE inhibitory activity

The observed values of ACE inhibition for the final hydrolysates (Table 1) ranged from 30.1% (single trypsin at 40ºC and substrate concentration 7.5 g/L) to 56.12% (single trypsin at 55ºC and substrate concentration 2.5 g/L). The ACE inhibitory activity of the final hydrolysate was fitted to the operation conditions by means of the crossed model expressed by Eq. 5. A set of 18 coefficients was estimated, whose statistical significance was evaluated by the associated probabilities. A backward elimination procedure was employed to remove those terms with associated probability higher than 5%. In contrast with the DH model, the reduced model for ACE inhibition was more

278 complex, comprising 11 terms and significant interactions of third ($X_1 \cdot X_2 \cdot S$, $X_1 \cdot X_2 \cdot T$) and fourth
279 order ($X_1 \cdot X_2 \cdot T^2$).

$$ACE = 0.3708 \cdot X_1 - 2.61 \cdot 10^{-4} \cdot X_1 \cdot S \cdot T + 4.28 \cdot 10^{-5} \cdot X_1 \cdot T^2 + 0.0139 \cdot X_2 \cdot T - 1.77 \cdot 10^{-3} X_2 \cdot S \cdot T + 5.38 \cdot 10^{-3} X_2 \cdot S_2^2$$
$$+ 0.0266 \cdot X_1 \cdot X_2 - 5.49 \cdot 10^{-3} \cdot X_1 \cdot X_2 \cdot S - 4.19 \cdot 10^{-4} X_1 \cdot X_2 \cdot T + 3.71 \cdot 10^{-5} \cdot X_1 \cdot X_2 \cdot S \cdot T + 3.84 \cdot 10^{-4} \cdot X_1 X_2 \cdot S^2 \quad (7)$$

282 As expected, the goodness of fit of this model was higher than that of the DH. Indeed, the
283 coefficient of determination was R^2=0.9972 (R^2 adjusted to the degrees of freedom = 0.9963). As
284 for the residuals, they presented an average value of 2.4333 ± 1.7581 %. The contour plots shown in
285 Figures 3a-3c illustrate the influence of operation conditions on the ACE inhibitory activity of the
286 final hydrolysate. The highest levels of ACE inhibition were observed at 2.5 g/L of substrate, 55ºC
287 and only trypsin in the enzyme preparation (56.12%, experiment 35 in Table 1). This value was
288 confirmed by the regression model. Indeed, the contour plot at 2.5 g/L (Fig. 3a) shows that the ACE
289 inhibition increased with both the percentage of trypsin in the enzyme mixture and the temperature,
290 reaching an absolute maximum of ACE inhibition (55.3%) at 2.5 g/L, 55ºC and 100% of trypsin.
291 Under these conditions (i.e. trypsin at 55ºC) the extent of the proteolysis was limited, with final DH
292 around 9%. As shown in the contour 3a, levels of ACE inhibition above 50% were detected for
293 tryptic hydrolysates processed above 48ºC. Increasing amounts of subtilisin in the enzyme mixture
294 allowed obtaining similar inhibitory levels, at the expense of higher processing temperatures (e.g.
295 50% ACE inhibition at 55ºC and 18.5% of subtilisin).

296 The ACE inhibitory effect of a given hydrolysate is favored by the presence of peptides containing
297 hydrophobic residues (e.g. Pro, Phe, Tyr) in the tripeptide sequence at the C-terminal end, since it
298 facilitates the interaction with the active site of the Angiotensin I Converting Enzyme [7]. To this
299 regard, it is reported that subtilisin cleaves preferably peptide bonds with participation of
300 hydrophobic residues [40], while trypsin does specifically with those containing arginine and lysine
301 residues.[37] Although both amino acids are charged positively, they are reported to favor ACE
302 inhibition when placed at the C- terminus [7]. Furthermore, Cheison et al.[41] affirmed that trypsin
303 behaves like chymotrypsin at high temperatures (i.e. above 50ºC). As a result, new peptides with
304 hydrophobic residues (Tyr, Trp, Phe) at the carboxyl side are released to the medium. In our case,

these latter may be responsible for the enhanced inhibitory potency observed for the tryptic hydrolysates above 45ºC.

At low temperatures, where trypsin displays the highest specificity, the inhibitory activity of the hydrolysate was favored by increasing levels of subtilisin in the enzyme preparation (e.g. 46% ACE inhibition at 40ºC and 21.6% of subtilisin). According to the predictive model, a local maximum (48.2% of ACE inhibition) was detected at 40ºC by employing a mixture 1:1 of both enzymes. As observed in the contour in Figure 2a, these conditions favored the extensive hydrolysis of the substrate, attaining values of final DH above 15.8%. Although the potential ACE inhibitory effect of a given peptide depends on a range of factors (e.g. residue composition, hydrophobicity), most of the active peptides identified to date are di- and tripeptides.[42,43] It is expectable that increasing DH would decrease the average size of the resulting peptides and thus contribute to the presence of potentially ACE inhibitors.

The levels of ACE inhibitory activity of the final hydrolysates obtained at substrate concentration 5 g/L and 7.5 g/L (contours in Figures 3b and 3c, respectively) were significantly lower, ranging from 32% to 40%. Under these conditions, the extent of the proteolysis was limited (contours in Figures 2b and 2c), which could be related to the lower inhibitory activity of the resulting hydrolysates.

In vitro digestion of the hydrolysates

The hydrolysates with the highest ACE inhibitory activity for each reaction temperature assayed (i.e. those produced by experiments no. 3, 20 and 35 in Table 1) were selected for assessing the effect of gastrointestinal enzymes on the ACE inhibitory activity. The size distribution profiles of both the crude hydrolysates (solid line) and their digests (dotted line) are shown in the Figures 4a to 4c. The SEC profiles identified four main fractions, whose percentages areas are listed in Table 2.

The effect of digestive enzymes on the ACE inhibitory activity was assessed by determining the IC_{50} value of both the crude hydrolysates and their digests (Table 2). The IC_{50} values of the selected crude hydrolysates ranged between 253 and 330 µg/mL (experiments 35 and 3, respectively). These values were better than those obtained for Mediterranean horse mackerel using combination 1:1 of

trypsin and subtilisin [44]. In contrast, higher ACE inhibitory potential was found in thermolysin hydrolysates of salmon.[45] Since the variations between the crude and the digested hydrolysates were not significant (Table 2), it was concluded that the ACE inhibitory activity of the selected hydrolysates was not altered by digestive enzymes. This is a very interesting feature, since gastrointestinal digestion is one of the main processes reducing the bioavailability of bioactive peptides. Indeed, many peptides showing high levels of *in vitro* ACE inhibitory activity failed to show *in vivo* effect due to their degradation by digestive enzymes.[46] Only a few studies have dealt with the effect of digestive enzymes on the ACE inhibitory activity of fish hydrolysates. In line with our results, Hwang[47] reported that ACE inhibitory activity of tuna cooking juice did not change after gastrointestinal digestion. Similar trend was described by Samaranayaka et al.[48] for Pacific hake autolisates, while Cinq-Mars et al.[49] found that Pacific hake hydrolysates increased its ACE inhibitory activity after simulated digestion.

Regarding the variation in molecular size distribution, all the fractions in the hydrolysate no. 3 (50% subtilisin-50% trypsin) except fraction A were altered during the digestion process. However, the final IC_{50} value remained constant. This can be explained because the degradation of active peptides in fraction B and C would be balanced with the generation of new active peptides. This behavior has been also suggested by Samaranayaka et al.[48] for pacific hake hydrolysates.

The crude hydrolysates no. 20 and 35 presented different size exclusion profiles, except for the fraction C (337 – 172 Da) which accounted for 15% of the total area. However, both samples presented similar values of IC_{50}. It may be concluded that the ACE inhibitory activity of these hydrolysates would be mainly determined by the fraction C (337-172 Da), which might contain di- and tripeptides as major species. Indeed, small peptides of 2 or 3 amino acids are usually identified as potent ACE inhibitory peptides[45,50]. The IC_{50} values for both tryptic hydrolysates remained unaltered after the simulated digestion. Furthermore, the profiles in Fig 4b and 4c showed no significant differences in the percentage area of fractions B and C after digestion. These results agree with previous studies where Mediterranean horse mackerel was hydrolyzed employing subtilisin and trypsin. In this work, some low molecular active peptides, such as HLALT, RQLAGP

and ELSAP, were identified[44] as potential ACE inhibitors. In addition, the low ACEI variation between crude and digested hydrolysates has been previously explained by Salampessy et al.[51], who reported that the fractions of trevally hydrolysates containing di- and tripeptides maintained its inhibitory potency after gastrointestinal digestion. More generally, Seki et al.[52] concluded that short chain peptides derived from sardine resisted the digestion process without modification.

CONCLUSIONS

The crossed mixture process model chosen in this work fitted adequately the observed data of final DH and ACE inhibition, with determination coefficients R^2=0.9913 and R^2=0.9972, respectively. This model predicted a maximal DH (15,94%) at 2.5 g/L of substrate concentration, 40ºC and an enzyme mixture comprising 41.2% of subtilisin and 59.8% of trypsin. The hydrolysis curves confirmed that the reaction rate was inhibited by increasing levels of substrate concentration, as well as the higher proteolytic activity of subtilisin. Nevertheless, the addition of trypsin to the enzyme mixture allowed obtaining higher degrees of hydrolysis at lower temperatures than those achieved when only subtilisin was employed. Levels of ACE inhibition above 50% were obtained for trypsin hydrolysates at the lowest substrate concentration and temperatures over 48ºC, which was attributed to the increasing affinity of trypsin towards peptide bonds containing hydrophobic residues such as Tyr, Trp or Phe. At lower temperatures the selectivity of trypsin was restrained to Arg and Lys residues, and a maximum of 48.2% ACE inhibition was predicted at 40ºC employing a mixture 1:1 of both endoproteases. Size exclusion chromatograms confirmed that the fractions containing small-sized peptides (e.g. di- and tripeptides) were mainly responsible for the ACE inhibitory activity. Furthermore, the experiments of simulated digestion confirmed that these fractions were resistant to digestive enzymes. Indeed, no significant differences in the levels of ACE inhibition were detected between the raw hydrolysates and their digests.

ACKNOWLEDGEMENTS

This work was supported by the Regional Plan Junta de Andalucía (project P12-AGR-1993) and the Spanish National Plan I+D+i (project CTQ2014-53615-R). R. Morales-Medina acknowledges a FPI grant from the Spanish Ministry of Science and Innovation.

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TABLE AND FIGURE CAPTIONS

Table 1. Experimental design and measured values for the final DH and ACE inhibition.

Table 2. ACE inhibitory activity and area percentage of the SEC-fractions of the raw hydrolysates no. 3 (2.5 g/L, 40ºC, 50% subtilisin), no. 20 (2.5 g/L, 47.5ºC, 100% trypsin) and no. 35 (2.5 g/L, 55ºC, 100% trypsin) and their respective digests. Mean values with different superscript letters are significantly different ($P < 0.05$)

Figure 1. Effect of individual enzymes and reaction temperature on the hydrolysis curves: (a) 5 g/L of protein with subtilisin at enzyme-substrate ratio of 1% w/w; (b) 5 g/L of protein with trypsin at enzyme-substrate ratio of 1% w/w.

Figure 2. Contour plots for the final degree of hydrolysis (4 h) as a function of the reaction temperature and the percentage of subtilisin in the enzyme mixture at three levels of substrate concentration: (a) 2.5g/L (b) 5 g/L and (c) 7.5 g/L.

Figure 3. Contour plots for the ACE inhibition as a function of the reaction temperature and the percentage of subtilisin in the enzyme mixture at three levels of substrate concentration: (a) 2.5g/L (b) 5 g/L and (c) 7.5 g/L.

Figure 4. Molecular size distribution of the selected hydrolysates and their respective digests: (a) experiment no. 3 (2.5 g/L, 40ºC, 50% subtilisin), (b) experiment no. 20 (2.5 g/L, 47.5ºC, 100% trypsin) and (c) experiment no. 35 (2.5 g/L, 55ºC, 100% trypsin). Dotted lines represent the digested samples.

562 Table 1. Experimental design and measured values for the final DH and ACE inhibition

563

Experimental run	Substrate concentration S (g/L)	Temperature T(ºC)	Percentage subtilisin X_1(%)	Percentage tripsin X_2(%)	Final DH (%)	ACE Inhibition (%)
1	2.5	40	100	0	13.24	40.40
2	2.5	40	75	25	13.4	45.33
3	2.5	40	50	50	16.48	47.33
4	2.5	40	25	75	15.91	43.45
5	2.5	40	0	100	13.4	40.27
6	5	40	100	0	12.74	37.50
7	5	40	75	25	14.12	39.93
8	5	40	50	50	13.19	37.87
9	5	40	25	75	12.97	31.29
10	5	40	0	100	10.7	36.78
11	7.5	40	100	0	13.04	37.40
12	7.5	40	75	25	12.17	39.06
13	7.5	40	50	50	13.71	34.33
14	7.5	40	25	75	13.15	38.94
15	7.5	40	0	100	8.97	30.09
16	2.5	47.5	100	0	14.15	43.84
17	2.5	47.5	75	25	14.81	48.33
18	2.5	47.5	50	50	15.09	43.14
19	2.5	47.5	25	75	14.32	47.26
20	2.5	47.5	0	100	11.16	48.66
21	5	47.5	100	0	13.37	38.74
22	5	47.5	75	25	10.5	32.65
23	5	47.5	50	50	11.61	30.71
24	5	47.5	25	75	9.49	31.02
25	5	47.5	0	100	7.34	38.43
26	7.5	47.5	100	0	13.01	37.59
27	7.5	47.5	75	25	10.85	34.29
28	7.5	47.5	50	50	14.05	37.28
29	7.5	47.5	25	75	12.74	36.48
30	7.5	47.5	0	100	9.17	32.87
31	2.5	55	100	0	12.24	45.93
32	2.5	55	75	25	11.59	44.04
33	2.5	55	50	50	12.12	42.18
34	2.5	55	25	75	12.24	50.23
35	2.5	55	0	100	8.9	56.12
36	5	55	100	0	14.73	44.32
37	5	55	75	25	9.7	33.70
38	5	55	50	50	8.69	35.32
39	5	55	25	75	10.96	33.27
40	5	55	0	100	7.39	38.04
41	7.5	55	100	0	13.89	39.18
42	7.5	55	75	25	13.37	36.57
43	7.5	55	50	50	12.29	36.71
44	7.5	55	25	75	13.98	31.22
45	7.5	55	0	100	10.14	35.76

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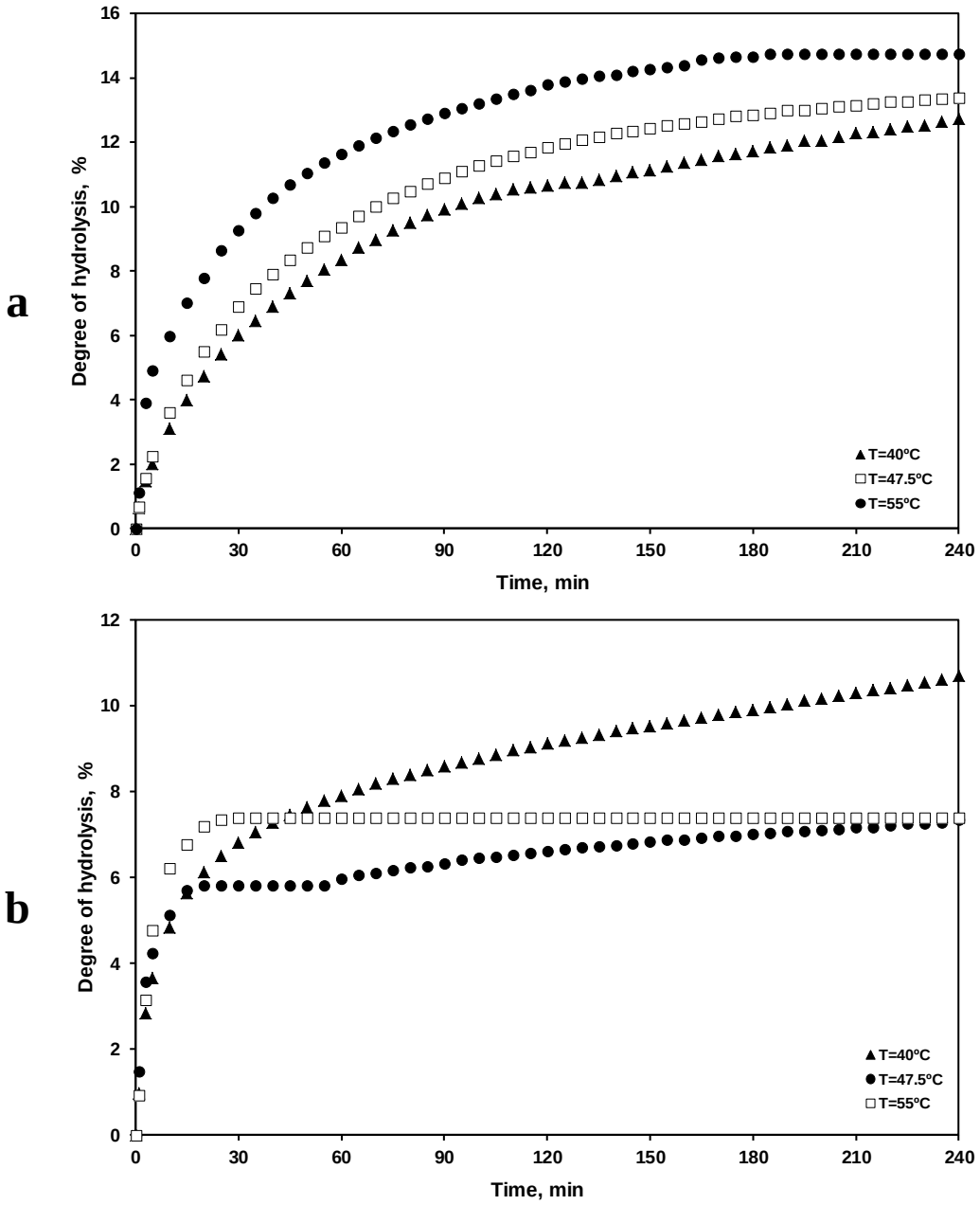
Table 2. ACE inhibitory activity and area percentage of the SEC-fractions of the raw hydrolysates number 3 (2.5 g/L, 40ºC, 50% subtilisin), 20 (2.5 g/L, 47.5ºC, 100% trypsin) and 35 (2.5 g/L, 55ºC, 100% trypsin) and their respective digests. Mean values in the same column with different superscript letters are significantly different ($P < 0.05$)

Sample	ACEI activity	Area percentage of fractions			
	IC_{50} (µg/mL)	Fraction A (>734 Da)	Fraction B (734-337 Da)	Fraction C (337-172 Da)	Fraction D (<172 Da)
3	330.2 ± 10.2^{a}	20.1 ± 1.7^{a}	21.8 ± 1.4^{a}	18.2 ± 0.7^{a}	39.8 ± 0.3^{a}
3d*	314.6 ± 14.2^{a}	22.5 ± 1.1^{ab}	18.9 ± 1.1^{b}	15.7 ± 1.4^{b}	42.9 ± 0.7^{b}
20	272.1 ± 11.1^{b}	27. ± 0.5^{c}	19. ± 0.5^{b}	15. ± 1.5^{bc}	39. ± 1.1^{a}
20d	261.3 ± 30.3^{b}	23.6 ± 0.6^{bd}	18.4 ± 0.5^{b}	13.4 ± 1.5^{c}	44.6 ± 0.2^{c}
35	253.0 ± 29.1^{b}	32.8 ± 1.0^{e}	16.4 ± 0.8^{c}	15.9 ± 0.5^{b}	34.9 ± 0.4^{d}
35d	265.6 ± 13.7^{b}	25.4 ± 1.8^{cd}	17.7 ± 0.1^{bc}	15.2 ± 0.3^{bc}	41.6 ± 0.8^{e}

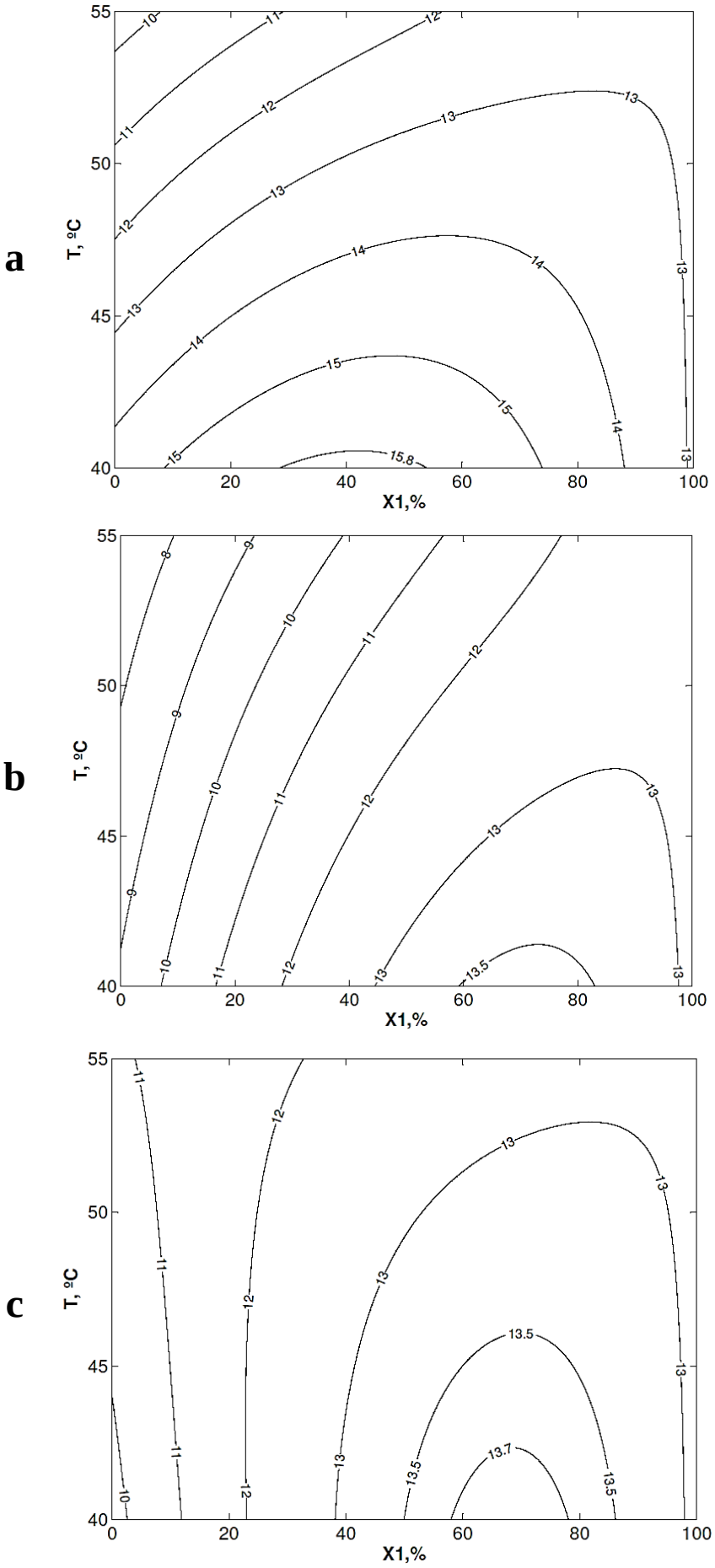
* Samples after simulated digestion.

586 Figure 1. Effect of individual enzymes and reaction temperature on the hydrolysis curves: (a) 5 g/L
587 of protein with subtilisin at enzyme-substrate ratio of 1% w/w; (b) 5 g/L of protein with trypsin at
588 enzyme-substrate ratio of 1% w/w.



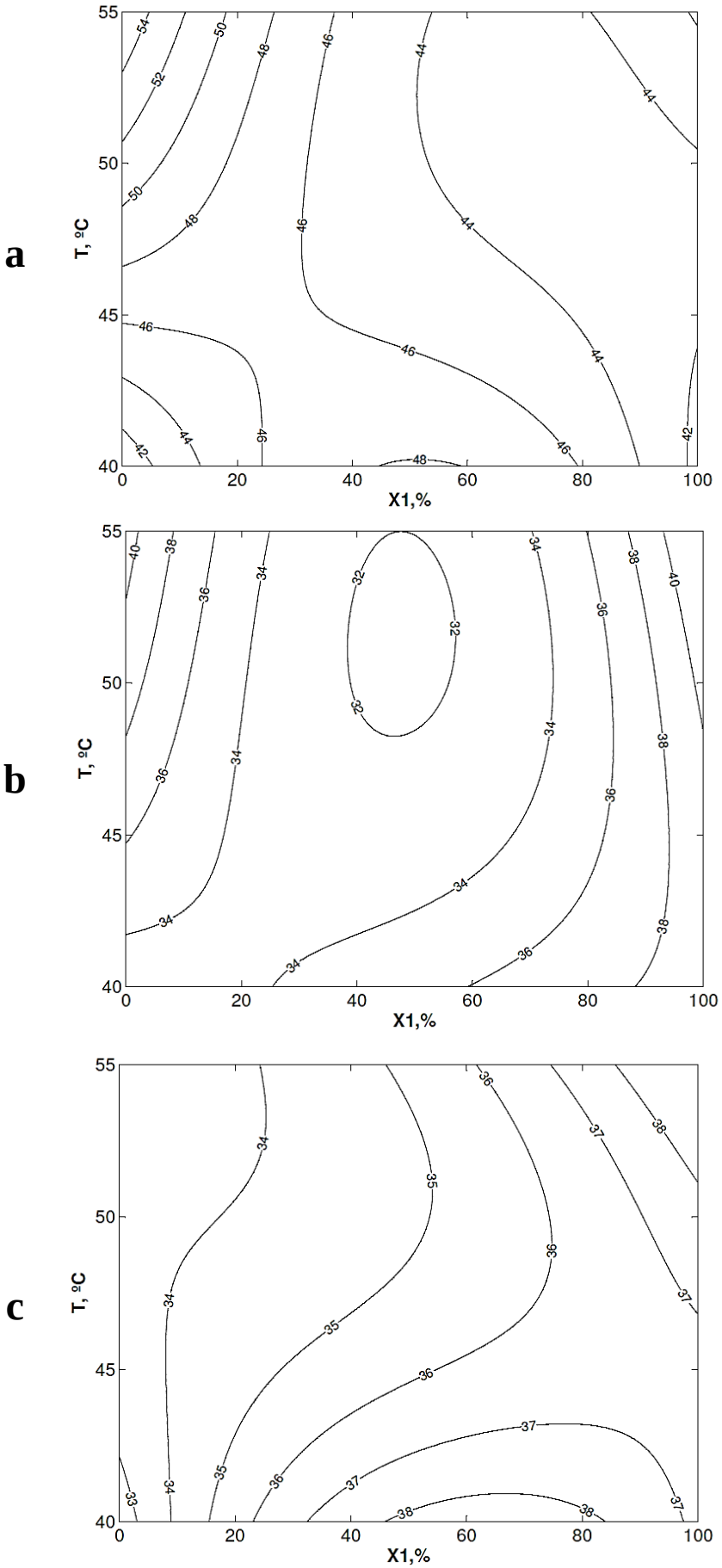
589

590 Figure 2. Contour plots for the final degree of hydrolysis (4 h) as a function of the reaction
591 temperature and the percentage of subtilisin in the enzyme mixture at three levels of substrate
592 concentration: (a) 2.5g/L (b) 5 g/L and (c) 7.5 g/L.



593

Figure 3. Contour plots for the ACE inhibition as a function of the reaction temperature and the percentage of subtilisin in the enzyme mixture at three levels of substrate concentration: (a) 2.5g/L (b) 5 g/L and (c) 7.5 g/L.



598 Figure 4. Molecular size distribution of the selected hydrolysates and their respective digests: (a)
599 experiment no. 3 (2.5 g/L, 40ºC, 50% subtilisin), (b) experiment no. 20 (2.5 g/L, 47.5ºC, 100%
600 trypsin) and (c) experiment no. 35 (2.5 g/L, 55ºC, 100% trypsin). Dotted lines represent the digested
601 samples.

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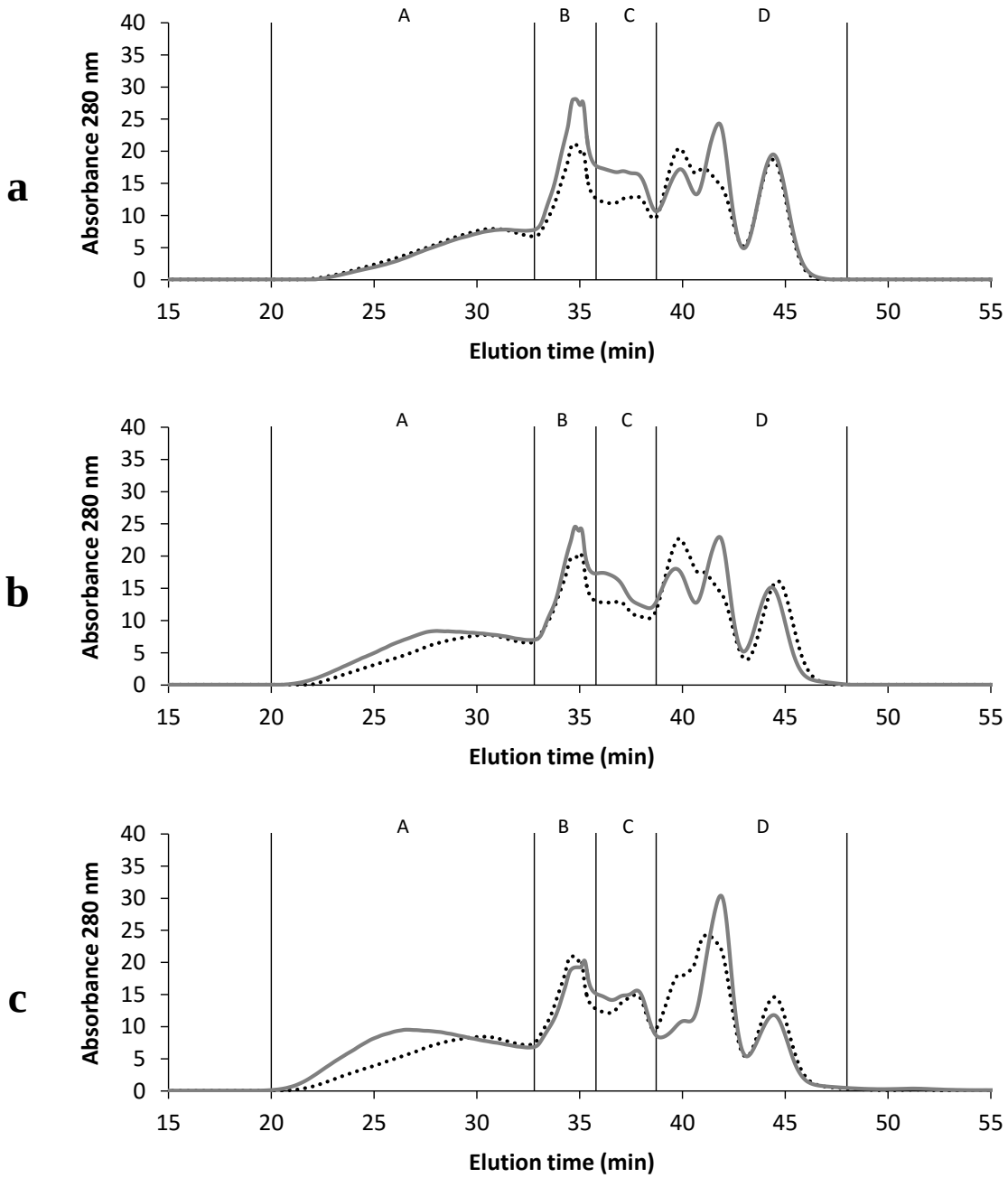

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