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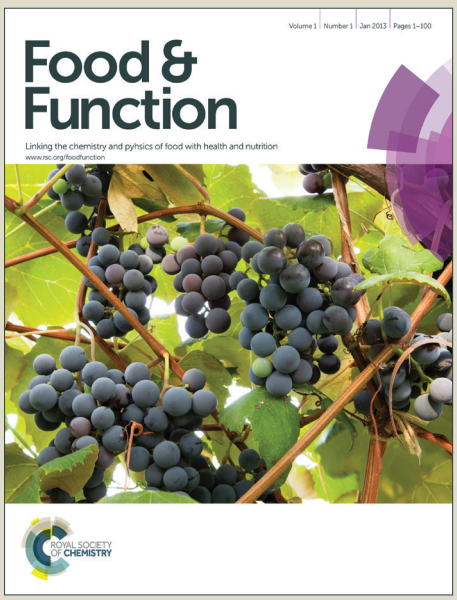

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Investigation of the Reactions of Acrylamide during in vitro Multistep Enzymatic Digestion of Thermally Processed Foods

Aytül Hamzalıoğlu, Vural Gökmen*

Department of Food Engineering, Hacettepe University, 06800 Beytepe, Ankara, Turkey

* Corresponding Author

Prof. Dr. Vural Gökmen

Tel: 90-312-297-7108

Fax: 90-312-299-2123

E-mail: vgokmen@hacettepe.edu.tr

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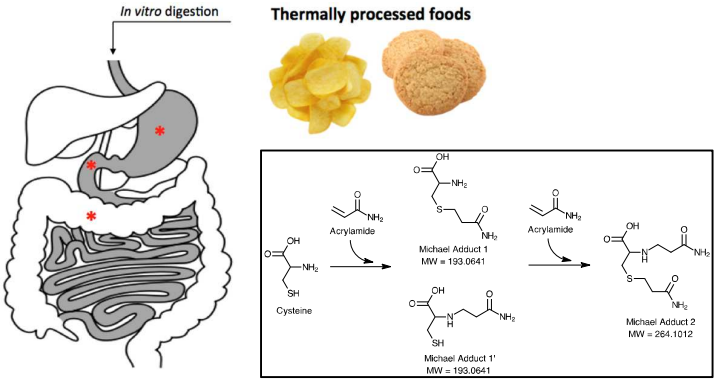

Highlights

- Nucleophile groups of amino acids binds to acrylamide during *in vitro* digestion.
- Acrylamide level in food decreases through duodenal and colon phases.
- Intermediates in fried potatoes may convert to acrylamide during gastric digestion.

Abstract

This study aimed to investigate the fate of acrylamide in thermally processed foods after ingestion. An in vitro multistep enzymatic digestion system simulating gastric, duodenal and colon phases was used to understand the fate of acrylamide in bakery and fried potato products. Acrylamide levels gradually decreased through gastric, duodenal and colon phases during in vitro digestion of biscuits. At the end of digestion, acrylamide reduction ranged between 49.2% and 73.4% in biscuits. Binary model systems composed of acrylamide and amino acids were used to understand the mechanism of acrylamide reduction. High-resolution mass spectrometry (HRMS) analyses confirmed Michael type addition of amino acids to acrylamide during digestion. Contrary to bakery products, acrylamide levels increased significantly during gastric digestion of fried potatoes. The Schiff base formed between reducing sugars and asparagine disappeared rapidly meanwhile acrylamide level increased during the gastric phase. This suggests that intermediates like the Schiff base accumulated in potatoes during frying are potential precursors of acrylamide under gastric conditions.

Introduction

Ingestion of food is considered as the major route of exposure to many contaminants in human health risk assessment. Acrylamide is one of the process contaminants formed in foods as a result of thermal processing such as roasting, frying or baking at elevated temperatures [1].Bakery products, fried potatoes and roasted coffee are the main dietary sources of acrylamide [2].

Total amount of a contaminant found in the ingested food does not always reflect the amount that is available to the body. Ingested food enters to the digestive tract that alters significantly its structure and chemical composition by means of varying pH and action of several enzymes in mouth, stomach and intestine. Thus, bioaccessibility is used to describe the proportion of the ingested contaminants in food that are released from the matrix into digestive juice in the gastrointestinal tract [3]. Therefore, determination of the bioaccessibility of a contaminant from the matrix, and the fate of ingested contaminant during digestion is an important issue for human health. For this purpose, applying *in vivo* methods, including animal and human subjects, is a better way to provide more accurate results, but also time consuming and expensive [4]. *In vitro* models that simulate the body conditions well are useful alternative to *in vivo* studies allowing a rapid screening of targeted compounds.

Number of studies has been previously carried out to determine the bioaccessibility of certain contaminants. Brandon et al. [5] used *in vitro* digestion models and tested the bioaccessibilities of lead, phthalates, and benzoic acid in

some commercial products. Yang et al. [6] tested the bioaccessibility of cadmium in uncooked rice from mining areas using an *in vitro* digestion model. De Angelis et al. [7] studied the bioaccessibility of *Fusarium* toxins, T-2 and HT-2 in two contaminated breads using a digestion model simulating the upper intestinal tract.

Acrylamide is one of the most widely encountered thermal process contaminants in foods. However, information about its fate during the digestion of processed foods is lacking. Owing to its potential reactivity, acrylamide present in foods may interact with certain components released from the food matrix under the varying conditions of gastrointestinal tract. Moreover, these conditions may favor the conversion of precursors into acrylamide. Therefore, this study aimed to investigate the fate of acrylamide present in thermally processed foods after ingestion using *in vitro* multistep enzymatic digestion system. The system simulating mainly gastric, duodenal and colon phases was used to digest actual foods (bakery and fried potato products). Binary models were also digested to understand better the reactions between acrylamide and amino acids (cysteine and lysine) under the conditions applied during digestion. The digested samples were analyzed by HRMS to confirm the Michael type addition of amino acids to acrylamide, and the conversion of Schiff base to acrylamide.

Results & Discussion

Bakery and fried potato products as main sources of dietary acrylamide were selected as typical examples of thermally processed foods. A total of 4 commercial biscuits (2 sweet and 2 non-sweet), one laboratory made biscuit, 2 potato chips and one potato fry were used for *in vitro* multistep enzymatic digestion test. Initial

acrylamide concentrations were found as 232±4 and 348±18 ng/g for non-sweet biscuits, 270±5 and 318±2 ng/g for sweet biscuits, and 173±3 ng/g for laboratory made sweet biscuit. Acrylamide concentrations of potato chips and potato fry samples were found as 178±8, 239±18 and 263±18 ng/g, respectively.

Table 1 gives the amounts of acrylamide remaining in the digests of biscuit samples after gastric and duodenal phases of the digestion process. There were significant reductions in the amounts of acrylamide in the digests of all sweet and non-sweet biscuits at the end of gastric and duodenal phases. Taking the entire enzymatic digestion process into account, acrylamide reduction ratio was found to range between 49.2% and 73.4% for biscuit samples. The reduction was significantly higher in the gastric and duodenal phases than in the colon phase. For biscuits, the ratio of acrylamide reduction ranged between 17.4% and 49.9% in the gastric phase, and between 23.9% and 58.1% in the duodenal phase.

Gastric phase was the simulation of stomach where pepsin hydrolyzed proteins into smaller peptides or amino acids at low pH conditions. Duodenal phase included the addition of bile salts capable of promoting digestion and absorption of lipids together with pancreatin having the activities of amylase, lipase and trypsin. In the colon phase, proteolytic enzymes of microbial flora of colon continued to favor the hydrolysis of proteins and peptides. So, the simulated digestion process created a pool of amino acids that might be interacting with acrylamide. It has been previously reported that Michael type addition of amino acids to acrylamide, which is a potential way to decrease acrylamide content of foods, can take place under certain conditions [8-9]. Due to its highly electrophilic nature, each molecule of

amino acid could form adducts with one or two molecules of acrylamide [10].

Three model systems, namely acrylamide (ACR), acrylamide-lysine (ACR-LYS), and acrylamide-cysteine (ACR-CYS) were used to understand the mechanism of acrylamide reduction. Table 2 gives the amounts of acrylamide remaining in the digests of these model systems after gastric, duodenal and colon phases. At the end of gastric phase, there were slight but statistically significant ($p<0.05$) reductions in the amounts of acrylamide for both ACR-CYS and ACR-LYS model systems. The amount of acrylamide remained relatively stable after duodenal and colon phases in the digest of the ACR-LYS model system. However, it tended to decrease significantly ($p<0.05$) after duodenal and colon phases in the digest of the ACR-CYS model system. These findings indicated the potential of acrylamide to react with nucleophilic groups (–SH, $–NH_2$) of amino acids side chains under the stated digestion conditions. Hidalgo et al. [10-11] reported a rapid reduction of acrylamide upon heating in the presence of N-acetyl-cysteine or lysine as a consequence of the Michael type addition the nucleophilic groups to the carbon–carbon double bond of acrylamide [11]. Cysteine owing to its highly nucleophilic -SH group was thought to be more favorable in reacting towards acrylamide [12].

Fig. 1 shows the proposed mechanism of acrylamide elimination through Michael type addition of cysteine during digestion process. According to this mechanism, cysteine may react with one or two moles of acrylamide from both nucleophilic groups (-SH or NH_2) forming Michael-adducts 1, 1’ or 2, respectively. Scan HRMS analyses of the digests of ACR-CYS model system confirmed the formation of these adducts with very high mass accuracy ($\Delta<2$ ppm) under the simulated digestion

131 conditions. Only the formation of Michael adduct 1 was observed in the digests of
132 ACR-LYS model system under the same conditions. These results suggest that
133 cysteine released through proteolytic activity of enzymes in the gastrointestinal
134 tract might be responsible for the elimination of acrylamide during digestion.

135 Table 3 gives the amounts of acrylamide remaining in the digests of fried potato
136 samples after gastric, duodenal and colon phases of the digestion process. Contrary
137 to biscuits, the amounts of acrylamide increased significantly ($p<0.05$) during
138 gastric digestion of fried potatoes. At the end of gastric phase, acrylamide levels
139 increased 3.95, 1.20 and 1.45 times in the digests of potato fry and potato chips,
140 respectively. Acrylamide levels tended to decrease significantly ($p<0.05$) in the
141 digests of fried potato samples after duodenal and colon phases. The ratio of
142 acrylamide reduction ranged between 78.2% and 96.8% in the duodenal phase,
143 and between 48.3% and 90.2% in the colon phase.

144 Eriksson et al. [13] determined the effects of pH and different enzymes on the
145 extraction of acrylamide from foods. According to their results, extraction yield of
146 acrylamide from foods at pH values ranging from 2.0 to 7.5, or with pepsin enzyme
147 was similar to the yield obtained with water. So, increase of acrylamide levels in
148 the digests of fried potato products during the gastric digestion cannot be
149 attributed to its increased extractability under acidic conditions. It is a fact that
150 asparagine and reducing sugars are the main precursors of acrylamide [14].
151 According to its formation, Schiff base, decarboxylated Schiff base and 3-
152 aminopropionamide form as critical intermediates upon heating a mixture
153 containing asparagine and glucose [15-17]. Since raw potato is relatively rich in

asparagine and reducing sugars, frying may form high quantities of above-mentioned intermediates together with acrylamide in fried potatoes. Therefore, these intermediates may be considered as potential precursors of acrylamide during the gastric digestion phase. To confirm this, a model system composed of asparagine and glucose (ASN-GLC) was prepared and heated at 180°C for 10 min. Then, heated model system of ASN-GLC was subjected to *in vitro* multistep digestion. As in fried potato samples, acrylamide amount significantly increased (41.3%) in the digest of ASN-GLC model system during the gastric phase while it significantly decreased (95.7%) after duodenal and colon phases. Prior to gastric digestion, scan HRMS analyses confirmed the presence of Schiff base formed between asparagine and glucose in heated ASN-GLC model system, as well as in fried potato samples with very high mass accuracy (Δ<1 ppm). In the gastric phase, decrease of the signal response of parent $[M+H]^+$ ion of the Schiff base was compatible with the increase of acrylamide level. More than 90% of the Schiff base present initially in the fried potatoes disappeared in their digests after the gastric phase. The results suggest that intermediates like the Schiff base formed between asparagine and reducing sugars accumulated in fried potatoes during frying are converted to acrylamide under gastric conditions as proposed in Fig. 2.

Experimental

Chemicals and consumables

Acrylamide (99%), L-cysteine (>99%), L-Lysine, L-Asparagine (98%), were purchased from Sigma (Deisenhofen, Germany). Potassium chloride, sodium chloride, magnesium chloride, ammonium bicarbonate, potassium dihydrogen

phosphate were purchased from Merck (Darmstadt, Germany). Potassium hexacyanoferrate (II) trihydrate and zinc sulphate heptahydrate were purchased from Merck (Darmstadt, Germany). Carrez I and Carrez II solutions were prepared by dissolving 15 g of potassium hexacyanoferrate in 100 mL of water, and 30 g of zinc sulfate in 100 mL of water, respectively. The enzymes: pepsin (≥250 U/mg solid) from porcine gastric mucosa, pancreatin (4 x USP) from porcine pancreas, protease from Streptomyces griseus (≥3.5 U/mg solid) and viscozyme L were purchased from Sigma–Aldrich (Deisenhofen, Germany). Bile extract, porcine was also purchased from Sigma Aldrich (Deisenhofen, Germany). Formic acid (98%), acetonitrile and methanol (HPLC grade) were purchased from J. T. Baker (Deventer, Holland). Oasis MCX solid phase extraction cartridges (1 mL, 30 mg), UPLC HSS T3 column (150 mm x 4.6 mm i.d.; 3 µm), Atlantis T3 column (250 mm x 4.6 mm id; 5 µm), and 0.45 µm nylon syringe filters were supplied by Waters (Millford, MA, USA).

Preparation of foods

Both commercial and laboratory made food samples were used to determine the fate of acrylamide during in vitro digestion. Commercial biscuits and potato chips were obtained from a local market, and dried potato samples from a fast food restaurant in Ankara.

Laboratory made biscuits were prepared using a recipe adapted from the American Association of Cereal Chemists (AACC) method 10-54. Ingredients were as follows; wheat flour (80 g), shortening (32 g), sucrose (35 g), glucose (0.6 g), fructose (0.6 g), nonfat milk powder (0.8 g), $NaHCO_3$ (0.8 g), NH_4HCO_3 (0.4 g), NaCl

(1 g), and water (17.6 g). All ingredients were thoroughly mixed in accordance with the procedure described in AACC method 10–54 using a dough mixer Artisan Kitchen Aid 5KSM150 (MI, USA). Dough was rolled in 4 mm thickness, cut into discs having 6 cm diameter. The discs were baked in a conventional oven (Memmert, UNE 400, Germany) set at 200 °C for 12 min. All food samples were grinded and freeze dried prior to the digestion process. In addition, they were analyzed to determine their initial acrylamide contents.

Preparation of model systems

Different model systems composed of acrylamide alone (ACR), acrylamide-cysteine (ACR-CYS), and acrylamide-lysine (ACR-LYS) were prepared to determine both the fate of acrylamide and its interaction with amino acids during in vitro digestion. For these model systems, 10 μmoles of reactants were dissolved in 10 mL of deionized water, and directly subjected to *in vitro* multistep digestion process.

Another model system composed of 10 μmoles of asparagine and glucose (ASN-GLC) was also prepared. It was heated 180°C for 10 min to form the Maillard reaction products including acrylamide and intermediate compounds like the Schiff base formed between asparagine and glucose. Then, heated model system was dissolved in 10 mL of deionized water and subjected to *in vitro* multistep digestion to determine changes in the levels of acrylamide and Schiff base formed between asparagine and glucose.

In vitro digestion

Digestion fluids simulating the saliva, gastric juice and duodenal juice were used to

mimic the conditions of gastrointestinal tract. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated duodenal fluid (SDF) were prepared according to the procedure described by Minekus et al. [18]. *In vitro* digestion procedure was adapted from procedure reported by Papillo et al. [19] . 5 grams of dry ground food or 10 mL of the model system were transferred to a glass flask with screw cap. For food samples, 5 mL of SSF was added and the flask was shaken for 2 min to simulate the oral passage. Liquid model system samples were not exposed to the oral phase, thus they were put directly into the gastric phase. After 5 mL of pepsin solution (12.5 mg/ml in 0.1 M HCl) and 10 mL of SGF were added, the mixture was adjusted to pH 2.0. Then, the acidified mixture was incubated at 37^{o}C by shaking for 2 h at an agitation speed of 60 strokes per min to simulate the gastric phase. Bile salts were dissolved in the SDF solution to a concentration of 10 mg/mL. The pH was adjusted to 7.5 after the gastric phase. After that, 20 mL of the mixture of SDF with bile salts and 5 mL of pancreatin solution (10 mg/ml in water) were added to the flask. The mixture was incubated at 37^{o}C by shaking for 2 h at an agitation speed of 60 strokes per min to simulate the duodenal phase. The colon phase was simulated by the addition of bacterial enzymes of the flora found in colon. It is supposed to be the consecutive hydrolysis of proteins and polysaccharides occur in the sample during colon phase. For this, 5 mL of protease solution (1mg/ml, pH 8.0) was added, and the mixture was incubated at 37^{o}C by shaking for 1 h. Then, 150 µL of Viscozyme L was added, and the mixture was incubated at 37^{o}C by shaking for 16 h at an agitation speed of 30 strokes per min. Aliquots of samples were withdrawn from the flask at the end of simulated gastric, duodenal and colon phases for the analyses of acrylamide, its precursors and

reaction products. All samples were digested with three parallels as described above.

Analysis of acrylamide in the digests by LC-MS/MS

Aliquots of the digests withdrawn from the samples of model systems were centrifuged at 11180 g for 5 min. The supernatant was filtered through 0.45 μm filter into an autosampler vial, and analyzed for acrylamide using LC-MS/MS. Aliquots from food samples were transferred to petri dishes and dried in an oven at 37^{o}C. Dried powder (100 mg) was double extracted with 10 mM formic acid (2 x 1 mL) by vortexing for 3 min. The combined extract was clarified by adding 0.125 ml of Carrez I and Carrez II solutions. The mixture was centrifuged at 10,000g for 5 min. The supernatant was passed through a preconditioned Oasis MCX solid phase extraction cartridge to further clean up the extract. The first 8 drops of the eluent were discarded. The rest was filtered through 0.45 μm filter into an autosampler vial, and analyzed for acrylamide using LC-MS/MS.

A Waters Acquity H Class UPLC system (Millford, MA) coupled to a TQ detector with electrospray ionization operated in positive mode was used to analyze the extracts for acrylamide. The chromatographic separations were performed on an Acquity UPLC HSS T3 column (150 mm x 4.6 mm i.d.; 3 μm) using 0.1 % formic acid in water with 0.1 % formic acid in acetonitrile as the mobile phase at a flow rate of 0. 5 mL/min. The column equilibrated at 40°C and Waters ACQUITY FTN Autosampler was held at 10°C during the analysis. The electrospray source had the following settings: capillary voltage of 0.80 kV; cone voltage of 22 V; extractor voltage of 4 V; source temperature at 120°C; desolvation temperature at 350°C;

desolvation gas (nitrogen) flow of 900 L/h. The flow rate of the collision gas (argon) was set to 100 L/h. Acrylamide was identified by multiple reaction monitoring (MRM) of two channels. The precursor ion $[M+H]^+$ 72 was fragmented and product ions 55 (collision energy of 9 V) and 44 (collision energy of 12 V) were monitored. The dwell time was 0.2 sec for all MRM transitions. Stock solution of acrylamide was prepared in water to a concentration of 1 mg/mL. Working solutions were prepared by diluting the stock solution with water. The concentration of acrylamide in samples was calculated by means of a calibration curve built in the range between 1 and 20 ng/mL (1, 2, 5, 10, 20 ng/mL).

Analysis of reaction products and precursors of acrylamide in the digests by high-resolution mass spectrometry (HRMS)

Aliquots of the digests withdrawn from model systems and food samples were transferred to petri dishes and dried in an oven at 37°C. Dried powder (100 mg) was extracted with 10 mL of water by vortexing for 2 min. Then, the extract was centrifuged at 11180 g for 5 min. After that, 2 ml of the supernatant was passed through a 0.45 μm filter into an autosampler vial prior to analysis of reaction products and precursors of acrylamide. HRMS was used to confirm Michael type addition of amino acids (cysteine and lysine) to acrylamide in the digests of model systems. In addition, it was used to confirm the presence of acrylamide precursors, specifically the Schiff base formed between asparagine and glucose in the digests of food samples.

A Thermo Scientific Accela UHPLC system (San Jose, CA) coupled to a Thermo Scientific Exactive Orbitrap HRMS was used to analyze the extracts. The HRMS

system was operated in positive electrospray ionization mode. The chromatographic separations were performed on Atlantis T3 column (250 mm x 4.6 mm id; 5 μm) using a gradient mixture of 0.05% aqueous formic acid and methanol as the mobile phase at a flow rate of 0.5 mL/min (30°C). The mobile phase gradient was programmed as follows: 70% of methanol for 8 min, linear increase to 95% of methanol within 4 min, 95% of methanol for 4 min, and linear decrease to 70% of methanol within 4 min. The scan analyses were performed in an m/z range between 50 and 400 at ultra-high resolving power (R=100,000). The data acquisition rate, the automatic gain control target and maximum injection time were set to 1 Hz, $1x10^6$ and 100 ms, respectively. The source parameters were as follows: sheath gas flow rate 30 (arbitrary units), auxiliary gas flow rate 10 (arbitrary units), discharge voltage 4.5 kV, discharge current 5 μA, capillary temperature 330°C, capillary voltage 47.5 V, tube lens voltage 115 V and vaporizer temperature 330°C. The corresponding ions were extracted from the total ion chromatograms to confirm the presence of the Michael addition products of cysteine and lysine to acrylamide, and of the Schiff base of asparagine in the digests.

Statistical analysis

The data were subjected to analysis of variance (one-way ANOVA). The SPSS 17.0 statistical package was used for the evaluation of statistical significance of the differences between mean values by Duncan test. $P<0.05$ was considered to be statistically significant for the results.

Conclusions

The conditions of simulated digestion favored the Michael type addition of amino acids to acrylamide. Owing to its two nucleophilic groups (–SH, $–NH_2$), cysteine becomes highly reactive toward acrylamide especially under the simulated duodenal conditions. As a result, acrylamide levels of baked or fried products decrease significantly during *in vitro* enzymatic digestion process. However, intermediates that are present in fried potatoes act as precursor increasing acrylamide levels under gastric conditions. Due to its elimination and formation potential during *in vitro* digestion process, levels of acrylamide ingested with foods may not directly indicate its absorption rate through gastric, duodenal and colon. Gastrointestinal conditions as well as ingested food composition seem effective on bioavailable acrylamide levels, and should be taken into consideration.

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368 ***Figure Captions***

369

370 **Fig. 1.** Proposed mechanism for the reduction of acrylamide during in vitro
371 enzymatic digestion through the formation of Michael adducts with cysteine

372 **Fig. 2.** Proposed mechanism for the formation of acrylamide during in vitro gastric
373 digestion from the precursors existing in fried potato

374

375 **Table 1.** Changes in the amount of acrylamide in the digests of biscuits during *in*

376 *vitro* multistep enzymatic digestion process

	Acrylamide per 5 g sample, μmol			
	initial	*gastric phase*	*duodenal phase*	*colon phase*
Non-sweet biscuits				
sample 1	16.32±0.40[a]	10.90±0.10[b]	6.68±1.39[c]	5.73±1.09[c]
sample 2	24.48±1.79[a]	12.27±0.45[b]	7.95±0.50[c]	7.60±0.56[c]
Sweet biscuits				
sample 1	18.96±0.45[a]	13.33±2.44[b]	5.59±0.05[c]	5.24±0.35[c]
sample 2	22.33±0.15[a]	11.57±2.44[b]	7.28±0.25[c]	5.94±0.35[c]
sample 3*	12.13±0.25[a]	10.02±0.25[b]	7.63±0.45[c]	6.16±0.25[d]

377 * Laboratory made biscuit sample.

378 [a–d] Values marked with different letters in each row are significantly different ($P < 0.05$).

379

380

381 **Table 2.** Changes in acrylamide content of different model systems during *in vitro*

382 enzymatic digestion

Model System	Acrylamide per model system, μmol			
	initial	*gastric phase*	*duodenal phase*	*colon phase*
ACR	10.25 ±0.56 [a]	9.29±0.19 [a,b]	8.10 ± 0.07 [b,c]	7.79 ± 0.05 [c]
ACR-LYS	10.25 ±0.56 [a]	8.22±0.01 [b,c]	8.27 ±1.25 [b,c]	8.36 ±0.43 [b,c]
ACR-CYS	10.25 ±0.56 [a]	8.31 ±0.13 [b,c]	5.32 ± 0.77 [d]	4.02 ± 0.41 [e]

383 [a–e] Values marked with different letters in each row are significantly different ($P < 0.05$).

384

385 **Table 3.** Changes in the amount of acrylamide in the digests of fried potatoes
386 during *in vitro* multistep enzymatic digestion process

	Acrylamide per 5 g sample, μmol			
	initial	*gastric phase*	*duodenal phase*	*colon phase*
Potato fry				
sample 1	18.47±1.74[a]	73.02±6.17[b]	2.32±0.40[c]	0.34±0.18[d]
Potato chips				
sample 1	12.49±0.75[a]	14.98±1.29[b]	1.20±0.70[c]	0.62±0.10[c]
sample 2	16.81±1.79[a]	24.41±0.10[b]	5.31±1.04[c]	0.52±0.03[d]

387 [a–d] Values marked with different letters in each row are significantly different ($P < 0.05$).

388