

Assessment of the *in vitro* digestibility of formulated snacks enriched with bioactive ingredients

CELAL DEGERLI and SEDEF NEHIR EL

Department of Food Engineering, Nutrition Section, Ege University, Bornova, Izmir 35100, Turkey

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Abstract. The present study aimed to formulate healthier snacks, and assess the effects of the foods and ingredients used in the formulation of these snacks on the *in vitro* hydrolysis of macronutrients in a real food matrix. A total of five different snacks were formulated based on Smart Snack criteria and various evaluations were conducted, including sensory analysis, approximate and fatty acid compositions, the degree of protein and lipid hydrolysis using pH-stat titration technique, and starch hydrolysis *in vitro*. The formulated snacks were found to comply with the criteria specified in the Smart Snack Standards. As regards protein hydrolysis, snacks 1, 2 and 3 exhibited values of 65.17, 84.84 and 63.12%, respectively ($P < 0.05$). For lipid hydrolysis, the values were 76.85, 62.63 and 74.65%, respectively ($P > 0.05$). The snacks were evaluated to have a moderate glycemic index. Snacks 4 and 5 were prepared as ready-to-eat; in these snacks, protein hydrolysis was found to be 57.37 and 84.46%, respectively ($P < 0.05$), while lipid hydrolysis values were 80.94 and 91.92%, respectively ($P < 0.05$). On the whole, the present study sought to ascertain whether healthier snacks could be developed within the framework of existing Smart Snacks standards.

Introduction

According to epidemiological and clinical studies reviewed in the report by the FAO and WHO, dietary preferences and physical activity exert critical effects on the risk of developing certain chronic diseases and cancers. By considering the energy density of foods in the diet, reducing the intake of added sugar, high fat/saturated fats and sodium, and increasing the intake of dietary fiber and bioactive compounds, can prevent the risk of developing chronic and degenerative diseases. This can aid individuals in the maintenance of their body weight, and can

help protect cognitive and physiological health (1). However, according to statistical data including 46 European countries, 1 in 3 children between the ages of 6 and 9 years is either overweight [body mass index (BMI), 25-30] or obese (BMI ≥ 30). The number of obese children in Europe was 41 million in 2016 and is expected to reach 70 million by 2025 (2). In the childhood and adolescence period, snacks with high-calorie and unbalanced nutrients are creating dietary issues. The majority of dietary guidelines describe all high-calorie foods and beverages consumed between main meals as 'snacks'. In some recommendations, 'snack foods' are defined as foods with a low nutrient density, but high calories, saturated fats and added sugar (3-6). Studies indicate a positive association between snack food consumption and the risk of developing chronic diseases, such as cardiovascular diseases, obesity, type-2 diabetes and insulin resistance (7). Nutrition in childhood plays a critical role in the prevention of obesity in both childhood and adulthood. The United States Department of Agriculture (USDA) has issued A Guide to Smart Snacks in School; in this standard, there are limits to the ingredients, nutrients and energy value of snacks (8). As a result of this new standard, it has become increasingly popular to employ snack designs that incorporate healthier recipes (containing added proteins, fibers, unsaturated fats, vitamins and mineral sources), as opposed to solely oil and/or sugar. Whey protein concentrate (WPC), natural sweeteners, dietary fibers, such as inulin and pectin, omega-3 fatty acids, resistant starch or certain bioactive compounds, such as gallic acid and ellagic acid are used in new product designs or enrichment studies (9-12). However, the nutritional quality of food does not only depend on its nutrient profile, but the matrix and physicochemical properties of food are also important for the fate properties and metabolic responses of nutrients and bioactive compounds in the digestive system. Research has indicated that foods of the same composition have differences in their digestion and responses, such as on the glycemic index (GI) or satiety, depending on its structural properties and applied technological processes (13). Another study proposed a method to evaluate the impact of complex food matrix structures on digestion kinetics (14). This method combines the pH-stat technique with a static *in vitro* digestion protocol based on the INFOGEST guidelines (15). That method was used to investigate the digestion behavior of two emulsion-type food matrices that have the same compositions (10 wt% fat, 15 wt% whey proteins), but have different structures at the

Correspondence to: Mr. Celal Degerli, Department of Food Engineering, Nutrition Section, Ege University, Erzene Mahallesi, No. 172, Bornova, Izmir 35100, Turkey
E-mail: celal.degerli@yahoo.com

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macroscopic and microscopic scales. However, studies on the structural effects of real food matrices on both lipolysis and proteolysis kinetics and hydrolysis degrees are required (14).

The present study aimed to develop healthier snacks and evaluate the *in vitro* hydrolysis of macronutrients within a real food matrix, utilizing a combined approach of pH-stat titration technique and the INFOGEST *in vitro* digestion protocol. A total of five snack prototypes were formulated following the Smart Snack criteria (8). These prototypes were then assessed for their compliance with nutrient content claims permitted by Regulation (EC) No. 1924/2006 (<http://data.europa.eu/eli/reg/2006/1924/2014-12-13>). Various foods (dry nuts, dry fruits, kefir, eggs, butter and sunflower oil) or ingredients rich in bioactive compounds and nutrients (whey protein, chickpea flour, green tea, inulin, cinnamon and honey) were used in the formulations. Of note, three of the snacks were baked and two of them were prepared as ready-to-eat. In all the snacks, the sensory evaluation, the approximate and fatty acid compositions, the degree of protein and lipid hydrolysis *in vitro* using pH-stat and *in vitro* starch hydrolysis were determined.

Materials and methods

Foods, ingredients and chemicals. All foods and ingredients were purchased from local suppliers. Goat kefir was purchased from Baltali Food Company. The 80% purity WPC (WPC80) was provided by Dr. Oetker Türkiye Food Company (<https://www.droetker.com.tr/kunye>). The vacuum dried vegetables; red pepper, beet roots, spinach, leeks, zucchini and carrot were a kind gift from Eregli AgroSan Company. Pullulanase (EC-3.2.1.41), pepsin (≥ 2500 U/mg protein, EC 232-629-3), pancreatin (EC 232-468-9), α -amylase (EC 232-565-6), α -chymotrypsin (C 4129), trypsin (T 030) and bile salt (B 8638) were purchased from MilliporeSigma. The D-glucose measurement kit was purchased from Megazyme.

Lyophilized goat kefir, resistant starch flour and brewed green tea. Goat kefir was freeze-dried using a freeze-dryer (Model-FT33, Armfield) and the dry matter ratio of goat kefir was increased from 12 to 96%, while the total fat ratio was increased from 4 to 28%.

Chickpea flour was diluted at a ratio of 1:8 with water and pre-gelatinized for 10 min. The autoclave was then held for 15 min at 121°C. Subsequently, pullulanase (80 U/g sample) was added followed by incubation in a water bath (60°C, 6 h). The retrogradation process was then repeated and the samples were dried at 55°C for 7 h. The dried samples were kept at 4°C in powder form until the resistant starch content was analyzed (16).

Dried green tea leaves (0.8 g) were added to 250 ml of boiling water and kept for 4 min for infusion. The leaves were filtered, and the tea was stored at 4°C until use.

Snack preparation. The classic homemade shortbread (R) was used as a control and its recipe was used as the basis for the five snacks. The ingredient list of the prepared snacks is presented in Table I. For snacks 1-3, the dry ingredients were mixed and green tea was then added. The dough was kneaded (7 min) by hand and shaped 5.5 cm width, 15 cm length and 2.5 cm in height before the pre-baking process in the convection oven (9629

CMS, Beko) for 40 min at 150°C, without a fan. Subsequently, the pre-baked dough was removed from the oven and cut into rectangular shapes, which were 1-cm-thick, and baked again for 10 min at 150°C, with a fan. For snacks 4 and 5, dried nuts were ground in the kitchen grinder (BH259CG, Blue House) for 10 sec and transferred to a bowl. The dried fruits were then mixed with WPC80 for snack 5 and with inulin for snack 4. All ingredients in the bowl were then kneaded by hand for 180 sec with the addition of honey, cinnamon, glycerol or sunflower lecithin. Baking was not applied to these snacks and they were prepared as ready-to-eat. For this, the dough was formed into a bar and stored at 4°C for 2 h in the refrigerator, to obtain its firmness. All snacks were stored at -18°C until analyses were performed.

Proximate composition. Fat, total sugar, moisture and ash analyses were determined according to AOAC (17). The water activities (a_w) of the snacks were determined with a precision humidity measuring instrument (Testo 650, Testo SE & Co. KGaA). The total carbohydrate amount (CHO) was determined using the difference method. The energy values of the snacks were calculated using Atwater calorie constants (18). The fatty acid composition of the snacks was measured using gas chromatography (Agilent Technologies, Inc.) with a FID detector at 235°C.

Sensory evaluation. The standard hedonic scale (5-point scale; from 1, dislike; to 5, like, ISO 11136:2014; <https://www.iso.org/standard/50125.html>) was used to assess the sensory attributes and overall acceptance of the developed snacks. A total of 80 panelists (aged between 18-55 years) were randomly selected and asked to evaluate the snacks according to their taste preferences. Overall acceptability was calculated as the mean of scores describing each attribute and multiplied by the importance factor. Snacks >70% points were considered successful.

In vitro hydrolysis of protein and lipids using pH-stat. The *in vitro* digestion of snacks was performed according to the INFOGEST standardized static digestion protocol (15). The preparation of digestive solutions; simulated saliva fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) and enzyme activity assays were performed according to this protocol. The oral and gastric phases of the protocol were applied the same as in INFOGEST protocol (15). According to this protocol, samples containing starch are mixed with saliva containing α -amylase enzymes and treated for 2 min. At this stage, starch digestion begins and dextrin form is formed. The digestion continues with the stomach and intestinal phases. In the present study, after performing the mouth and stomach stages according to this protocol, the method of Mat *et al* (14) was used to monitor fat and protein hydrolysis with pH-stat (14). For oral digestion, 3 g of the sample was mixed with 4 ml SSF and 0.025 ml of 0.3 M CaCl_2 and the volume was adjusted to 10 ml using ultrapure water. Subsequently, the mixture was incubated in a shaking incubator operated at 200 rpm and 37°C for 2 min. Following mouth digestion, 8 ml SGF and 0.005 ml CaCl_2 were added to the bolus. The pH was then adjusted to 3 using 6 M HCl and 1 ml pepsin enzyme (2,000 U/ml) was

Table I. List of ingredients of the snacks (100 g samples).

Ingredients	Snack sample					
	R	1	2	3	4	5
Whole wheat flour	49.75	35.56	30.10	30.10	-	-
Chickpea flour (RS)	-	8.47	6.02	4.32	-	-
Butter (82% fat)	15.40	7.62	10.16	10.16	-	-
Sunflower oil	-	2.54	-	-	-	-
Egg (whole)	12.90	25.4	18.07	24.9	-	-
Desiccated coconut	-	-	-	-	8.55	8.55
Goat kefir powder	-	3.11	3.11	3.11	-	-
Sugar (beet)	12.15	-	4.81	3.00	-	-
WPC 80	-	6.35	6.35	3.18	-	21.40
Nut mix	7.35	-	4.51	4.51	28.06	27.36
Hazelnuts	2.45	-	-	-	14.03	-
Almonds	4.90	-	4.51	4.51	-	-
Walnuts	-	-	-	-	14.03	13.68
Peanuts	-	-	-	-	-	13.68
Dried fruits	2.45	-	6.30	9.16	44.70	38.50
Raisin (sultanas)	-	-	3.15	4.58	29.80	25.65
Blueberry	-	-	-	-	8.77	-
Cranberry	2.45	-	3.15	4.58	-	8.55
Sour cherry	-	-	-	-	6.13	4.30
Dried vegetables	-	3.56	-	-	-	-
Inulin	-	3.05	3.65	2.53	13.80	-
Brewed green tea	-	4.23	6.82	4.23	-	-
Cinnamon (powder)	-	-	-	0.70	-	-
Salt (sea)	0.10	0.10	0.10	0.10	-	-
Honey (pine)	-	-	-	-	-	1.24
Other ingredients (glycerol, sunflower lecithin)	-	-	-	-	4.89	2.95

R, control; samples 1, 2 and 3 were baked samples; samples 4 and 5 were unbaked samples; RS, resistant starch.

added to the mixture. The gastric digestion was performed in a shaking incubator operated at 200 rpm and 37°C for 2 h. Following the gastric phase, some modifications were made in the SIF formulation (with NaCl instead of NaHCO₃) to prevent buffering effect during intestinal digestion (14). The volume of gastric bolus was completed to 20 ml with SIF and pancreatin added with pancreatic lipase (100 U/ml trypsin activity and 2,000 U/ml lipase activity in the final mixture). For the intestinal phase, an automatic titrator (Kyoto KEM AT-510, Kyoto Electronics Manufacturing Co., Ltd.) with a pH-stat program was then used to record the reaction kinetics by maintaining the pH at 7.0 with addition of 0.1 N NaOH as a titrant and determined with the action of enzymes release protons, and subsequently a decrease in pH. The analysis was performed in two steps. In the first step, the determination of the total degree of hydrolysis (DH_{total}) (proteolysis + lipolysis), intestinal digestion was performed with the action of pancreatin (100 U/ml final solution based on trypsin activity), which can hydrolyze both proteins (peptides and remaining proteins after gastric phase) and lipids. The volume of NaOH consumed during the titration was recorded

(V1). In the second step, for the determination of only the degree of proteolysis, intestinal digestion was conducted with trypsin (100 U/ml final mixture) and chymotrypsin (25 U/ml final mixture). The volume of NaOH consumed during the titration was recorded (V2). Following 2 h of digestion of the small intestine, the degree of proteolysis was calculated using the following equation (Eq. 1):

$$DH_{protein} = 100 \times \frac{V_2(NaOH) \times N(NaOH)}{\alpha(RNH_2) \times m_{protein} \times h_{tot}} \quad \text{Eq.1}$$

where V₁ (NaOH) is the total volume of NaOH consumed (mL), N (NaOH) is the normality of NaOH (Eq/L), m_{protein} is the protein content of the sample (g), α(RNH₂) is the mean degree of dissociation of α-amino groups, h_{tot} is the number of peptide bonds with respect to the origin of the protein (meqv/g protein). Since the recorded data from the pH-stat device in the intestinal phase is the result of all physical and enzymatic actions throughout the digestion, it is assumed that both total degree of hydrolysis and degree of proteolysis calculations represent the entire digestion procedure, consisting of oral, gastric, and intestinal phases (14). For the

Table II. Chemical composition and energy value of the snacks.

Sample	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Water activity (a_w)	Total sugar (%)	Total CHO (%)	Energy (kcal/30 g)
R	3.60±1.01	1.22±0.04	8.7±0.26	21.90±0.34	0.354±0.08	17.42±2.48	62.7±0.74	148
1	3.10±1.01	2.70±0.06	20.5±0.31	18.30±0.60	0.308±0.07	2.20±0.93	55.2±0.34	134
2	3.29±1.12	2.18±0.04	17.7±0.68	16.05±0.11	0.309±0.09	10.95±2.72	59.5±0.22	127
3	3.34±0.43	1.97±0.15	16.5±0.26	17.10±0.34	0.323±0.06	8.66±5.95	61.0±0.22	129
4	7.30±0.29	2.20±0.08	5.7±0.69	24.30±0.51	0.520±0.04	17.08±3.45	60.2±0.71	119
5	7.70±0.26	2.20±0.04	22.6±0.21	19.00±2.01	0.509±0.03	15.40±2.32	48.3±2.06	110

R, control; samples 1, 2 and 3 were baked samples; samples 4 and 5 were unbaked samples; CHO carbohydrate.

in vitro degree of lipolysis calculation, the following equation was used (Eq. 2):

$$DH_{lipid} = 100 \times \frac{[V(1)-V(2)] \times N(NaOH) \times M(lipid)}{\alpha(FFA) \times m_{lipid} \times 2} \quad \text{Eq.2}$$

where V_2 (NaOH) is the total consumption of NaOH in the presence of pancreatin enzymes at the intestinal phase. V_1 (NaOH) is the total amount of NaOH consumed (ml) during the intestinal phase of *in vitro* proteolysis, N (NaOH) is the normality of NaOH, m_{lipid} is the oil mass of the sample (g), M_{lipid} is the molecular weight of the triglycerides in the oil fraction of the sample (g/mol) and $\alpha(FFA)$ is the mean degree of dissociation of the carboxylic groups of FFA.

In vitro starch hydrolysis and estimated GI (eGI). All stages, namely oral, gastric and intestinal were applied according to the method suggested in the study by Minekus *et al* (15). Starch digestion begins in the mouth with α -amylase and dextrin is formed. The glucose measurements were taken by sampling at the beginning of the oral phase (t_0) and every 30 min of the intestinal phase for each snack. The end of the oral stage (t_0) was accepted as the initial moment for glucose measurement, to subtract the naturally available glucose (if accessible) derived from raw materials in the recipe, such as dry fruits. Additionally, these data would be used to subtract from the further glucose measurements during digestion to calculate starch hydrolysis. With this approach, it is expected to prevent a potential error in the calculation of starch hydrolysis stemming from the available glucose in the recipe. Enzymatic reactions in the samples were then terminated by the addition of 1 M HCl and centrifuged at 6,500 x g, 20°, 20 min. The samples were diluted with water then treated using GOPOD reactive solution and incubated in a water bath for 20 min at 45°C. The D-glucose analysis kit (cat. no 700004297, Megazyme) was used for the determination of the absorbance of sample at a 510 nm wavelength in UV visible spectrophotometer (Cary 50 Scan, Varian). Glucose was calculated according to following equation (Eq 3) (19):

$$\text{Glucose (\%)} = \frac{A_t \times V \times C \times D}{A_s \times W} \quad (\text{Eq.3})$$

where A_t is the absorbance of the sample, A_s is the absorbance of the glucose standard solution, V is the volume of the measured sample (ml), C is the concentration of the glucose

standard solution (mg/ml), D is the dilution factor, W is the weight of the sample (mg).

The rate of released glucose from sample starch digestion was expressed at different times and the hydrolysis index (HI) was obtained by dividing the area under the curve of the sample to the area of control (white bread). The eGI was calculated using equations 3, 4 and 5 respectively (20). The HI of white bread was accepted as 100. Calculations using equations 4 and 5 are the best correlated formulas for *in vitro* measurements when they are compared with *in vivo* glycemic responses according to reference study (21).

$$TN_t = G_t (g) \times 0.90 \quad (\text{Eq.4})$$

$$eGI_t = 39.71 + 0.549 \times HI \quad (\text{Eq.5})$$

where HI is the percentage of total starch hydrolysis, TN_t is the total amount of hydrolyzed starch at the time of t (g), and G_t is the amount of glucose in the sample at the time of t (g).

Statistical analysis. All analyses were performed in triplicate. Data were analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc test using SPSS Statistics 20 software (IBM Corp.). The confidence interval was selected at 95%. All results are presented as the mean $\pm$ standard deviation. A P-value <0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Proximate composition. The chemical composition and energy value of the snacks are presented in Table II. The percentages of cooking loss of R (recipe), and snacks 1, 2 and 3 were 16, 28, 18 and 17%, respectively. The final percentage calculations of chemical compositions and caloric values for the snacks were adjusted to account for the mass loss incurred during the cooking process. The energy values of all snacks apart from R (control) were <135 kcal when they were evaluated on a serving size (30 g) basis. While the amounts of total CHO in the snacks varied between 14.4 and 16.56 g, the reference was found as 18.3 g per serving (Table II). The highest total sugar content was determined at 17.4% for the control formula (R). The main fatty acids were palmitic (16:0), oleic (18:1) and linoleic acid (18:2) in all snacks (Table III). The values of water activity (a_w) of all snacks were measured <0.6 (Table II), which

Table III. Fatty acid composition of the samples (%).

Fatty acids	Snack samples				
	1	2	3	4	5
C10:0	1.99	4.13	2.44	0.20	0.95
C12:0	2.84	4.23	ND	5.67	8.31
C14:0	9.73	13.20	8.62	2.01	3.71
C16:0	29.90	32.44	28.32	9.49	6.09
C18:0	8.04	8.66	9.34	ND	ND
C18:1	33.89	23.72	35.41	41.97	42.39
C18:2	10.18	5.62	10.59	32.83	30.41
C18:3	ND	ND	ND	6.17	5.51

Samples 1, 2 and 3 were baked samples; samples 4 and 5 were unbaked samples; ND, not detected.

Table IV. *In vitro* protein, lipid hydrolysis, released glucose, HI and eGI values of the snack samples.

Hydrolysis (%)	White bread	Snack sample					
		R	1	2	3	4	5
DH _{proteolysis}	-	-	65.17±3.91 ^a	84.84±5.39 ^b	63.12±10.95 ^a	57.37±3.44 ^c	84.46±7.96 ^b
DH _{lipolysis}	-	-	76.85±1.76 ^d	62.63±12.68 ^d	74.65±7.07 ^d	80.94±1.54 ^e	91.92±5.07 ^f
RG%	77.83	64.02	50.84	65.46	64.13	90.13	79.93
HI	100	105.57 ^g	61.54±1.76 ^h	105.12±12.68 ⁱ	107.19±7.07 ⁱ	NM	NM
eGI	70	73.04 ^j	56.21 ^k	69.03 ^l	68.33 ^l	NM	NM

Different letters within the same row indicate statistically significant differences in terms of DH_{proteolysis} (a-c), DH_{lipolysis} (d-f), HI (g-i) and eGI (j-l) (P<0.05). Values represent the mean ± SD (n=6). R, control; samples 1, 2 and 3 were baked samples; samples 4 and 5 were unbaked samples. White bread was also used here. RG, released glucose; HI, hydrolysis uindex; eGI, estimated glycemic index (≤55: Low; 56<eGI<69: Moderate; 70≥eGI: High); NM, not measured.

provides the minimum limit to prevent microbial growth and plays a crucial role in biochemical reactions during storage. The water activities of the baked snacks (snacks 1-3) were found to be lower than those of the ready-to-eat snacks (snacks 4 and 5) (Table II), resulting from the heat treatment applied to snacks 1-3 and the glycerol and lecithin involved in the ready-to-eat snacks.

Sensory evaluation. The results of sensory analysis on the hedonic scale are expressed with a score >100. For snacks 1, 2, 3, 4 and 5, the scores were 70.9, 80.8, 81.8, 71.5 and 77.1, respectively. The snack preferences were in the following order: 3>2>5>4>1. The consumer preference for snacks 3 and 2 may be attributed to the presence of dried fruits and a higher butter content. Additionally, the lower utilization of retrograded chickpea flour in these snack samples compared to snack 1 may contribute to a more pleasant aroma. Conversely, the lower preference for snacks 4 and 5 is likely due to their higher content of dietary fiber (13.80% in sample 4) and WPC (21.40% in sample 5) compared to the preferred samples (2 and 3). These ingredients may have resulted in a less favorable sensory perception. Finally, snack 1, containing retrograded chickpea flour with no added sugar or dried fruits, may have

been perceived as the least favorable due to its relative dryness and potentially strong salty taste.

Degree of proteolysis. Proteolysis was evaluated by decreasing the pH value of the protons of the carboxyl ends of amino acids and peptides, which are released following GI digestion. According to the data obtained from pH-stat during the intestinal digestion of the snacks, the kinetics of all snacks, apart from snack 2 were similar in shape, exhibiting a rapid rising rate at the beginning (first 45 min) followed by a gradual attenuation, leading to a plateau. Snack 2 reached the highest degree of hydrolysis with an increasing speed up to 115 min, but did not exhibit a tendency to plateau; probably the reaction was continuing at this moment (Fig. 1). Apart from snack 4, all snacks had >40% proteolysis within 30 min. At the end of the intestinal digestion, the degree of hydrolysis was estimated >80% for snacks 2 and 5. The degree of hydrolysis of all the snacks at the end of the intestinal phase is presented in Table IV. There was no significant difference between snacks 1 (65.17%) and 3 (63.12%) (P>0.05), while snack 4 had the lowest degree of hydrolysis (57.37%), and snacks 2 and 5 had high degrees of hydrolysis (84.84 and 84.46%, respectively; P<0.05). In the recipe for snack 4, a high amount (13.8%) of inulin was used;

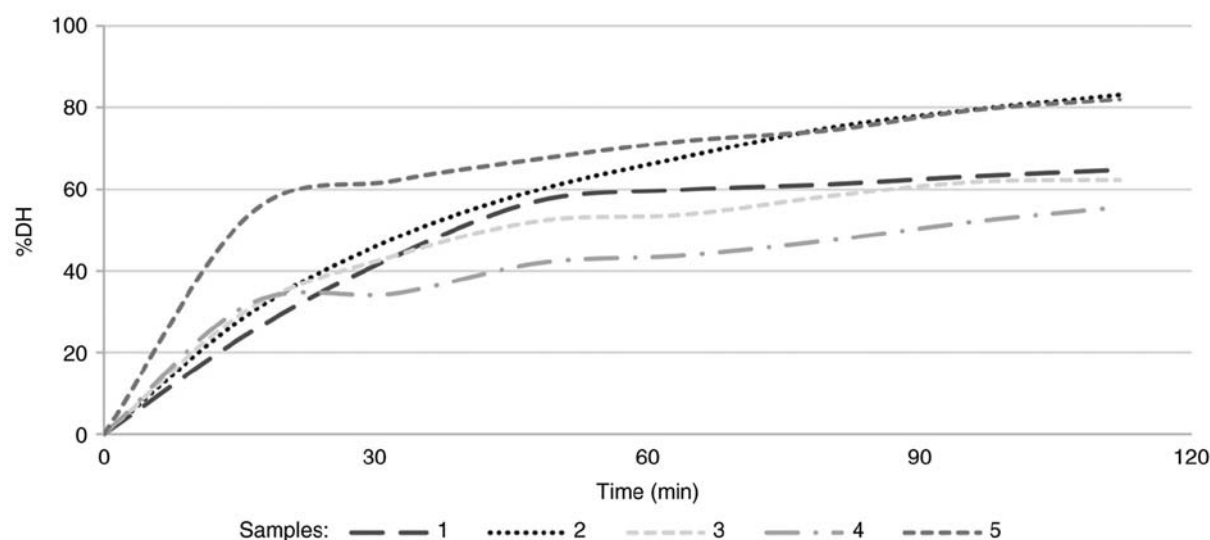


Figure 1. *In vitro* proteolysis curves of the samples. R, control; samples 1, 2 and 3 were baked samples; samples 4 and 5 were unbaked samples. DH, degree of hydrolysis.

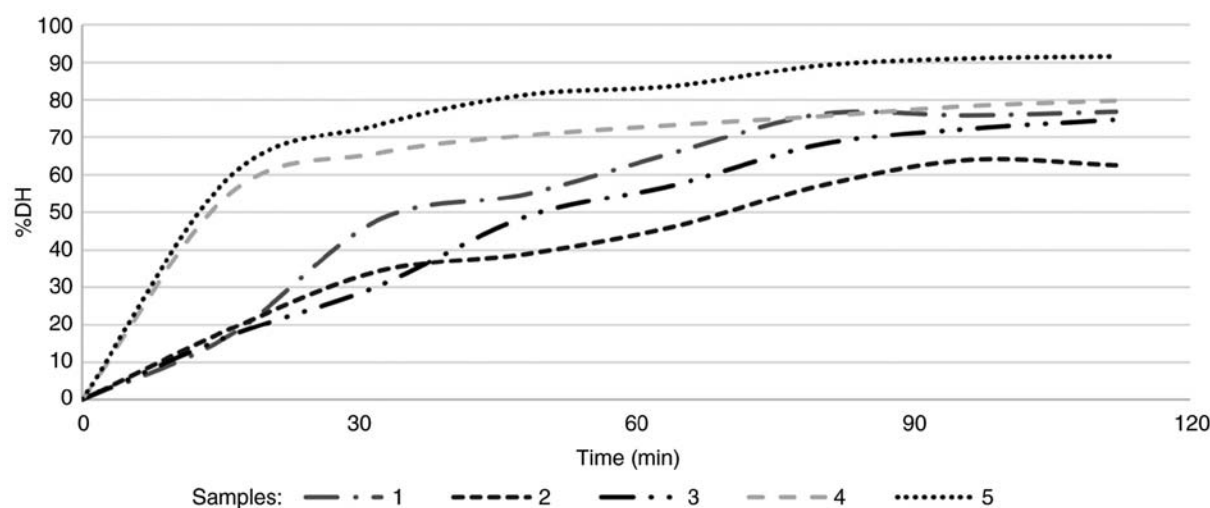


Figure 2. *In vitro* lipolysis curves of the samples. R, control; samples 1, 2 and 3 were baked samples; samples 4 and 5 were unbaked samples. DH, degree of hydrolysis.

thus, it could be a distinct difference compared to snacks 1, 2 and 3 (inulin contents: 3.05, 3.65 and 2.53%, respectively). In snack 5 which had the highest degree of hydrolysis, inulin was not used. On the other hand, it could be said that resistant starch had no effect on protein hydrolysis in snacks 1, 2 and 3 ($P < 0.05$). Sciarini *et al* (22) examined the *in vitro* digestibility of gluten-free bread enriched with inulin. They noted that the protein hydrolysis of bread decreased when inulin was added at 10% to the bread and increased at the level of 5%. The addition of fiber can cause a different structure between protein and starch in bread, which could make starch and protein less accessible to enzyme hydrolysis and influence protein aggregation (13,23). Inulin as a viscous soluble dietary fiber increases digesta viscosity and decreases the kinetics of enzyme reactions due to reducing the rate of mixing with proteolytic enzymes released in the stomach or small intestine during digestion (23,24). However, this physicochemical barrier for enzyme reactions can be evaluated with the view

that it provides a low GI due to lower carbohydrate digestion. This effect will be explained in further detail below in the subsection entitled 'Degree of starch hydrolysis and eGI'.

Starch hydrolysis. Another reason for the highest hydrolysis degree found in snack 5 may be the presence of WPC in the recipe; snack 5 had the highest amount of WPC (21.40%) among the snacks. This result is consistent with the findings in the literature (13,25), reporting that the denatured form of the whey protein structure is easily hydrolyzed with intestinal protease enzymes. However, a previous study found that the proteolysis results of biscuits enriched with 20% whey protein and pea protein is was not altered compared with the control (23). Furthermore, different proteolysis rates have been reported when heat-induced whey protein gel structures are subjected to simulated intestinal digestion. It has been observed that the gel structure may affect the reaction rate and hydrolysis mechanisms, and the differences in the diffusion of the enzyme

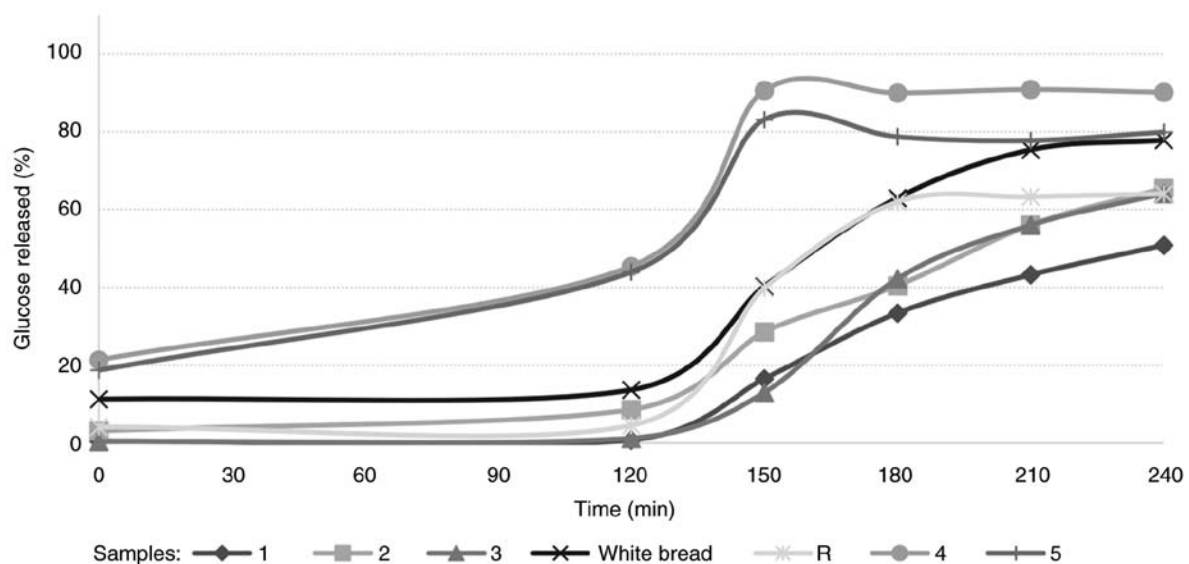


Figure 3. Released glucose of the samples during *in vitro* digestion. R, control; samples 1, 2 and 3 were baked samples; samples 4 and 5 were unbaked samples. White bread was also used here.

into the chyme during proteolysis may result from the steric barrier created by the gel structure (26,27). In the present study, the use of enriched whey protein without the use of heat treatment may correspond to high levels of enzyme activity.

Degree of lipolysis. Lipolysis was calculated using data obtained by the subtraction of the DH values obtained in the presence of proteolytic enzymes, from DH values obtained in the presence of pancreatin enzymes (Fig. 2). In snacks 4 and 5 (ready-to-eat samples), a lipolysis >80% was observed, which was higher than that of the baked snacks (snacks 1, 2 and 3) ($P<0.05$; Table IV); snacks 4 and 5 exhibited rapid digestion at the beginning; however, the release rate of fatty acids then gradually decreased, and the lipolysis rate remained relatively constant after 75 min, until the end of intestinal digestion. However, in the baked snacks, the rate of lipolysis was slower, with hydrolysis occurring in the first 25 min at ~30%; this value was >65% in snacks 4 and 5. There were no marked differences between snacks 1, 2 and 3 ($P>0.05$); however, the lipolysis values of snacks 4 and 5 differed significantly ($P<0.05$) (Table IV). The difference between snacks 4 and 5 can be explained by the degree of lipolysis being negatively affected due to the interaction of dietary fibers with molecules, such as bile salts, fatty acids and calcium during gastro-intestinal digestion (28). Furthermore, the lower lipolysis values of the baked samples compared with the ready-to-eat snacks may be based on the presence of starch that negatively affects lipolysis. A previous study investigated the influence of intestinal conditions, non-lipid components and food matrix on lipolysis with a selection of 52 real foods. The results were then statistically processed using factors such as energy, protein, total lipid, starch, saturated fatty acid, monounsaturated fatty acid, polyunsaturated fatty acid, fiber, iron, calcium, sodium content of the foods; and lipid structures (complex solid, continuous aqueous phase and continuous lipid phase) in the food matrix (28). The authors of that study pointed out that lipid-protein and

lipid-starch interactions negatively affected lipolysis in real foods when using the INFOGEST (15) static *in vitro* digestion method. Notably, high-fat foods were not affected by these macronutrient interactions (29). In the present study, snacks 4 and 5 that had a total lipid content of 24 and 19%, respectively, the lipolysis values were higher than those of the baked snacks ($P<0.05$). It was found that snack 5, which had the highest protein content (22.6%), had the highest lipolysis value (91.9%; $P<0.05$; Table IV) compared with the other snacks. Additionally, the high unsaturated fatty acid content in the snacks positively affected the lipolysis values, resulting in the uncooked snacks (ready-to-eat) having higher lipolysis values than the baked snacks ($P<0.05$; Table IV). On the contrary, the content of mono or polyunsaturated fatty acids has been reported to have no effect on lipolysis in real foods (29).

Degree of starch hydrolysis and eGI. The glucose released during the hydrolysis of the sample during simulated gastrointestinal digestion is illustrated in Fig. 3. Starch hydrolysis was examined in R (control recipe), white bread and baked snacks (numbered as 1, 2 and 3) that contain a certain amount of complex carbohydrates (Fig. 3). The calculated HI and eGI values of the samples are presented in Table IV. The eGI values were correlated to 70 (white bread) (≤ 55 : Low; $56 < \text{eGI} < 69$: Moderate; $70 \geq \text{eGI}$: High) and although the control (R) was on the border, it was found to have a high GI, while snacks 1, 2 and 3 were evaluated as having a moderate GI. The hydrolysis rate (HI) of snack 1 was significantly lower than that of the other snacks ($P<0.05$). According to the eGI calculations, all baked snacks differed significantly compared with R ($P<0.05$). This is particularly crucial for assisting the regulation of the blood glucose level for insulin resistance, type-2 diabetes and obesity (20). The glucose released during *in vitro* digestion of snacks was measured by sampling at the beginning of the oral phase (t0) and every 30 min of the intestinal phase. According

to the results (Fig. 3), glucose was not detected in snack 1 at the beginning (t120) of the intestinal phase. The release of glucose molecules was finished in unbaked snacks after the first 30 min (t150) of the intestinal phase. However, glucose hydrolysis continued in baked snacks until the time interval of t210 and t240. This difference may have been caused by inhibitory and delaying effects of ingredients such as whole wheat, resistant starch, and inulin (30). Besides, the food matrix could affect starch digestibility by creating interactions such as starch-protein, starch-fiber and starch-lipid (31). In snacks 1, 2 and 3, brewed green tea was used in ratios 4.23, 6.8 and 4.23%, respectively. In another study, gallic acid and epigallocatechin gallate in green tea extract may have inhibitory effects on enzymes that hydrolyze starch (32). However, that inhibitory effect was not observed in our study. Using brewed tea instead of extract could be the reason for this result.

Evaluation of snacks as 'Smart Snacks, SS' and nutritional claims. According to the Smart Snacks (SS) standard (8), SS must meet certain criteria. There must be a grain product with whole grains as the main ingredient ($\geq 50\%$ by weight) or contain fruit and vegetables, milk, dairy products or protein as the main ingredient, or a combination of foods that provides at least 0.25 cups of fruit and/or vegetables by weight. Additionally, there are five more nutritional requirements based on the snacks' composition (8). When comparing the data from the present study with these requirements, all snacks met the mandatory criteria outlined in the Smart Snacks standard. These criteria include having ≤ 200 calories, with $\leq 35\%$ of the calories derived from fat, $< 10\%$ of the calories derived from saturated fat, and 35% of the weight derived from total sugars. The sources of sugar and fat in the snacks complied with the exceptions specified in the standard, such as dried fruits, dried vegetables, nuts and seeds.

Furthermore, according to the Annex of Regulation (EC) No. 1924/2006 (<http://data.europa.eu/eli/reg/2006/1924/2014-12-13>), on labeling standards, all snacks can be nutritionally claimed as a source of omega-3, containing a high amount of monounsaturated fatty acids, rich in polyunsaturated fatty acids, and as a source of protein. For snacks 1, 4 and 5 as 'no sugar added', 'high amount of protein' was amended by the Regulation (EU) No. 1047/2012 (<https://eur-lex.europa.eu/eli/reg/2012/1047>).

The calorie target of the snacks in the present study was set at 135 calories per serving. Additionally, investigating the bioaccessibility of macronutrients in the samples was critical for understanding the impact of the food matrix. Chriqui *et al* (33) observed in their study that implementing Smart Snacks policies in school food environments effectively influenced the consumption of fruits, vegetables, and healthier choices among young individuals. The snacks developed in the present study were well-liked by the panelists, which is a crucial criterion for replacing fatty, sugary, or salty snacks with healthier alternatives.

In the present study, sensory successful products with Smart Snack criteria, which can be sold in school canteens, were developed considering the portion size. In the future, a better approach perhaps would be to develop these standards considering the bioaccessibility/bioavailability of proteins,

lipids and carbohydrates, and understanding the effects of the food matrix and interactions of these macronutrients in food.

The healthy eating recommendations strive to enhance overall well-being by focusing on specific dietary improvements. These include reducing saturated fat intake, while increasing the consumption of complex carbohydrates, such as dietary fiber and bioactive compounds found in legumes, cereals, vegetables and fruits. To further promote health and nutrition, the introduction of Smart Snack standards has revolutionized snack choices by evaluating them based on their positive impact. These new criteria ensure that snacks available in school canteens align with health goals. To address the diverse needs of consumers, the present study successfully developed sensory appealing products that meet the Smart Snack criteria, taking into account appropriate portion sizes. However, to enhance these standards further, it would be beneficial to consider the bioaccessibility/bioavailability of essential nutrients, such as protein, lipids and carbohydrates along with further standard updates on vitamins, minerals and trace elements. This would involve the understanding of how these macronutrients interact within the food matrix and their effects on the body. By incorporating bioaccessibility/bioavailability factors and assessing the intricate interactions among macronutrients, future standards can better optimize the nutritional value of food products. This approach will ensure that the snacks offered in school canteens not only meet the smart snack criteria but also provide optimal nutrient absorption and utilization. Consequently, individuals will benefit from improved health outcomes and overall well-being.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CD and SNE worked collectively on the study design. CD conducted the literature review, all the experiments, the statistical analysis and participated in the discussion of the results. SNE participated in the discussion of the results and contributed to the structure and grammar of the whole article. CD and SNE confirm the authenticity of all the raw data in that study. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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