



Analytical Methods

Development of a standardised human *in vitro* digestion protocol based on macronutrient digestion using response surface methodology[☆]Sylvie Hollebeeck^a, Florianne Borlon^a, Yves-Jacques Schneider^a, Yvan Larondelle^{a,*}, Hervé Rogez^b^a Institut des Sciences de la Vie & UCLouvain, B-1348 Louvain-la-Neuve, Belgium^b Universidade Federal do Pará & Centre for Agro-food Valorisation of Amazonian Bioactive Compounds (CVACBA), 66.095-780 Belém-PA, Brazil

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ABSTRACT

Bioaccessibility studies should be taken into account when evaluating the physiological effects of ingested compounds at the intestine level. Several *in vitro* digestion protocols have been described, with a wide range of experimental conditions but no optimised protocol exists. In order to fill in this gap, we evaluated the influence of three continuous factors (pH, incubation time, and enzyme concentrations), in the range of values found in literature, on the digestion of standard macronutrients (starch, albumin, triolein) alone or in mixture. Three central composite designs, using response surface methodology, were employed to model the three abiotic steps of pre-colonic digestion. A validated *in vitro* digestion was eventually set up for the salivary step (pH 6.9, 5 min, 3.9 units α -amylase/ml), the gastric step (pH 2, 90 min, 71.2 units pepsin/ml), and the abiotic duodenal step (pH 7, 150 min, 9.2 mg pancreatin and 55.2 mg bile extract/ml).

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1. Introduction

Many *in vitro* digestion models are currently used as alternatives to *in vivo* experiments to study the intestinal bioaccessibility of food, xenobiotics and drugs. Nevertheless, a standardised human *in vitro* digestion system that correlates with *in vivo* results is still lacking. Indeed, a large range of digestion durations, pH levels, concentrations and compositions of digestive enzymes, and food matrices tested has been used. Since these factors have a significant influence on the results obtained with *in vitro* digestion protocols, the published results are difficult to compare (Hur, Lim, Decker, & McClements, 2011), as already highlighted by Oomen et al. (2003) for the bioaccessibility of soil contaminants.

Abbreviations: BSA, bovine serum albumin; CCD, central composite design; C_{enz} , enzymatic concentration; FFA, free fatty acid; FID, flame ionisation detector; GC, gas chromatography; PBS, phosphate buffer saline; SPE, solid phase extraction; RSM, response surface methodology; TCA, trichloroacetic acid; t_{dig} , time of digestion; TG, triacylglycerol; USP, United States Pharmacopeia.

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Almost all authors have worked with times ranging from 5 min to 3 times that length (15 min) for salivary digestion, from 30 min to 6 times that length (180 min) for gastric digestion, and from 60 min to 6 times that length (360 min) for duodenal digestion (Hur et al., 2011). In general, the pH levels reported in the literature for salivary, gastric, and duodenal digestion have presented a low variation, ranging from 5.0 (Minekus, Marteau, Havenaar, & Huis in't veld, 1995) to 6.9 (Lebet, Arrigoni, & Amando, 1998) for the salivary step, from 1.1 (Oomen et al., 2003) to 2.8 (Alexandropoulou, Komaitis, & Kapsokafalou, 2006) for the gastric step, and from 6.3 (Alexandropoulou et al., 2006) to 7.8 (Oomen et al., 2003) for the duodenal step. As for the digestive enzymes, that are usually considered as the most important factors in the *in vitro* digestion protocols, large differences in the concentration and composition have been noted between protocols, in addition to a lack of precision about enzymatic activity. As recently reviewed by Hur et al. (2011), the most frequently used enzymes or enzymatic mixtures are (i) α -amylase for the salivary step, (ii) pepsin for the gastric one and (iii) pancreatin, trypsin, chymotrypsin, peptidase, and lipase for the duodenal one. The enzymatic concentrations used are expressed in mg of solid per ml, making the protocols difficult to compare because no information is available on enzymatic activity in units per mg of enzymatic protein. Enzymatic concentrations are better expressed in units/ml (King & Campbell, 1961) within the standard conditions defined by the supplier.

The aim of this study was to set up a standardised, rapid, and low-cost *in vitro* digestion protocol consisting of three consecutive steps—the salivary, gastric, and duodenal steps—to mimic, in a simple way, the physiological pre-colonic human digestion in normal conditions. For this purpose, three standard macronutrients were selected and tested alone for the protocol set up (starch for the salivary step, bovine serum albumin (BSA) for the gastric step, and triolein for the duodenal step) and then in a mixture for validation purposes. The nutrient concentrations were chosen to take into account the volumes of ingested and secreted fluids, as well as the relative proportions of the three major classes of dietary macronutrients. For a standard meal, without any distinction for the three meals, it can be considered that an adult should eat approximately 120 g of carbohydrates, 25 g of protein, and 25 g of lipids, leading to an energetic daily contribution of 60% for carbohydrates, 12% for protein, and 28% for lipids if an average energetic consumption of ca. 2400 kcal is considered. In 24 h, a human drinks roughly 2 l, and secretes 1 l of saliva, 2 l of gastric juice, 1 l of bile, 2 l of pancreatic juice, and 1 l of intestinal juice. Considering an average of three meals per day, the volume of chyme content in each digestive compartment can be easily calculated (Tortora & Grabowski, 1996).

Three key factors involved in digestion were examined: pH, duration of incubation (t_{dig}), and digestive enzyme concentration (C_{enz}). First, different values of these three factors, within the range of values found in the *in vitro* protocols described in the literature, were used with three separate central composite designs (CCD) (for the three steps of digestion), with the goal of evaluating the impact of these variations on the results of digestion of the respective substrate, *i.e.* starch, BSA or triolein. The enzymes and biological fluids used were the followings: α -amylase for the salivary step, pepsin for the gastric step, and a mixture of pancreatin and bile salts for the duodenal step. Second, values of these three factors were selected using response surface methodology (RSM) to approach as much as possible a standard human digestion. Finally, after verifying the adequacy of the selected values for each step independently, the three successive digestion steps were validated by digestion of the simple food matrix (the mixture of starch, BSA and triolein). This well-defined and easy-to-perform *in vitro* digestion protocol could be particularly useful for a rapid and inexpensive evaluation of the abiotic part of the gastro-intestinal fate of food items and natural extracts.

2. Materials and methods

2.1. Digestive enzymes

All digestive enzymes and biological fluids were purchased from Sigma–Aldrich (Saint-Louis, MO). α -Amylase from human saliva (type IX-A, 210 units/mg solid, 2400 units/mg protein) was dissolved in the phosphate buffer saline (PBS) solution to obtain a stock solution of 90 units/ml and was stored at -20°C until use (no decrease of enzymatic activity was observed in these conditions, data not shown). Porcine pepsin from gastric mucosa (1:10,000, 460 units/mg solid, 1020 units/mg protein) was dissolved in HCl 0.1 M. Because pepsin activity may decrease over time, a solution was freshly prepared for each experiment. Pancreatin from porcine pancreas (activity equivalent to 4 $\times$ United States Pharmacopeia [USP] specifications) was used as a source of lipase and colipase. Pancreatin was mixed with a porcine bile extract mixture at a constant ratio of 1:6 and was freshly prepared before use. Pancreatin contains several enzymes, including amylase, trypsin, lipase, ribonuclease, and protease. Bile extract contains glycine and taurine conjugates of hyodeoxycholic acid, and other bile salts.

2.2. Standard macronutrients and simple food matrix

For the salivary step, the substrate used was starch from wheat (Merck, Darmstadt, DE). At this step, the carbohydrates are considered to be in contact with 1 l of fluid per meal, corresponding to the beverage and the salivary juice, which leads to a physiological representative starch concentration of 90 mg/ml PBS. In our experiments, the initial starch concentration was however reduced to 30 mg/ml to allow a complete dissolution in PBS. This solution was heated at 90°C with closed cap until complete gelatinisation on the day before the experiment, and was immediately stored at 4°C until used. A new solution was prepared before each experiment. For the gastric step, the substrate used was BSA from Sigma–Aldrich (Cohn V fraction). At this step, the proteins are considered to be in contact with fluids provided by the beverages, as well as by the salivary and gastric juice inputs, corresponding to a theoretical physiological volume of 1.67 l per meal, and leading to an initial BSA solution of 15 mg/ml PBS. For the duodenal step, the substrate used was triolein (Sigma–Aldrich). At this step, the lipids are considered to be in contact with fluids provided by the beverages, as well as by the salivary, gastric, pancreatic, and intestinal juices, corresponding to the theoretical volume of 3 l per meal, and leading to an initial triolein suspension of 8.33 mg/ml PBS.

The simple food matrix was a mixture of the three macronutrients containing 30 mg of wheat starch, 15 mg of BSA, and 8.33 mg of triolein per ml of PBS.

2.3. *In vitro* digestion

Each digestion step was optimised separately. All incubations were performed in 25 ml amber bottles. For all steps, the fixed parameters were the respective initial concentrations of the substrates of digestion, the temperature (37°C), and the applied constant magnetic stirring of 350 rpm. For the salivary step, the incubation volume was kept constant at 10.43 ml. The pH was adjusted to the experimental values defined in the CCD, by adding HCl 1 M. The α -amylase concentrations were achieved by the addition of a certain volume of the stock enzymatic solution (90 units/ml). For the gastric step, the incubation volume was kept constant at 12.30 ml. Different pepsin solutions were prepared in HCl 0.1 M and combined with the BSA solution to reach the expected initial C_{enz} . The pH was adjusted with HCl 1 M. For the duodenal step, the incubation volume was kept constant at 8.36 ml. Different enzymatic solutions, containing pancreatin and bile extract, were prepared in NaHCO_3 0.1 M and combined with the triolein suspension to obtain the expected C_{enz} just before the experiment. The pH was adjusted with NaHCO_3 1 M. For the salivary step, the bottles were left with air to mimic aerobic conditions, while anaerobic conditions were mimicked for the gastric and duodenal steps by flushing N_2 . For the salivary and gastric steps, samples were withdrawn, transferred to glass tubes, and dipped into a 90°C water bath for 10 min to stop the enzymatic reactions, cooled at room temperature, and immediately analysed. For the duodenal step, samples were withdrawn, transferred to Pyrex tubes, and directly dipped into a liquid nitrogen bath to stop the enzymatic activities before being stored at -80°C for further analyses.

2.4. Analyses of final products of digestion

2.4.1. Starch digestion

Starch digestion was monitored by the Bernfeld assay (Bernfeld, 1955), a colorimetric method that measures the reducing capacity of sugars, using a calibration curve with different maltose concentrations. Maximal digestion of starch corresponded to a calculated thorough hydrolysis of the starch solution into maltose moieties.

For the different experimental conditions of the salivary CCD, samples were expressed in percentage of maximal starch digestion. The reducing capacity of the initial starch solution was measured and subtracted from each sample value.

2.4.2. Bovine serum albumin digestion

BSA digestion was monitored by the absorbance at 280 nm of the supernatant left after protein precipitation with trichloroacetic acid (TCA) (Sigma–Aldrich). A TCA solution in water (20%, w/v) was added to the incubation sample to reach a final concentration of 0.2 g/ml, which allowed an optimal protein precipitation (Sivaraman, Kumar, Jayaraman, & Yu, 1997). The mixture was left on ice for 10 min, and centrifuged at 10,580g and 4 °C for 10 min. Previously, kinetic curves were obtained with the three initial pepsin concentrations used in the CDD (15, 45, and 125 units/ml) at pH 2. After different durations of incubation, the same maximum of absorption was reached with the three pepsin concentrations and was considered as the maximal BSA digestion. For the different experimental conditions of the gastric CCD, samples were expressed in percentage of maximal hydrolysis of the peptidic bonds of BSA.

2.4.3. Triolein digestion

Triolein digestion leads to the release of free oleic acid (C18:1, cis9), which was assayed by gas chromatography (GC). Before GC analyses, total lipids were extracted, following the method of Bligh and Dyer (1995) with a chloroform:methanol:water (2:2:1.8, v:v:v) solvent solution. The free fatty acid (FFA) fraction was then separated from the rest of the lipid fraction (also containing neutral lipids and phospholipids) by SPE (Bond Elut–NH2, 200 mg, 3 ml; Varian, Palo Alto, CA) according to Kaluzny, Duncan, Merritt, and Epps (1985). Methylation of the FFA fraction was carried out with the addition of 1 ml KOH 0.1 M in methanol, incubation for 1 h at 70 °C, with regular vortexing, followed by the addition of 0.4 ml of 1.2 M HCl in methanol for 15 min. A final addition of 2 ml of hexane allowed the extraction of fatty acid methyl esters, which were then separated by GC. The GC Trace system (Thermo Finnigan, Milan, IT) was equipped with a flame ionization detector (FID) and a GC PAL autosampler (CTC analytics, Zwingen, CH). Separation was carried out on a RT2560 capillary column (100 m × 0.25 mm internal diameter, 0.2 µm film thickness; Restek, Bellefonte, PA) using hydrogen as the carrier gas at a constant velocity of 35 ml/min and a pressure of 200 kPa. The injection volume was 1 µl. The FID temperature was 255 °C. During the process, the temperature was initially 80 °C; it increased up to 175 °C at a 25 °C/min^{−1} progression, and it was held at 175 °C for 25 min, followed by an increase of 10 °C/min until it reached 205 °C. This temperature was maintained for 4 min until a new 10 °C/min progression up to 225 °C. This temperature was held at 225 °C for 20 min before decreasing at a 20 °C/min progression until the initial temperature of 80 °C was reached. Prior to injection, the samples were spiked with a defined concentration of the external standard, methylated undecanoic acid (C11:0). Free oleic acid was identified by comparison with a standard injection of oleic acid methyl ester, and was quantified using peak area.

2.5. Experimental design for the response surface procedure

For each step of digestion, a three-factor CCD was obtained with the help of the Minitab® version 15 software (trial version) to study the effects of the three continuous factors under investigation, pH, t_{dig} , and C_{enz} , on the substrates of digestion (starch, BSA, and triolein). Each model was a one-block face-centred ($\alpha = 1$) CCD for three numerical factors, conducted to a 2³ factorial design with six additional axial points coded $\pm\alpha$ and three central points. Three CCDs were then designed, respectively, for the salivary, gastric, and

Table 1

Central composite design setting in original and coded forms^a of the independent variables (X_{S1} , $\text{Log}X_{S2}$, and $\text{Log}X_{S3}$) and experimental results for the salivary (Y_S) digestion of starch.

Run order	X_{S1}^b	$\text{Log}X_{S2}^c$	$\text{Log}X_{S3}^c$	Y_S [%]
1	−1 (5)	−1 (0.699)	−1 (−0.523)	1.23
2	−1 (5)	−1 (0.699)	+1 (0.431)	5.83
3	−1 (5)	0 (0.937)	0 (−0.045)	3.32
4	−1 (5)	+1 (1.176)	−1 (−0.523)	2.15
5	−1 (5)	+1 (1.176)	+1 (0.431)	13.97
6	0 (6)	−1 (0.699)	0 (−0.045)	3.15
7	0 (6)	0 (0.937)	−1 (−0.523)	2.15
8	0 (6)	0 (0.937)	0 (−0.045)	4.21
9	0 (6)	0 (0.937)	0 (−0.045)	3.73
10	0 (6)	0 (0.937)	0 (−0.045)	4.83
11	0 (6)	0 (0.937)	+1 (0.431)	12.33
12	0 (6)	+1 (1.176)	0 (−0.045)	7.15
13	+1 (7)	−1 (0.699)	−1 (−0.523)	1.62
14	+1 (7)	−1 (0.699)	+1 (0.431)	9.19
15	+1 (7)	+1 (1.176)	−1 (−0.523)	3.25
16	+1 (7)	0 (0.937)	0 (−0.045)	5.37
17	+1 (7)	+1 (1.176)	+1 (0.431)	21.23

^a Values between brackets are the original forms of the variables.

^b X_{S1} , pH; X_{S2} , t_{dig} (min); X_{S3} , C_{enz} (α -amylase) (units/ml).

^c Log values correspond to 5, 8.7, and 15 min for X_{S2} and to 0.3, 0.9, and 2.7 units/ml for X_{S3} .

duodenal steps. The experimental domains of the 3 variable factors were defined for each step of digestion, depending on the ranges of values cited in the literature, and they are presented in Table 1 for the salivary step, in Table 2 for the gastric step, and in Table 3 for the duodenal step. The fixed factors were the followings: temperature of digestion (37 °C), agitation rate (350 rpm), and initial concentration of the substrates of digestion (see Section 2.2). Tables 1–3 also show the experimental conditions in coded and original values, tested randomly for each step of digestion. Each point was carried out once, 3 repetitions on 3 independent days were performed for the central point (samples 8, 9, and 10).

For each of the three digestion steps under investigation, an analysis of the experimental results aimed at constructing a predictive model that estimates the percentage of digestion in the ranges of the three tested factors. That model is represented by the following second-order equation (Eq. (1)):

Table 2

Central composite design setting in original and coded forms^a of the independent variables (X_{G1} , $\text{Log}X_{G2}$, and $\text{Log}X_{G3}$) and experimental results for the gastric (Y_G) digestion of bovine serum albumin.

Run order	X_{G1}^b	$\text{Log}X_{G2}^c$	$\text{Log}X_{G3}^c$	Y_G [%]
1	−1 (1)	−1 (1.477)	−1 (1.176)	2.41
2	−1 (1)	−1 (1.477)	+1 (2.097)	11.21
3	−1 (1)	0 (1.866)	0 (1.636)	9.90
4	−1 (1)	+1 (2.255)	−1 (1.176)	8.45
5	−1 (1)	+1 (2.255)	+1 (2.097)	26.15
6	0 (2)	−1 (1.477)	0 (1.636)	9.45
7	0 (2)	0 (1.866)	−1 (1.176)	7.39
8	0 (2)	0 (1.866)	0 (1.636)	14.85
9	0 (2)	0 (1.866)	0 (1.636)	16.07
10	0 (2)	0 (1.866)	0 (1.636)	15.02
11	0 (2)	0 (1.866)	+1 (2.097)	24.04
12	0 (2)	+1 (2.255)	0 (1.636)	22.56
13	+1 (3)	−1 (1.477)	−1 (1.176)	3.37
14	+1 (3)	−1 (1.477)	+1 (2.097)	13.31
15	+1 (3)	+1 (2.255)	−1 (1.176)	7.86
16	+1 (3)	0 (1.866)	0 (1.636)	9.85
17	+1 (3)	+1 (2.255)	+1 (2.097)	25.94

^a Values between brackets are the original forms of the variables.

^b X_{G1} , pH; X_{G2} , t_{dig} (min); X_{G3} , C_{enz} (pepsin) (units/ml).

^c Log values correspond to 30, 73.5, and 180 min for X_{G2} and to 15, 43.3, and 125 units/ml, respectively, for X_{G3} .

Table 3

Central composite design setting in original and coded forms^a of the independent variables (X_{D1} , $\text{Log}X_{D2}$, and $\text{Log}X_{D3}$) and experimental results for the duodenal (Y_D) digestion of triolein.

Run order	X_{D1}^b	$\text{Log}X_{D2}^c$	$\text{Log}X_{D3}^{c,d}$	Y_D [%]
1	−1 (6.5)	−1 (1.778)	−1 (−0.699)	0.00
2	−1 (6.5)	−1 (1.778)	+1 (1.398)	97.91
3	−1 (6.5)	0 (2.167)	0 (0.349)	47.51
4	−1 (6.5)	+1 (2.556)	−1 (−0.699)	8.48
5	−1 (6.5)	+1 (2.556)	+1 (1.398)	94.63
6	0 (7)	−1 (1.778)	0 (0.349)	48.80
7	0 (7)	0 (2.167)	−1 (−0.699)	9.43
8	0 (7)	0 (2.167)	0 (0.349)	55.48
9	0 (7)	0 (2.167)	0 (0.349)	47.72
10	0 (7)	0 (2.167)	0 (0.349)	49.05
11	0 (7)	0 (2.167)	+1 (1.398)	94.33
12	0 (7)	+1 (2.556)	0 (0.349)	61.49
13	+1 (7.5)	−1 (1.778)	−1 (−0.699)	7.00
14	+1 (7.5)	−1 (1.778)	+1 (1.398)	80.11
15	+1 (7.5)	+1 (2.556)	−1 (−0.699)	25.67
16	+1 (7.5)	0 (2.167)	0 (0.349)	62.97
17	+1 (7.5)	+1 (2.556)	+1 (1.398)	70.23

^a Values between brackets are the original forms of the variables.

^b X_{D1} , pH; X_{D2} , t_{dig} (min); X_{D3} , C_{enz} (pancreatin) (mg/ml).

^c Log values correspond to 60, 146.7, and 360 min for X_{D2} and to 0.2, 2.2, and 25 mg/ml, respectively, for X_{D3} .

^d Constant bile:pancreatin ratio of 1:6.

$$Y_i = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i2} + \beta_3 X_{i3} + \beta_{11} X_{i1}^2 + \beta_{22} X_{i2}^2 + \beta_{33} X_{i3}^2 + \beta_{13} X_{i1} * X_{i3} + \beta_{23} X_{i2} * X_{i3}, \quad (1)$$

where Y_i is the response variable ($i = S, G$ or D for the salivary, gastric or duodenal step, respectively); X_{i1} , X_{i2} , and X_{i3} are the independent variables; and β_0 , β_1 , β_2 , β_3 , β_{11} , β_{22} , β_{33} , β_{12} , β_{13} , and β_{23} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively. X_{i2} and X_{i3} (t_{dig} and C_{enz}) were transformed into log values, while X_{i1} (pH) was not. The interaction term between pH and t_{dig} ($X_{i1} * X_{i2}$) had no sense in these experiments and was thus not taken into account. The response variable (% of digestion) was fitted to the model and the coefficient of determination R^2 indicated whether the regression model fitted the experimental results. The predicted R^2 (R^2_{pred}) indicated whether the model correctly predicted the new observations' responses. The lack of fit of the regression model was indicated by a p -value <0.05.

2.6. Selection of the conditions of digestion

For each of the three steps of digestion, values of the three factors, i.e. pH, t_{dig} , and C_{enz} , were selected using the corresponding model and the corresponding physiological percentage of digestion of each substrate (starch for the salivary step, BSA for the gastric step, and triolein for the duodenal step). The adequacy of the selected values was tested by performing each step of digestion independently with its appropriate substrate. Three experimental replicates were performed for each step of digestion, and experimental and predicted values of digestion were compared.

2.7. Validation of the digestion model

Finally, the validity of the optimised digestion protocol was verified by submitting a simple food matrix, made of appropriate proportions of starch, BSA and triolein, to the three successive steps of the digestion protocol under their respective optimal conditions of pH, t_{dig} , and C_{enz} . One ml samples were withdrawn from the initial solution (10 ml) as well as at each step of digestion in order to quantify the digestion products (starch digestion after the salivary step, BSA digestion after the gastric step, and triolein digestion after the duodenal step). The withdrawn volumes were taken into

account when adjusting the enzymatic concentrations. Three experimental replicates of the entire digestion protocol were performed. Experimental and predicted values of digestion were compared after each digestion step.

3. Results and discussion

3.1. Effects of pH, time of digestion, and enzymatic concentrations

For each step of digestion, i.e. the salivary, gastric, and duodenal steps, a CCD was designed with the three continuous factors, pH, t_{dig} , and C_{enz} , and the ranges of values for these variables were defined according to the values encountered in the literature. The experimental data were fitted to a second-order polynomial model (Eq. (1)), and ANOVA statistical analysis was used to determine the values of the regression coefficients and their associated p -values. The respective models in coded values allowed for the comparison of the effects (linear, quadratic, and interaction) of the three independent factors on the substrates of digestion (Table 4). The visualisation of these effects was facilitated by the use of response surface plots (Fig. 1).

3.1.1. Salivary step

The substrate of digestion of the salivary step was a starch solution at 30 mg/ml. The range of values for the 3 factors is shown in Table 1, as is the experimental percentage of starch digestion, which varied from 1.23% to 21.23%.

The experimental results seemed to indicate the greatest influence of the C_{enz} factor ($\text{Log}X_{S3}$), compared to the t_{dig} ($\text{Log}X_{S2}$) and the pH (X_{S1}) factors, because fixed α -amylase caused less variation in the results of starch digestion. Indeed, the results ranged from 1.2% to 3.2% for the lowest C_{enz} (α -amylase) (0.3 units/ml), from 3.1% to 7.1% for the middle C_{enz} (0.9 units/ml), and from 5.8% to 21.2% for the highest C_{enz} (2.7 units/ml). Concerning the effects of pH, the results ranged from 1.2% to 14.0% for the lowest pH (5), from 2.1% to 12.3% for the middle pH (6), and from 1.6% to 21.2% for the highest pH (7). Similarly, the results ranged from 1.2% to 9.2% for the shortest time of incubation (5 min), from 2.1% to 12.3% for the middle time (8.7 min), and from 2.1% to 21.2% for the longest time (15 min).

The regression coefficients of the model of salivary digestion are presented on Table 4, and they adequately fit the experimental data. The variables pH (X_{S1}) and t_{dig} ($\text{Log}X_{S2}$) showed significant linear ($p < 0.001$) model terms. C_{enz} (α -amylase) ($\text{Log}X_{S3}$) was significant in the linear ($p < 0.001$) and quadratic ($p < 0.001$) terms, as well as in the terms for interaction with pH ($p < 0.01$) and t_{dig} ($p < 0.001$). The significant regression coefficients of the polynomial model in coded values showed decreasing influence of each parameter as follows: $\text{Log}X_{S3} > \text{Log}X_{S2} > (\text{Log}X_{S3})^2 > \text{Log}X_{S2} * \text{Log}X_{S3} > X_{S1} > X_{S1} * \text{Log}X_{S3}$.

The response surface plots of salivary digestion (Fig. 1A) were generated by fixing the third factor at the central value of the CCD ($X_{S1} = 6$, $\text{Log}X_{S2} = 0.937$, and $\text{Log}X_{S3} = -0.045$). They showed a slight increase of starch digestion with an increase in pH and t_{dig} , but the most important linear effect was attributed to the C_{enz} (α -amylase) (linear effect reinforced by a positive quadratic effect). Positive interaction effects between pH and t_{dig} , and between t_{dig} and C_{enz} showed that starch digestion increased when the values of these factors were concomitantly increasing. According to the values of the regression coefficient and the surface plots, C_{enz} (α -amylase) was a determining factor in mimicking digestion *in vitro*.

3.1.2. The gastric step

For the gastric step of digestion, the autodigestion of pepsin at very acidic pH values cannot be ruled out. Strugala and coworkers

Table 4
Regression coefficients and standard errors of the three predicted second-order models for the response variables of salivary (Y_S), gastric (Y_G), and duodenal (Y_D) digestion.

Model parameters	Y_S		Y_G		Y_D	
	Regression coefficient	S.E.	Regression coefficient	S.E.	Regression coefficient	S.E.
Intercept	4.46 ^a	0.38	15.01 ^a	0.52	53.64 ^a	2.85
<i>Linear</i>						
X_{i1}	1.41 ^a	0.28	0.22 ^a	0.39	−0.26 ^{NS}	2.11
$\text{Log } X_{i2}$	2.67 ^a	0.28	5.12 ^a	0.39	2.67 ^{NS}	2.11
$\text{Log } X_{i3}$	5.22 ^a	0.28	7.12 ^a	0.39	38.66 ^a	2.11
<i>Quadratic</i>						
X_{i1}^2	−0.27 ^{NS}	0.55	−4.90 ^a	0.76	−0.54 ^{NS}	4.07
$(\text{Log } X_{i2})^2$	0.53 ^{NS}	0.55	1.23 ^{NS}	0.76	−0.64 ^{NS}	4.07
$(\text{Log } X_{i3})^2$	2.62 ^a	0.55	0.94 ^{NS}	0.76	−3.92 ^{NS}	4.07
<i>Interaction</i>						
$X_{i1} * \text{Log } X_{i3}$	1.14 ^b	0.32	0.19 ^{NS}	0.44	−8.30 ^b	2.36
$\text{Log } X_{i2} * \text{Log } X_{i3}$	2.20 ^a	0.32	2.13 ^a	0.44	−5.04 ^{NS}	2.36
Lack of fit	$p = 0.260$ $R^2 = 0.9858$		$p = 0.197$ $R^2 = 0.9860$		$p = 0.101$ $R^2 = 0.9781$	

$i = S, G$ or D for the salivary, gastric or duodenal step, respectively.

S.E., standard error.

NS, non-significant.

^a $p < 0.001$ (Statistically significant).

^b $p < 0.01$ (Statistically significant).

indicated that pepsin is stable at a pH of 2.5 (Strugala, Kennington, Campbell, Skjak-Bræk, & Dettmar, 2005) but more acidic conditions are used in the present study. The pepsin C_{enz} values given in this work should thus be considered as initial values. The substrate of digestion used for this step was a BSA solution at 5 mg/ml PBS. BSA contains 608 peptidic linkages, and pepsin is theoretically able to cleave a maximum of 179 fragments (http://web.expasy.org/peptide_cutter/), leading to the determination of maximal digestion at approximately 30% of the total peptidic linkages found in BSA. Preliminary kinetic studies allowed us to find the maxima of absorption measured for the three values of initial C_{enz} (pepsin) in the CDD, i.e. 15, 43.3, and 125 units/ml of incubate (data not shown). These kinetic curves reached a common steady state of absorption, considered as our maximum of BSA digestion.

The experimental conditions of the CDD and the respective experimental percentages of gastric digestion, varying from 2.41% to 26.15%, are shown in Table 2. All three factors, i.e. pH (X_{G1}), t_{dig} ($\text{Log } X_{G2}$) and initial C_{enz} ($\text{Log } X_{G3}$) seemed to have an influence on digestion, with the greatest effects coming from $\text{Log } X_{G3}$. Indeed, the results of BSA digestion ranged from 2.4% to 26.1% for the lowest value of pH (1), from 7.4% to 24% for the middle value (2), and from 3.4% to 26% for the highest value (3). For the effects of the incubation time, the results ranged from 2.4% to 13.3% for the shortest duration of digestion (30 min), from 9.4% to 22.6% for the middle one (73.5 min), and from 11.2% to 26.1% for the longest one (180 min). For the effects of the initial enzymatic concentration (pepsin), the results showed less variation since they ranged from 2.4% to 8.5% for the lowest initial C_{enz} (15 units/ml), from 9.5% to 22.6% for the middle initial C_{enz} (43.3 units/ml), and from 11% to 26% for the highest one (125 units/ml).

As shown in Table 4, pH presented significant linear and quadratic effects ($p < 0.001$), and t_{dig} and C_{enz} showed significant linear ($p < 0.001$) model terms. The interaction terms between t_{dig} and C_{enz} (pepsin) ($\text{Log } X_{G2} * \text{Log } X_{G3}$) also showed significant effects ($p < 0.001$). The significant regression coefficients of the gastric polynomial model in coded values showed decreasing influence of each parameter as follows: $\text{Log } X_{G3} > \text{Log } X_{G2} > X_{G1}^2 \gg \text{Log } X_{G2} * \text{Log } X_{G3} > X_{G1}$.

The response surface plots are represented in Fig. 1B (central value of $X_{G1} = 2$, $\text{Log } X_{G2} = 1.866$, and $\text{Log } X_{G3} = 1.636$). They showed an increase in BSA digestion when t_{dig} and C_{enz} increased, with the most important linear effect attributed to C_{enz} . A negative

quadratic effect of pH indicated that there was a maximum of BSA digestion at the value of 2. Positive interaction effects between t_{dig} and C_{enz} showed that BSA digestion increased when the values of these factors concomitantly rose. According to the values of the regression coefficients and the surface plots, all three factors appear to be of importance for mimicking gastric *in vitro* digestion.

3.1.3. The duodenal step

To model the duodenal step of digestion, a suspension of triolein of 8.33 mg/ml of incubate was used as a substrate and was digested by a mixture of pancreatin and bile extract. Concerning the concentration of bile extract, sufficiently high levels of bile salts are necessary to promote lipase activity (Mun, Decker, & McClements, 2007). A bile:pancreatin ratio of 6:1 is frequently used in *in vitro* digestion protocols (Alexandropoulou et al., 2006; Garrett, Failla, & Sarama, 1999; Laurent, Besançon, & Caporiccio, 2007) and was therefore used in this study. The experimental percentage of triolein digestion was obtained by subtracting the FFAs quantified in the bile extract, and calculations were based on the assumption that 1 mol of triglycerides leads to 3 mol of FFAs. In addition to pancreatic lipase, pancreatin contains indeed also non-specific esterases (Sek, Porter, & Charman, 2001) that were verified to be active in our case through the digestion of 2-oleylglycerol (data not shown).

The experimental conditions of the CDD are presented in Table 3, as well as the experimental percentage of triolein digestion obtained, varying from 0.00% to 97.91%. The experimental results of triolein digestion showed the high impact of the C_{enz} ($\text{Log } X_{D3}$), as compared to either pH (X_{D1}) or t_{dig} ($\text{Log } X_{D2}$). Indeed, the results of triolein digestion ranged from 0.00% to 97.91% for the lowest value of pH (6.5), from 9.43% to 94.33% for the middle value (7), and from 7.00% to 80.11% for the highest value (7.5). For the effects of the incubation time, the results ranged from 0.00% to 97.91% for the shortest duration of digestion (60 min), from 0.00% to 94.33% for the middle one (146.7 min), and from 8.48% to 94.63% for the longest one (360 min). For the effects of the enzymatic concentration (pancreatin), the results ranged from 0% to 9% for the lowest C_{enz} (0.2 mg/ml), from 48% to 63% for the middle C_{enz} (2.2 mg/ml), and from 70% to 98% for the highest C_{enz} (25 mg/ml).

As shown in Table 4 and illustrated in Fig. 1C (central value of $X_{D1} = 7$, $\text{Log } X_{D2} = 2.167$, and $\text{Log } X_{D3} = 0.349$), the only significant regression coefficients of the duodenal model was the

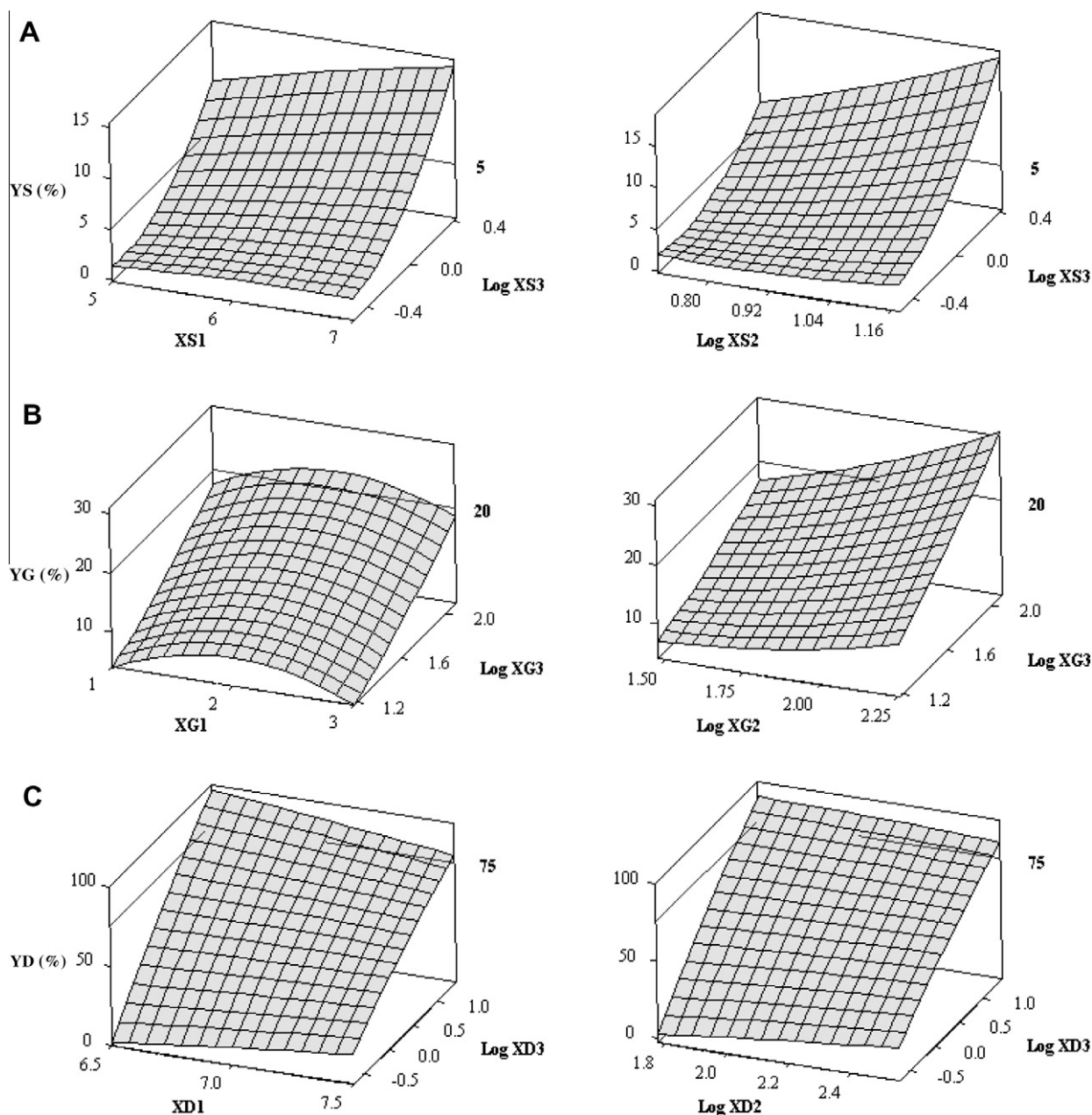


Fig. 1. Response surface plots for salivary (A), gastric (B), and duodenal (C) digestion as functions of pH (X_{i1}), time of digestion ($\text{Log}X_{i2}$), and enzymatic concentration ($\text{Log}X_{i3}$) ($i = S, G$ or D for the salivary, gastric or duodenal steps, respectively). The value of the missing independent factor in each plot was kept at the central value. The line represents the expected percentage of substrate digestion needed to approach physiological digestion.

C_{enz} (pancreatin) in its linear term ($p < 0.01$) and its interaction term with pH ($X_{D1} * \text{Log}X_{D3}$) ($p < 0.05$). The negative interaction effect between pH and C_{enz} was however very small (-8.30) in comparison to the linear effect of C_{enz} ($+38.66$).

3.2. Selection of the conditions of digestion

The modelisation approach presented above was used to select values of the three factors under investigation, i.e. pH, t_{dig} , and C_{enz} , for each of the three steps of digestion considered. A special focus was put on C_{enz} , found as the most influential parameter. Once determined, the adequacy of the selected values was verified for each step of the digestion.

3.2.1. Setup of salivary conditions

Most of the existing protocols only apply successive gastric and abiotic duodenal digestion, and very few consider the salivary step. It might however be of importance for certain foodstuffs (as for starch-containing food) and has thus been considered here. Values for the three factors were defined to mimic the physiological starch digestion results. In humans, the short period of residence of starch in the mouth allows for salivary hydrolysis of approximately 5% of the total osidic linkages (Guyton & Hall, 1996). By using the salivary polynomial model, several factor values permitted to achieve 5% of digestion, as illustrated by the line in the response surface plot in Fig. 1A. Human saliva was fixed at 6.9, a value commonly used in the literature on *in vitro* salivary digestion (Laurent et al., 2007; Lebet et al., 1998). Several pairs of t_{dig} and C_{enz} (α -amylase)

allowed for the achievement of a predicted 5% of starch digestion, as illustrated in Fig. 2A. The value of t_{dig} was then fixed at 5 min in order to correspond to the physiological time (Guyton & Hall, 1996). As a result, the predictive model led to the determination of a C_{enz} (α -amylase) value of 1.3 units/ml to achieve 5% of starch digestion. The adequacy of the selected values of pH, t_{dig} and C_{enz} , i.e. 6.9, 5 min and 1.3 units α -amylase/ml, was then verified experimentally by digesting the starch solution in these conditions. The experimental value obtained was $4.6 \pm 0.1\%$ of starch digestion.

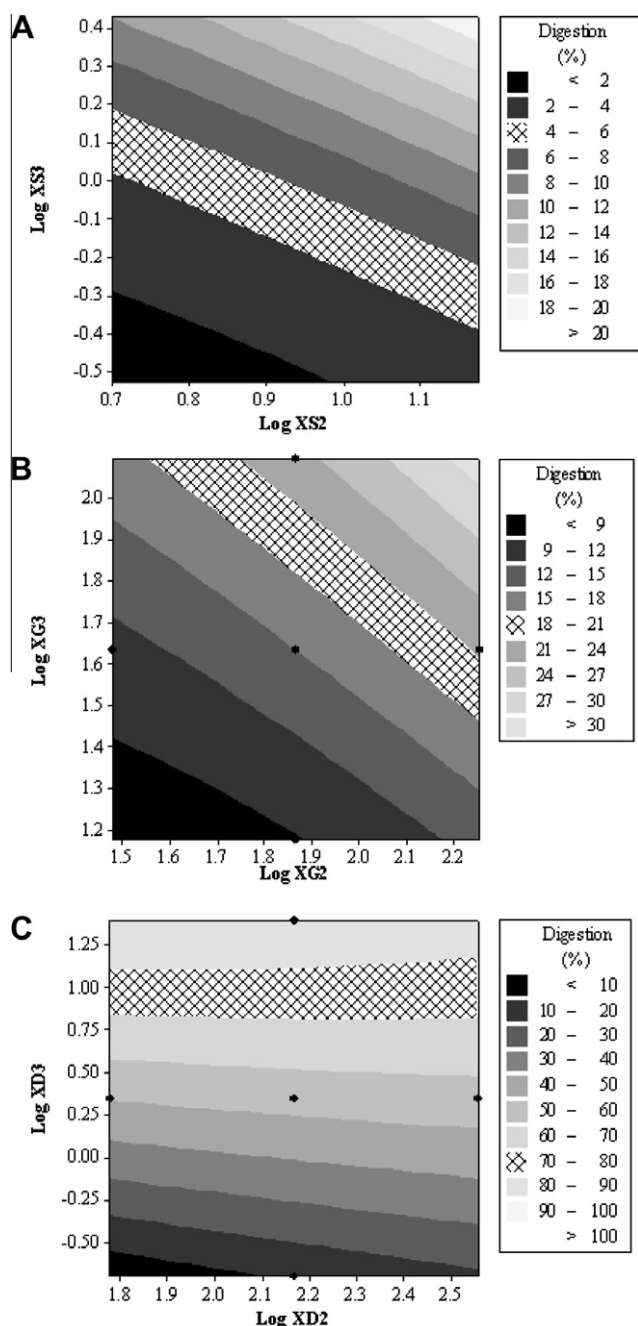


Fig. 2. Contour plots for salivary (A), gastric (B), and duodenal (C) digestion as functions of duration of digestion ($\text{Log} X_{i2}$) and enzymatic concentrations ($\text{Log} X_{i3}$) ($i = S, G$ or D for the salivary, gastric or duodenal steps, respectively). The pH values were fixed at 6.9, 2, and 7, respectively, for the salivary, gastric, and duodenal steps. The grid area represents the zone containing the targeted percentages of digestion, i.e. 5% for salivary digestion of starch, 20% for gastric digestion of bovine serum albumin, and 75% for duodenal digestion of triolein.

Taking into account that the initial starch concentration (30 mg/ml) was 3-fold lower than the theoretical *in vivo* value (90 mg/ml), we extrapolate that the C_{enz} should be adjusted to 3.9 units/ml for further studies of bioaccessibility. This enzymatic value is difficult to compare with the literature, because of the lack of information concerning α -amylase specific activity. In our study, 3.9 units/ml corresponded to $1.625 \mu\text{g}$ α -amylase/ml, which is a concentration approximately 180 times lower than the concentration of $290 \mu\text{g}$ α -amylase/ml used in the study of Oomen et al. (2003).

3.2.2. Setup of the gastric conditions

Knowing that pepsin is able to cleave a maximum of roughly 30% of the total peptidic linkages found in the BSA (see Section 3.1.2), the common maximal absorbance value obtained with the three kinetic curves was considered to correspond to 30% of peptidic linkage hydrolysis. In humans, pepsin begins the process of protein digestion. It has been estimated to hydrolyse 10–20% of the dietary protein peptidic linkages in the stomach (Guyton & Hall, 1996). In our study, values of the factors under consideration were selected to achieve a BSA peptidic hydrolysis percentage of 20% corresponding thus to 2/3 of the maximal reachable absorbance value. Several pairs of factor values were predicted to achieve this objective with the polynomial model used for the gastric step (Fig. 2B). Because C_{enz} (pepsin) had the greatest effect in our model, pH and t_{dig} were fixed at physiological values by taking into account the negative quadratic effect of pH, leading to a maximum of digestion at pH 2 (Table 4).

A pH level of 2.0 is a very commonly used value in the literature on *in vitro* gastric digestion (Laurent et al., 2007; Lebet et al., 1998; Pérez-Vicente, Gil-Izquierdo, & Garcia-Viguera, 2002). It can indeed be considered as an adequate intermediate value since the physiological gastric pH of a fasting person has been shown go down to 1.3 whereas it can reach 4.9 for someone who has eaten recently (Russell et al., 1993). The residency time of the chyme in the human stomach is approximately 90 min (1.954 in log value). This value has thus been chosen. Based on a pH of 2.0 and a t_{dig} of 90 min, the predictive model allowed for the determination of an initial C_{enz} (pepsin) of 71.2 units/ml of incubate. The adequacy of the selected values of pH, t_{dig} and C_{enz} was verified by digesting the BSA solution in these conditions. The experimental value obtained was $18.4 \pm 0.9\%$ of hydrolysis of the BSA peptidic linkages.

Regarding pepsin concentrations, the comparison with the literature is quite difficult since most authors express C_{enz} in mg/ml. In the study by Pérez-Vicente et al. (2002), gastric digestion was undertaken with pH 2 and $t_{\text{dig}} = 150$ min. This corresponded to a need for 44.0 units/ml in our predictive model, while they used 32 units/ml. Mandalari et al. (2010) used a pH 2.5 and $t_{\text{dig}} = 120$ min, corresponding to 61.1 units/ml in our model, while they used 146 units/ml. McDougall, Fyffe, Dobson, and Stewart (2005) used a pH 1.7 and $t_{\text{dig}} = 120$ min, corresponding to 57.9 units/ml in our model, while they used 315 units/ml.

3.2.3. Setup of the duodenal conditions

Triolein was chosen as model nutrient to set up the digestion conditions of the duodenal lumen. In humans, lipid digestion begins in the stomach, with roughly 10–30% of ester bond cleavage occurring due to the activity of gastric lipase (Mu & Høy, 2004), but the hydrolysis goes to completion in the duodenum through the activity of pancreatic lipase and unspecific esterases (Lowe, 2002; Mu & Høy, 2004). In this study, it was targeted to reach 75% of ester bond hydrolysis in the *in vitro* duodenal digestion, considering a mean ester bond hydrolysis of 20% in the stomach and a total hydrolysis of 95%. Several pairs of factor values were predicted to achieve this objective with the polynomial model used for the duodenal step (Fig. 2C). The regression model showed that C_{enz} (pancreatin) was a highly important factor in comparison to

pH and t_{dig} . The physiological pH in the duodenum is between 4 and 5.5, that of the jejunum is between 5.5 and 7, and that of the ileum is between 7 and 7.5. The enzymes are poured into the duodenum, where they are mainly active for 30–45 min (Daugherty & Mrsny, 1999). In addition, they continue their activity in the jejunum, the main site of absorption, where the chyme remains for approximately 90–120 min (Daugherty & Mrsny, 1999). The *in vitro* intestinal step considered here corresponds to the abiotic duodenal step because neither epithelial nor microbiological influence was taken into account. Nevertheless, we decided to select a longer duration of residency as compared to the theoretical one in order to take into account the activity of duodenal enzymes in the jejunum segment. The pH and t_{dig} were fixed at the central values of the CCD, i.e. pH of 7 and t_{dig} of 150 min. The predictive model allowed us then to determine a C_{enz} (pancreatin) of 9.2 mg/ml to achieve 75% of triolein digestion (concomitantly with a 6-fold higher bile extract concentration, i.e. 55.2 mg/ml). The adequacy of the selected values of pH, t_{dig} and C_{enz} was verified by digesting the triolein suspension in these conditions. The experimental value obtained was $77.9 \pm 8.7\%$ of hydrolysis of the ester bonds.

The C_{enz} (pancreatin) values in the literature vary from 0.2 (Boyer, Brown, & Liu, 2005) to 3 mg/ml of digestion mixture (Hack & Selenka, 1996), except for one study that used 20 mg/ml (Sek et al., 2001). It is however difficult to compare our results with those in the literature because the type of pancreatin used must be taken into account. Most of the time, the activities of the enzymes contained in pancreatin (if specified) vary from 1 (Hack & Selenka, 1996) to 8 X USP specifications (Sek et al., 2001). The pancreatin activity used for this study was equivalent to 4 X USP specifications. It is also important to note that pancreatic lipase is occasionally added to pancreatin in order to increase the lipase activity at this step of digestion (Hur, Lim, Park, & Joo, 2009).

3.3. Validation of the digestion model

Once the values of the factors were selected thanks to the use of the respective predictive models, and verified for each step of *in vitro* digestion, the simple food matrix was used as a substrate submitted to the three consecutive steps, and samples of the salivary, gastric, and duodenal digestion steps were taken to analyse the respective products of digestion. Three independent *in vitro* digestions were conducted with three equivalent substrate mixtures and each enzymatic solution was prepared just before use. For each test, the following controls were performed: enzymatic digestion without substrate and non-enzymatic digestion of the food standard matrix. The experimental percentages of digestion were of $4.9 \pm 0.9\%$ of starch digestion after the salivary step, $18.6 \pm 3.8\%$ of BSA digestion after the gastric step, and $71.1 \pm 12.0\%$ of triolein digestion after the duodenal step, which satisfactorily matches the expected values, i.e. 5% for starch digestion, 20% for BSA digestion, and 75% for triolein digestion. No digestion was observed, either in the absence of enzymes or in the absence of the simple food matrix substrate (data not shown).

4. Conclusions

In this study, we evaluated the influence of three continuous factors, i.e. pH, t_{dig} and C_{enz} , in the range of values found in literature, on the digestion of a simple food matrix (starch, BSA, and triolein). Three CCDs were employed to model the salivary, gastric, and duodenal digestion steps, and response surface methodology was used, highlighting the impact of these three factors on the results of digestion. The most relevant factor was the enzyme concentrations for all three steps of *in vitro* digestion under investigation. Interestingly enough, this factor is precisely the

one for which large ranges are encountered in the literature. Moreover, most publications suffer from a lack of data about enzymatic activities. Values of the three factors were selected for each step using the corresponding model and the corresponding physiological percentage of substrate digestion, and the adequacy was verified with the selected values and the appropriate substrate. The *in vitro* digestion was eventually validated under the successive optimal conditions of pH, t_{dig} , and C_{enz} with the simple food matrix submitted to the three successive steps of the digestion protocol.

This study allowed us to establish a simple, well-defined protocol of *in vitro* pre-colonic digestion, which approaches as closely as possible physiological human digestion in a rapid and low-cost way, and without excessive use of enzymes. For the salivary step, 5% of starch digestion was reached using α -amylase from human saliva at 3.9 units/ml of incubate, a pH of 6.9, and a 5 min incubation under aerobic conditions. For the gastric step, 20% of hydrolysis of BSA peptidic bonds was achieved using porcine pepsin from gastric mucosa at 71.2 units/ml of incubate, a pH of 2, and a 90 min incubation under anaerobic conditions. For the duodenal step, 75% of hydrolysis of triolein ester bonds was achieved using a mixture containing 9.2 mg/ml of pancreatin from porcine pancreas and 55.2 mg/ml of porcine bile extract, at a pH of 7, during 150 min under anaerobic conditions. A constant magnetic stirring at 350 rpm was applied for the three steps of digestion and the temperature was set at 37 °C. This study highlighted that the α -amylase and pepsin concentrations may be reduced in some existing digestion protocols, while pancreatin concentrations should be increased in most protocols when no additional lipase is present.

As it approaches as closely as possible the physiological human digestion in a rapid and low-cost way, the proposed protocol should be used in the bioaccessibility tests that need to be included in the evaluation of the physiological effects of ingested compounds at the intestinal level. It is of interest both for the study of bioavailability of food items present in complex food matrices and as a first evaluation step of digestion of natural extracts, xenobiotics and drugs associated to simple matrices as in the case of food supplements. For the study of bioavailability of complex food matrices and processed foodstuffs, we recommend using the proposed methodology but the optimal conditions should be specifically determined with the food matrix of choice instead of the simplified matrix used in the present work.

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