Multivariate optimization of microwave-assisted enzyme digestion of α-casein for generation of bioactive peptides

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ABSTRACT

Caseins are the principal milk proteins and serve as an important source of bioactive peptides with diverse beneficial effects for human health, such as antihypertensive, immunomodulating, anti-inflammatory, and antithrombotic. The objective of this study was to optimize, using the design of the experiment, the production of bioactive peptides from α-casein applying microwave-assisted enzyme digestion (MAED). The optimal MAED conditions (time, temperature, and enzyme/protein ratio) were established for pepsin (digestion time 4 min, temperature 41°C, and E:P ratio 1:40) and trypsin (digestion time 10 min, temperature 37° C and E:P ratio 1:200.) enzymes. Digestion yields and the intensity of different bioactivities, *i.e.*, antimicrobial, antioxidant, and acetylcholinesterase and α-glucosidase inhibitory activities, evaluated by the HPTLC-bioassay technique were used as response variables. Trypsin-formed α-casein peptides showed antimicrobial, antioxidant, and acetylcholinesterase inhibition activities, while pepsin-formed showed antimicrobial, antioxidant, and α-glucosidase inhibition activities. These results demonstrate that MAED is a fast and effective technique for bioactive peptide production from casein proteins.

Keywords: Bioactive peptides, α-casein, EDA-HPTLC-bioassay, α-glucosidase, acetylcholinesterase, antioxidant.

1. INTRODUCTION

Caseins are the predominant milk protein accounting for 80% of the total protein of almost all mammalian species. Caseins are complex phosphoproteins with unique structural properties like a high proline content and phosphoserine residues [1,2]. These insoluble proteins present an open and flexible chemical structure that facilities its digestion and in consequence the formation of bioactive peptides [3], which are categorized according to their corresponding subunit source such as κ-casein, α-casein, and β-casein [4]. α-casein is the most abundant casein subunit (65%), and chemically corresponds to a monomeric phosphoprotein composed of two subunits of α_{S1}-casein (23.62 kDa) and α_{S2}-casein (25.23 kDa) [5]. This protein presents a high biological value, and its enzymatic digestion generates several bioactive peptides with beneficial properties for human health [4,6]. The activity of peptides depend on their amino acid composition, sequence and length [7], which is commonly between 3 to 20 amino acid residues, with a mass lower than 6 kDa [8]. Several bioactivities have been reported, e.g., antihypertensive [9], antioxidant [10], antimicrobial [11], antidiabetic [12], and immunomodulatory [13]. Although bioactive peptides can be naturally generated during gastrointestinal digestion or lactic fermentation process [6], several chemical and biotechnological methods have been developed to obtain bioactive peptides for functional foods and/or nutraceuticals production [14]. Some advances techniques like ultrasound-assisted (UAED) and microwave-assisted enzyme digestion (MAED) have been applied to efficiently carry out the proteins digestion mainly focused in establishing shot-time processes with lower enzyme amounts requirement [15,16]. Both, UAED and MAED methods changes protein conformation improving the accessibility and susceptibility of peptide bonds to enzymes catalytic action, allowing faster protein hydrolysis, increasing peptide formation [17]. Thus, the efficiency is highly enhance in terms of time [18; 19], enzyme amount and reproducibility [17]. Regarding peptides determination, the most commonly technique is chromatography [20], e.g., gel chromatography [21], ion-exchange chromatography (IEC) [20], and reverse-phase high-performance liquid chromatography (HPLC) [15]. Another versatile technique is high-per-formance thin layer chromatography (HPTLC) used as an alternative or complementarily tool to HPLC for separating hydrophilic and small peptides based on parameters such as size, charge, and polarity [22-24]. Due to the lack of important chromophore groups, peptides detection requires pre- or post-chromatography derivatization the direct coupling with mass spectrometry however neither process can detect bioactivity, of the peptides generated [25,26]. The most traditional approach to study the peptide biological activities such as antioxidant and enzymes inhibition, is the evaluation of the entire digested, and in the case of observing an inhibitory effect, a large and time-consuming procedure begins including fractioning and purification processes to isolate different peptide groups, which requires the use of special techniques like semi-preparative chromatography. All peptide groups are individually reevaluated to find which group (s) possess the biological activity. To shorten this process some hyphenation methods coupling analytical separation and bioassays have been developed, for example HPLC and effect-directed analysis. The main limitation is the use of organic solvents and other additives, which are commonly incompatible with biological and biochemical assays [27]. Under this scenario HPTLC coupled with effect-directed analysis (EDA) and mass spectrometry (MS) arise like an excellent alternative [28]. EDA is a study strategy that combines chemical analysis with biological effects, it has increasingly attracted attention in the field of food science to evaluate functional foods, food safety, foodborne diseases, and health care [29,30]. EDA-HPTLC consist of two principal steps, first is necessary to separate the molecules from food matrix (chromatography), and then the obtained fractions are evaluated for their biological activity (effect) directly on the plate (*in situ*) without any incompatibility with mobile phase because all solvents are fully evaporated before carrying out the bioassay [31]. The hyphenation of HPTLC and EDA is well known in both domains, autography coupled with chemical analysis (e.g., DPPH) and bioautography (bioassay) coupled with biological system or biological evaluation (e.g., enzymatic inhibition) [31,32]. The biological activities of milk caseins are well documented [33-37], but only few studies describe the bioactivity of peptides produced by MAED [38]. The objective of this study was to optimize, using design of the experiment the production of bioactive peptides from α-casein applying MAED. The intensity of peptides formation as well as different bioactivities, e.g., antimicrobial, antioxidant, and acetylcholinesterase and α-glucosidase inhibition activity, evaluated by HPTLC-bioassay technique was used as response variable.

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2. MATERIALS AND METHODS

Reagents, chemicals, and solvents

Trypsin from bovine pancreas, porcine pepsin, bovine *α*-casein, acetylcholinesterase (AChE) from Electrophorus electricus (EC 3.1.17), α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), 1-naphthyl-acetate, caffeic acid (≥98%), oxytetracycline hydrochloride (≥95%), thiazolyl blue tetrazolium bromide (MTT) (98%), Fast Blue B Salt (~95%) and 2,2.-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl) aminomethane (Tris) (>99.7%), ammonium hydrogen carbonate (>99%), hydrochloric acid (37%), sodium chloride (>99%), potassium chloride (>99%), calcium chloride, potassium dihydrogen phosphate (≥98%), sodium phosphate dibasic, triethylamine 10 % v/v, fluorescamine, ethanol, methanol, 2-propanol, n- butanol, and ammonia (32%) were obtained from Merck (Darmstadt, Germany). 2-naphthyl-α-D-glucopyranoside (>99%) was acquired from Goldbio (St. Louis, MO, USA). Donepezil hydrochloride (secondary pharmaceutical standard) was donated from a local pharmaceutical company. Ultrapure water (18.2 MΩcm at 25ºC) was produced using a Simplicity system from Millipore (Bedford, MA,USA).The *α*-glucosidase enzyme solution (1.25 U/mL) was prepared in 100 mM potassium phosphate buffer (pH 7.4), and acetylcholinesterase enzyme solution (0.75 U/mL) was dissolved in 50 mM Tris–hydrochloric acid (HCl) buffer (pH 7.8). Chromatography was performed on 20 x 10 cm and 10 x 10 cm HPTLC plates from Merck, coated with a 200 µm silica gel 60 F Z

aein microwave-assisted enzymatic digestion (MAED)

Casein digestion was performed following the method described by Bove et al., [39] with some slight modifications in digestion time and temperature reported by Miquel et al., [40]. Briefly, casein was prepared in 0.05 M ammonium bicarbonate (pH 7.8) with careful stirring until its complete dissolution. Pepsin and trypsin enzymes were prepared in simulated gastric GS and intestinal IS buffer according to the method described previously [41]. The enzymes and proteins mixture was directly loaded into the microwave high-pressure vessel for a final volume of 12 mL. The pepsin-casein mixture was adjusted to pH 3 with 1 M HCl. After MAED, the reaction was ended by applying a heat-shock treatment at 95ºC for 10 min. All MAED experiments were performed on Milestone (Sorisole, BG, Italy) Ethos X with SK-15 rotor with contact-less sensors of temperature and pressure using a microwave constant power of 800 W. MAED optimal conditions for pepsin were: enzyme to protein (E:P) ratio of 1:40, digestion time of 4 min and 41ºC, while for trypsin were: E:P ratio of 1:200, 10 min and 37ºC.

Optimization of MAED

MAED was optimized using a face-centered central composite design (CCD) with two central points. The experimental design was established with three factors: a) temperature (°C), b) reaction time (min), and c) E:P ratio. The response variable was the sum of the band intensities for each digestion condition and the intensity of bioactive peptides bands (i.e., peptides with antimicrobial, antioxidant, and acetylcholinesterase and/or α-glucosidase inhibition activities), both evaluated by fluorescence after derivatization with fluorescamine (0.02% w/v). Each digestion condition (temperature, time, and E:P ratios) was prepared according to the experimental plan and performed in duplicate (n=*2*) in random order to minimize the effects of uncontrolled factors.

High-performance thin-layer chromatography

Digested casein solutions were applied on HPTLC plates silica gel 60 F^{254} using CAMAG (Muttenz, Switzerland) Automatic TLC Sampler 4 (ATS4) with the following settings: band length 7 mm, track distance 8 mm, dosage speed 150 nL/s and first application x-axis and y-axis at 10 mm. Application volumes were 15 and 22 µL/band for pepsin and trypsin digestion, respectively. Chromatography was performed in CAMAG twin-trough chamber up to a migration distance of 70 mm using a mobile phase composed of 2-propanol: n-butanol: ammonia: water (57: 20: 20: 3 v/v/v/v). After separation the plate was dried for 30 min at 60 °C on CAMAG TLC plate heater. All digested were applied in duplicate dividing the HPTLC plate in two sections: the first section was used for derivatization with 0.02% w/v fluorescamine in acetone (3s and then dried the plate at 95ºC for 20 min), by means of CAMAG immersion device. Optionally the plate can be subjected to a second immersion into a 10% v/v triethylamine in acetone to increase and stabilize the fluorescence. The second section was directly used to carry out the bioassay. Plate images were photo-documented under white and UV (254 and 366 nm) light illumination (reflectance) using a CAMAG Reprostar 3 documentation system. All the instruments were controlled through CAMAG WinCats 1.4.7 software.

HPTLC-bioassay: AChE inhibition

AChE-inhibiting peptides were detected following the protocol described by [31]. Briefly, the plate was immersed into 50 mM Tris-HCl buffer, pH 7.8, and then dried on a plate heater for 10 min at 115°C. Three milliliters of 1.5 mg/mL 1-naphthyl acetate dissolved in methanol were sprayed onto the plate by means of Merck TLC sprayer and the plate was then dried on a plate heater for 10 min at 60 °C. Enzyme reaction was carried out by spraying on the plate 3 mL of AChE solution (0.75 U/mL of AChE enzyme in 50 mM Tris-HCl buffer, pH 7.8) and incubated at 37ºC for 10 min over a horizontal stand inside a closed moisture chamber containing ca. 50 mL of ultrapure water. Enzyme product (2-naphthol) was detected by reaction with freshly prepared Fast Blue B salt aqueous solution (1.5 mg/mL) to obtain a purple background stable for at least 24 h, which made possible to observe the colorless inhibition bands (2 min reaction). The plate image was photo-documented under white light illumination.

HPTLC-bioassay: α-glucosidase inhibition

α-glucosidase-inhibiting peptides were detected applying the protocol reported by Galarce-Bustos et al., [42] with slight modifications Aranda et al., [43]. Briefly, the plate was immersed into 100 mM phosphate buffer, pH 7.4, and dried on a plate heater for 10 min at 115 °C. ca. 3 mL of 1 mg/mL 2-naphthyl-α-D-glucopyranoside in methanol were sprayed on the plate and dried at 60°C for 10 min. Three milliliters of enzyme solution (1.25 U/mL of α-glucosidase enzyme in 100 mM phosphate buffer, pH 7.4) were sprayed on the plate and incubated at 37ºC for 10 minutes over a horizontal stand inside a closed moisture chamber containing ca. 50 mL of ultrapure water. Inhibitors were detected as colorless bands over purple background after spraying 3 mL of 1.5 mg/mL Fast Blue B salt aqueous solution. The plate image was photo-documented under white light illumination.

HPTLC-bioassay: antibacterial compounds

Antibacterial peptides were evaluated by direct bioautography with *Bacillus subtilis* following the method described by Jamshidi-Aidji and Morlock [44], with slight modifications. Briefly, plates were immersed (3.5 cm/s) for 6 s into $1x10^7$ CFU/mL Muller-Hinton bacterial culture through CAMAG immersion device. The plate was then incubated at 37°C for 2 h, under aerobic conditions, over a horizontal stand inside a closed moisture chamber containing ca. 50 mL of ultrapure water. After incubation, a proper volume of 0.2 % w/v phosphate-buffered (pH 7.4) MTT staining solution was sprayed on the plate employing a Merck TLC-sprayed and incubated again at 37°C for 30 min. After this second incubation, the plate was dried on a TLC plate heater for 5 min at 50°C. Antibacterial compounds were observed as colorless bands on purple background (formazan formation). The plate image was photo-documented under white light illumination.

HPTLC-assay: antioxidant analysis by DPPH

Radical scavenging peptides were detected following the protocol described by Oresanya et al., [29] with some modifications. Briefly, the plate was immersed into a 1 mg/mL methanolic DPPH solution. The plate was then dried in the dark at room temperature for 20 min. Antioxidant peptides were observed as

colorless bands on a purple background. Plates were photo documented under white light.

Digital image processing

The bioactive peptide bands intensities were quantified by image processing using ImageJ software version 1.53a (Wayne Rasband, National Institutes of Health, USA). All images were processed in TIFF format with a resolution of 500 dpi, and pre-treatment was performed using Adobe Photoshop CC version 2017.1.1 software (Adobe System Incorporated San José, CA, USA), obtaining an 8-bit image. Each target band was outlined by means of the "Rectangle" selected tool, considering a uniform selection size and covering the whole band. The band profile plots were obtained using the "Gels" option under the "Analyze" menu, then selecting "First Lane" to define the first target band and "Next Lane" for the following bands, whose selection process was closed with "Plot lanes". Each band peak was defined using the "Straight" tool, which allows drawing a straight line at the base of the peak to subsequently select the whole area drawn from the "Wand" tool. Finally, the area was obtained by applying the "Measure" option under the "Analyze" menu. These area values were used to obtain the optimal conditions using Modde 7 software.

Statistical analysis

Data were evaluated using descriptive statistics as mean, standard deviation (SD), and relative standard deviation (RSD). Central composite designs were prepared and analyzed using MODDE version 7.0.0.1 software (Umetrics, Umea, Sweden). All the above statistical analyses were carried out with a significance level (α) of 0 .0 5 using GraphPad (San Diego, CA, USA) Prism 8.0 software.

3. RESULTS AND DISCUSSION

Optimization of MAED

MAED conditions were optimized using a face-centered central composite design with two central points; this chemometric technique is one of the most used due to its high efficiency regarding the number of experiments and the information obtained [45,46]. From the factors that could affect pepsin and trypsin MAED, the most relevant ones were selected according to preliminary assays and published reports [47,48]. Thus, the following factors were studied: temperature (X_1), time (X_2), and E:P ratio (X_3), keeping reaction volume constant (12 mL), minimum level for MAED. Since enzyme concentration is a critical aspect due to the high cost of trypsin and pepsin enzymes, it is essential to determine the minimum enzyme concentration capable of generating an adequate amount of bioactive peptides detectable by HPTLC (bioassay and fluorescence). Following Pavón-Perez et al., report [15] three trypsin concen-trations (0.01, 0.1, and 1 mg/mL) were evaluated, achieving proper results with the lowest concentration, which also helped to avoid enzyme autolysis [49].

According to these preliminary results, the following factor ranges were studied for trypsin MAED: temperature-X_1 (37 to 65°C), digestion time-X_2 (from 3 to 30 min), and E:P ratio-X_3 (1:20 to 1:200) (Table 1). For pepsin digestion, the same approach was performed assaying different enzyme concentrations (0.1, 0.5, 1.0, and 1.5 mg/mL) following the method described by Bove et al., [39]. Unfortunately, only with the higher enzyme concentration was possible to obtain detectable amounts of bioactive peptides. Thus, the following factor ranges were studied for pepsin MAED: temperature-X_1 (37 to 55 °C), digestion time-X_2 (from 3 to 20 min), and E:P ratio-X_3 (1:12 to 1:40) (Table 2). The response/variable studied was digestion yields in terms of peptides and bioactive peptides generation. For the first case it was established as the sum of all peptide bands intensities determined by fluorescence, and for the second was considered only the intensities of bioactive peptides determined by HPTLC-bioassay.

Table 1. Experimental runs for a central composite design with the corresponding round of experiments, designated variables, and response in the fluorescence intensity of peptides and inhibition intensity bioactive peptides (DPPH assay) for α-casein digestion with trypsin.

Run	Temperature (°C)	Time (min)	E:P ratio	DPPH assay Inhibition intensity %
1	37	3	1:20	0
2	37	3	1:200	100.0
3	37	30	1:20	0
4	37	30	1:200	112.1
5	65	3	1:20	0
6	65	3	1:200	45.5
7	65	30	1:20	0
8	65	30	1:200	49.0
9	51	3	1:110	24.0
10	51	30	1:110	24.6
11	37	17	1:110	16.9
12	51	17	1:20	0
13	51	17	1:110	0
14	51	17	1:200	119.5
15	65	17	1:110	0
16	51	17	1:110	0

Table 2. Experimental runs for a central composite design with the corresponding round of experiments, designated variables, and response in the fluorescence intensity of peptides and inhibition intensity bioactive peptides (enzymatic, antibacterial, and DPPH assay) for α-casein digestion with pepsin.

Run	Temperature (°C)	Time (min)	E:P ratio	α-glucosidase assay (Inhibition intensity %)	DPPH assay (Inhibition intensity %)	*B. subtilis* assay (Inhibition intensity %)
1	30	3	1:13	100.0	100.0	100.0
2	30	20	1:13	97.7	99.2	106.9
3	55	3	1:13	57.5	111.0	103.3
4	55	20	1:13	72.1	127.6	139.1
5	30	3	1:40	168.3	132.4	150.4
6	30	20	1:40	123.2	141.2	136.9
7	55	3	1:40	116.6	143.8	145.4
8	55	20	1:40	100.1	123.9	139.1
9	43	3	1:27	93.7	131.3	114.0
10	43	20	1:27	107.6	127.5	131.0
11	30	12	1:27	90.9	118.7	123.5
12	55	12	1:27	106.7	117.5	111.2
13	43	12	1:13	94.6	140.9	139.4
14	43	12	1:40	183.3	133.7	102.8
15	43	12	1:27	124.0	126.9	88.3
16	43	12	1:27	127.8	129.9	82.0

An experimental plan composed of sixteen runs was established for each enzyme (Table 1 and Table 2), assayed at least in duplicate (n = 2) in randomized order to minimize the effects of uncontrolled factors. An analysis of variance (ANOVA) with a significance level (α) of 0.05 was carried out to determine which experimental factors significantly affect the variable "band intensity". According to the results (Table 1), the E:P ratio (P=0.001) and temperature (P=0.002) significantly affected the peptide formation measured by intensity, while the factor time (P=0.06) did not have a significantly effect nevertheless the p-value obtained was closed to the significance level (0.06 vs 0.05). The optimal conditions provided by the model for trypsin MAED were a digestion time of 10 min, a temperature of 37°C and an E:P ratio of 1:200 (Figure 1). Regarding pepsin, the only factor that significantly affected the peptide formation was the E:P ratio (P=0.01), while time (P=0.88) and digestion temperature (P=0.12) did not significantly affect the process (Figure 2). The optimal conditions provided by the model for pepsin MAED were a digestion time of 4 min, a digestion temperature of 41°C, and an E:P ratio of 1:40.

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Figure 1. HPTLC chromatogram of design of experiments of casein digested with trypsin on ProteoChrom HPTLC plate, using a mobile phase composed of 2-propanol: n-butanol: ammonia: water (57: 20: 20: 3 v/v/v/v)

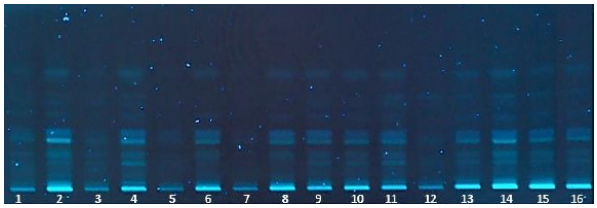
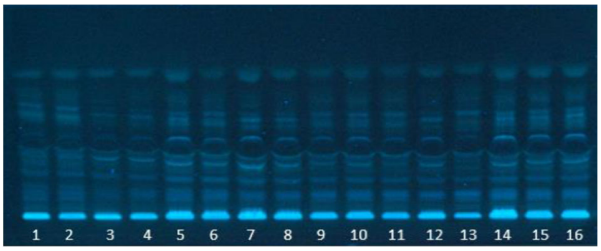


Figure 2. HPTLC chromatogram of design of experiments of casein digested with pepsin on ProteoChrom HPTLC plate, using a mobile phase composed of 2-propanol: n-butanol: ammonia: water (57: 20: 20: 3 v/v/v/v).



Bioassays

The bioassay for AChE inhibitory pepsin-digested peptides detection showed positive results, but the inhibition bands rapidly disappeared after a few seconds. This phenomenon could be related to higher plate moisture that could produce a spreading effect, overlapping the inhibition band. To solve the problem, the drying temperature was increased from 95ºC to 120ºC without positive results. Contrarily, trypsin-digested peptides showed two bands with inhibitory activity over AChE (Figure 3). In the case of α-glucosidase, only pepsin-digested peptides showed two inhibitory bands at *h*Rf=0.43 (Figure 4). Antioxidant peptides were observed with both enzymes in all experimental plan conditions; however, the effect was more intense for pepsin-digested peptides. In terms of antibacterial activity, both enzymes exhibited the ability to produce antibacterial peptides against B. subtilis. As mentioned earlier, the pepsin MAED yielded a higher number of peptide bands, among which a noteworthy band with a high inhibitory activity was observed at *h*Rf=0.53 (Figure 5 A y Figure 5 B). Trypsin-digested peptides only showed antibacterial activity when high protein concentrations were used. As can be seen in Figure 5C, at least a couple of antibacterial bands are clearly observed at *h*Rf=0.31.

Figure 3. HPTLC- chromatograms of digested peptides with trypsin. HPTLC-AChE bioassay of bioactive zones at t4, t6, and t8 corresponding to digested peptides with trypsin. Photo-documented under white light (A) and 254 nm (B).

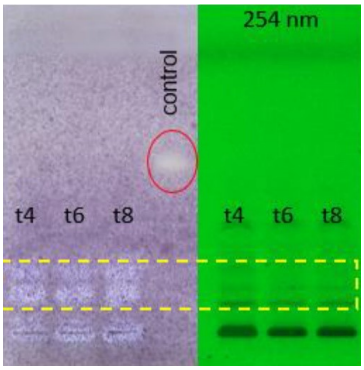


Figure 4. HPTLC chromatograms of digested peptides with pepsin on silica gel 60 F^{254} plates using a mobile phase composed of 2-propanol: n-butanol: ammonia: water (57: 20: 20: 3 v/v/v/v). HPTLC- α -glucosidase bioassay photo-documented under 254 nm (A) and white light (B).

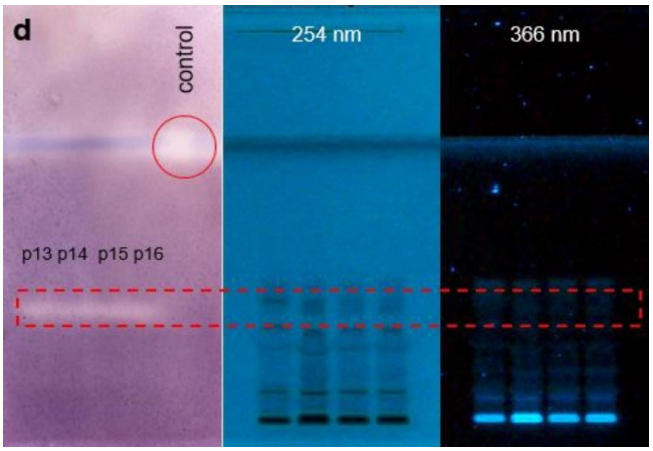
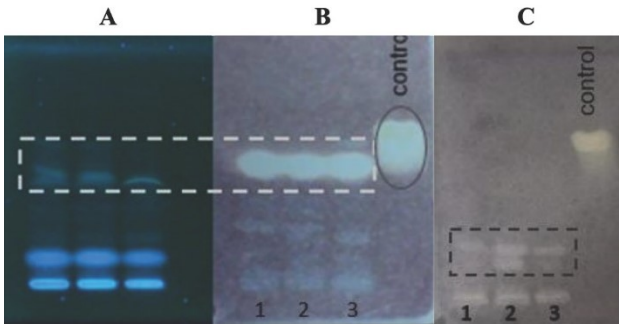


Figure 5. HPTLC chromatogram after derivatization with fluorescamine reagent at FLD 366 nm (A), *Bacillus subtilis* bioautogram of casein digested with pepsin (B), and trypsin (C) (each digested was applied 20 µL/area). Positive control, oxytetracycline 4 µL.



Optimization of MAED conditions for obtaining bioactive peptides

An analysis of variance (ANOVA) with a significance level (α) of 0.05 was performed to determine which factors significantly affect the intensity (quantity) of bioactive peptides. Optimization of bioactive peptides generation from trypsin digestion was evaluated by measuring antioxidant capacity (DPPH). The optimal conditions suggested by the model were a digestion time of 30 minutes, a temperature of 37°C, and an E/P ratio of 1/200. The factors that significantly ($P < 0.05$) influence the intensity of the peptide were the temperature ($P = 0.04$), E/P ratio ($P = 0.0002$), and the interaction between temperature and E/P ratio ($P = 0.04$). In the case of pepsin-digested bioassays, the response variable included antioxidant capacity (DPPH), antimicrobial properties, and α-glucosidase inhibitors.

For antioxidant peptides, the optimal conditions proposed by the model were a digestion time of 3 minutes, a temperature of 42°C, and an E/P ratio of 1/40. Only the last factor significantly affects the peptides intensity (P = 0.04), the other factors and interactions did not show any statistically significant effect. The optimal pepsin digestion conditions to obtain peptides with antimicrobial activity determined through B. *subtilis* inhibition were a digestion time of 3 min, a temperature of 30°C, and E/P ratio of 1/40.
Regarding α-glucosidase inhibitor's peptides, the optimal conditions recommended by the model were a digestion time of 5 minutes, a temperature of 35°C, and an E/P ratio of 1/40. Only the E/P ratio (P = 0.004), showed a statistically significant effect over bioactive peptide formation. and the most influential factor in pepsin digestion was the ratio of enzyme to protein.
Previous studies have demonstrated that MAD can yield peptides with AChE inhibitory, α-glucosidase inhibitory, and antimicrobial activities. Hall and Liceaga [50] conducted a study on Cryllodes sigillatus and observed antihypertensive and antidiabetic effects using a 10-minute microwave-assisted digestion (MAD) method. In contrast, Srinivas and Prakash [5] examined the inhibitory activity of AChE, antioxidant capacity, and antimicrobial activity of peptides derived from α-casein. However, their digests were obtained through conventional digestion, which involved a minimum of 2 hours for the digestion process.

4. CONCLUSIONS

This study presents a novel approach utilizing MAED and HPTLC-bioassay to obtain and detect bioactive peptides from α-casein digestion with pepsin and trypsin enzymes. The optimization of α-casein digestion was achieved through chemometric tools employing a central composite design, allowing to establishment a high throughput, precise, and sustainable MAED method to generate bioactive peptides from α-casein digestion. HPTLC-bioassay proved to be a unique technology capable of detecting different in situ bioactivities. Through this analytical technique, it was possible to detect peptides generated from pepsin- and trypsin digestion with very promising bioactivities, i.e., AChE inhibitory peptides, α-glucosidase inhibitors, antioxidant, and antimicrobial peptides. These peptides will be identified soon to be studied as functional ingredients or nutraceuticals.

5. ACKNOWLEDGEMENTS

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