

# Acrylamide bioaccessibility in potato and veggie chips. Impact of *in vitro* colonic fermentation on the non-bioaccessible fraction

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## ABSTRACT

Potato-based products contribute largely to the daily intake of acrylamide. In addition to potato crisps, the European Commission has included veggie crisps in the list of foods that should be monitored for their acrylamide content. In the present study, acrylamide content in potato and veggie chips (sweet potato, beetroot and carrot) and their bioaccessibility after *in vitro* digestion was assessed. The non-bioaccessible fraction was also submitted to *in vitro* fermentation under colonic conditions. Faecal samples from volunteers of three age groups (children, adolescents and adults) were used to evaluate the microbiota effect on the acrylamide availability. Sweet potato chips exhibited the highest acrylamide content (2342 µg/kg), followed by carrot (1279 µg/kg), beetroot (947 µg/kg) and potato chips (524 µg/kg). After *in vitro* digestion, acrylamide bioaccessibility was significantly lower in veggie chips (59.7–60.4 %) than in potato chips (71.7 %). Potato and sweet potato chips showed the significantly lowest acrylamide content in the non-bioaccessible fraction (22.8 and 24.1 %, respectively) as compared with beetroot chips (28.4 %). After the fermentation step, acrylamide percentage in the soluble fraction of veggie chips ranged from 43.03 to 71.89 %, the highest values being observed in sweet potato chips fermented with microbiota from children. This fact would involve that the acrylamide was released from the non-bioaccessible fractions by the microbiota. These findings point out that the levels of potentially absorbable acrylamide after the complete gastrointestinal process could be modulated by both the food matrix composition and the microbiota. These factors should be further considered for a more precise risk assessment of dietary acrylamide in humans.

## I. INTRODUCTION

Snacks are defined as a small amount of food consumed between meals, including chips and other salty snacks, sweets, candies, chewing gum, chocolate bars, chocolates and nuts. Snack consumption has increased in recent decades (Younginer et al., 2016), with potato chips and other salty snacks standing out as the most consumed products within this category (MAPA, 2021). Despite potato chips have a dominance position on the market, they are often perceived as unhealthy foods (Nguyen et al., 2022). Nowadays, consumer's preferences move towards healthier options for snacking boosting food companies to search for healthier and added-value alternatives, often based on novel vegetables, cereals, roots or legumes (Niva, 2007; Breitling-Utzmann and Hankele, 2019; Mesías et al. 2019). However, knowledge about the potential toxicological effect of the chemical contaminants formed during the thermal treatment applied to novel snacks is still scarce (Nguyen et al., 2022). Acrylamide is a chemical process contaminant generated through the Maillard reaction between reducing sugars and free asparagine in foods treated at temperatures above 120 °C and in low moisture conditions (Mottram & Wedzicha, 2002). Potato chips together with other processed potatoes, coffee and cereal-based products are the main food sources of exposure to acrylamide (EFSA, 2015). The European Commission Regulation (EC) 2017/2158 established control measures and reference levels for acrylamide

content in these foodstuffs (EC, 2017) and later, the European monitoring programme of acrylamide was extended to other food products, including vegetable chips (EC, 2019).

The high-water solubility of acrylamide promotes its rapid absorption after consumption and consequently its distribution by the systemic circulation to all human tissues (Fuhr et al., 2006; Koszucka et al., 2020). However, not all ingested acrylamide is necessarily bioavailable, since several factors involved in the digestive process such as mastication, variations of the pH and action of numerous digestive enzymes in mouth, stomach and intestine could affect the bioavailability through structural and chemical changes (Hamzaloglu & Gökmen, 2015; Sansano et al., 2017). Additionally, another key factor affecting acrylamide accessibility is the food matrix effect, as has been recently demonstrated by our research group (González-Mulero et al., 2022). *In vitro* approaches have facilitated nutrient bioaccessibility studies due to its high reproducibility and low cost. Minekus et al. (2014) reported an international standardised *in vitro* digestion protocol with three static phases considering physiological enzyme activities and salt concentrations based on human *in vivo* data. Depending on the target compounds, additional steps to this protocol should be added. In this sense, Pérez-Burillo et al. (2018) showed that considering only the soluble fraction obtained after *in vitro* digestion could underestimate the antioxidant capacity of foodstuffs, since the undigested fraction may be metabolised by the gut microbiota releasing compounds with additional bioactivity. Therefore, a fermentation step of the undigested fraction could be necessary to get more precise information on the fate of bioactive compounds during the gastrointestinal transit.

Previous studies have evaluated the acrylamide bioaccessibility from the soluble portion after *in vitro* digestion of certain processed foods such as coffee, cereal and potato-based foods (Hamzaloglu & Gökmen, 2015; Sansano et al., 2017; Badoud et al., 2020). However, there is no information concerning the effect of colonic fermentation on the acrylamide trapped in the non-soluble portion after digestion. In this sense, the objective of this research was to evaluate the acrylamide bioaccessibility in four different snack samples, including potato and veggie chips, applying the standardised *in vitro* digestion protocol INFOGEST, but also including an *in vitro* fermentation procedure of the undigested fractions using faecal samples from different population groups. Acrylamide was monitored in both soluble and non-soluble fractions after *in vitro* digestion and at the end of the fermentation process to analyse the possible influence of the different colonic microbiota in the final acrylamide potentially absorbable.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

All chemicals, solvents and reagents used for *in vitro* digestion and fermentation, and acrylamide determination were of analytical grade and purchased from Sigma-Aldrich unless otherwise mentioned. All solutions were prepared using deionised water from a Milli-Q Integral 5 water purification system (Millipore, Billerica, MA, USA).

Specific enzymes used for *in vitro* digestion analyses were  $\alpha$ -amylase from human saliva (A1031), pepsin from porcine gastric mucosa (P6887), pancreatin from porcine pancreas (P7545) and bile salts (B8756). The reagents used for *in vitro* fermentation analysis were sodium phosphate monobasic dihydrate (cat. no. 567550), peptone (cat. no. T7293), L-cysteine (cat. no. 168149), resazurin sodium salt (cat. no. 199303) and sodium sulphide hydrate (cat. no. 14738). For acrylamide determination, acrylamide standard (99 %), potassium hexacyanoferrate (II) trihydrate (98 %, Carrez-I), zinc acetate dihydrate (>99 %, Carrez-II) were used. Acrylamide labelled by  $^{13}\text{C}_3$  (99 %) was acquired from Cambridge Isotope Laboratories (Andover, MA, USA). Formic acid (98 %), methanol (99.5 %) and hexane were acquired from Panreac (Barcelona, Spain). Cellulose syringe filter units (0.22 and 0.45  $\mu\text{m}$ ) were obtained from Análisis Vínicos (Tomelloso, Ciudad Real, Spain) and Oasis-HLB cartridges (30 mg, 1 mL) were purchased from Waters (Milford, MA, USA).

## 2.2. Food samples

Four different fried snacks (potato, sweet potato, beetroot and carrot chips) were selected and purchased in a local supermarket of Granada (Spain). Samples were coded, photographed, weighed and, as the entire portion was edible, directly homogenised with a hand blender (Taurus, Vital CM, Spain). Finally, each ground sample was aliquoted and stored at  $-20\text{ }^{\circ}\text{C}$  until analysis. Fig. 1 depicts external appearance of samples. The nutritional composition of the snacks was obtained from the manufacturer declarations recorded in the package labelling (Table 1).

## 2.3. *In vitro* digestion of the snacks

The *in vitro* digestion method used for the fresh ground snacks was adapted from the INFOGEST protocol, composed by an oral phase, a gastric phase and an intestinal phase (Fig. 2). Simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluids were prepared according to Minekus et al. (2014). Fresh sample (4 g) was weighted in polypropylene tubes and Milli-Q water was added to complete a final weigh of 5 g. The enzyme concentrations were maintained as established by the standardised INFOGEST protocol. For the oral phase, 4.5 mL of SSF (without  $\alpha$ -amylase) including 25  $\mu\text{L}$  of 0.3 M  $\text{CaCl}_2$  was added to all the samples to make a paste-like consistency. Next, samples were sonicated (Vibracell VCX 130; Sonics & Materials Inc, Danbury, USA) to facilitate the accessibility of lipids during the next steps of the digestion protocol (Teixeira et al., 2022), thus avoiding the addition of the lipase enzyme in the intestinal phase. Next, 0.5 mL of  $\alpha$ -amylase enzyme solution was added to the samples (concentration adjusted as established by the standardised protocol to reach 75 U  $\alpha$ -amylase/ml SSF) and the mix was incubated at  $37\text{ }^{\circ}\text{C}$  for 2 min with mild and continuous agitation (model Julabo ED, Julabo GmgH, Germany). After that, 10 mL of the SGF solution with pepsin and 5  $\mu\text{L}$  of 0.3 M  $\text{CaCl}_2$  were added and the pH was lowered to 3.0 by adding 1 M HCl (final mix pepsin concentration was adjusted to 2000 U/mL). The mixture was again incubated at  $37\text{ }^{\circ}\text{C}$  for 2 h with mild and constant agitation. Finally, 20 mL of the SIF solution containing pancreatin, bile salts and 40  $\mu\text{L}$  of 0.3 M  $\text{CaCl}_2$  were added to the samples, followed by the pH raising to 7.0 using 1 M NaOH (final mix pancreatin concentration was adjusted to 13.37 mg/mL, while bile salts were adjusted to 10 mM). Samples were one last time incubated at  $37\text{ }^{\circ}\text{C}$  for 2 h with mild and continuous agitation and then, to halt the enzymatic reactions, were rapidly frozen in liquid nitrogen and stored at least overnight at  $-20\text{ }^{\circ}\text{C}$ . All samples were defrosted and centrifuged at 3220g and  $4\text{ }^{\circ}\text{C}$  for 45 min to carefully separate the soluble and the residual fractions (undigested or non-soluble pellet). Part of these separated soluble and non-soluble fractions of each sample were lyophilised and stored at  $4\text{ }^{\circ}\text{C}$  until acrylamide analysis, and the rest were stored at  $-20\text{ }^{\circ}\text{C}$  until the following *in vitro* fermentation. The procedure was repeated by triplicate.

Blanks of the *in vitro* digestion were prepared with 5 mL of Milli-Q water and the complete protocol was developed. They were shown to be free of acrylamide.

## 2.4. *In vitro* colonic fermentation

The *in vitro* fermentation method used was established by Pérez-Burillo et al. (2021). Faecal samples were obtained from four donors of three different age groups: children ( $6 \pm 1$  yr.-old), adolescents ( $13 \pm 2$  yr.-old) and adults ( $38 \pm 12$  yr.-old). All-donors were healthy, with average body mass indexes (range 18.5–24.5  $\text{kg}/\text{m}^2$ ) and followed a normal diet, not taking antibiotics in the previous year. Faecal samples were obtained from the donors in the morning of the analysis day. Faeces of volunteers of the same age group were homogenised to minimize interindividual variations, and pools were placed in sterile containers and stored at  $4\text{ }^{\circ}\text{C}$  until the preparation of the inoculum.

The fermentation culture medium (peptone solution pH 7.0) was mixed with resazurin (1 mg/ml), followed by an autoclave sterilization step. A reductive solution made of cysteine and sodium sulphide (since

cysteine is sensitive to temperature and cannot be autoclaved) and the faecal inoculum, consisting of a solution of 32 % faeces in phosphate buffer 100 mM, pH 7.0 was prepared.

Firstly, 0.5 g of the non-soluble fraction of each digested snack was weighted in a polypropylene tube. Considering that 10 % of the soluble fraction obtained after the digestive process passes to colonic fermentation along with the complete non-soluble fraction (Pérez-Burillo et al., 2021), the proportion of soluble fraction corresponding to the 0.5 g of non-soluble fraction was estimated for each digested snack and added to the tube (Fig. 2). An aliquot of 7.5 mL of fermentation culture medium and 2 mL of faecal inoculum were added to the sample tube to reach a final volume of 10 mL (considering also the supernatant volume added).

Next, an anaerobic atmosphere was created by bubbling N<sub>2</sub> for 1 min to the samples, which were then incubated at 37 °C for 20 h under mild oscillation. To stop the microbial activity, samples were immersed in ice immediately after the 20 h incubation and centrifuged at 4000g for 10 min. The resulted supernatant (soluble fraction that might be potentially absorbed after a fermentation process in the colon) was collected and stored at -80 °C. The solid residue, representing the non-absorbed fraction after fermentation, was also stored.

Blanks from the *in vitro* digestion were prepared as the food samples. Blank aliquots from the initial *in vitro* fermentation procedure were also prepared to assess the possible input of acrylamide from the faecal inoculum of each donor group tested. Samples (supernatant and solid residue after fermentation, digestion blank and fermentation blank) were lyophilised and stored at 4 °C until acrylamide determination. All blanks were shown to be free of acrylamide.

The study protocol was conducted in accordance with the ethical recommendations of the Declaration of Helsinki. Ethical approval to work with human faeces was obtained from the Ethic Committee of Human Research belonging to the University of Granada (Spain), registration num. 1080. An information sheet for faecal sample volunteer together with a collection consent were distributed and signed by participants or by parents/guardians in the case of children.

## 2.5. Acrylamide determination by LC-ESI-MS/MS

Acrylamide formed in the snacks during the industrial frying process was determined following the protocol established by González-Mulero et al. (2022). For the snack samples, 0.5 g were weighted in polypropylene centrifugal tubes and 9.4 mL of Milli-Q water were added. Two mL of hexane were also incorporated to remove the fat content. Samples were spiked with 100 µL of the acrylamide internal standard solution ((<sup>13</sup>C<sub>3</sub>)-acrylamide) and homogenised for 15 min (Ultra Turrax, IKA, Mod-T10 basic, Bohn, Germany). Then, 250 µL of both Carrez I (15 g of potassium ferrocyanide/100 mL of water) and Carrez II solutions (30 g of zinc acetate/100 mL of water) were added to the tubes and centrifuged at 4 °C, 9000g for 10 min. Hexane was removed.

For the soluble and non-soluble fractions from the *in vitro* digestion, 0.25 g of the lyophilised samples were weighted and mixed with 4.7 mL of Milli-Q water and 50 µL of the acrylamide internal standard solution. After homogenizing for 10 min, 125 µL of both Carrez I and Carrez II solutions were added, and the tubes were centrifuged at 4 °C, 9000g for 10 min. For the soluble and non-soluble fractions from the *in vitro* fermentation, the entire lyophilised samples were used (~0.1 g) and 2.35 mL of Milli-Q water were added. Tubes were spiked with 25 µL of the acrylamide internal standard solution, homogenised for 10 min and 62.5 µL of both Carrez I and Carrez II solutions were added. Then, samples were centrifuged at 4 °C, 9000g for 10 min.

All the supernatants were filtered through a 0.22 µm cellulose filter and cleaned loading 1 mL into an Oasis-HLB cartridge, previously conditioned with 1 mL of methanol and 1 mL of distilled water, at a flow rate of 2 mL/min. First drops were discharged, and the rest collected into an amberlite LC-MS vial.

Acrylamide was determined by an Agilent 1200 liquid chromatograph coupled to an Agilent Triple Quadrupole MS detector (Agilent Technologies, Palo Alto, CA, USA) using an Inertsil ODS-3 column (250 × 4.6 mm, 5 µm; GL Sciences Inc., Tokyo, Japan) at 30 °C. Mobile phase was formic acid in water (0.2 mL/100 mL) at a flow rate of 0.4 mL/min with an isocratic elution. The injection volume was 5 µL, and the needle was set at 1.0 kV. The positive ionisation mode with electrospray ionisation was used. Under these chromatographic conditions, the acrylamide molecule eluted at 6.1 min. Nitrogen was used as the nebulizer gas (12.0 L/min), and the source temperature was set at 350 °C.

The signal at m/z 72.1–m/z 55.1 and m/z 72.1–m/z 27.1 were isolated for acrylamide and m/z 75.1–m/z 58.1 for (<sup>13</sup>C<sub>3</sub>)-acrylamide. The fragmentation was set at 76 V and the collision energy at 8 V for the transition m/z 72.1 > m/z 55.1, 9 V for m/z 75.1 > m/z 58.1 and 11.0 V for m/z 72.1 > m/z 27.1. Multiple reactions monitoring (MRM) were used to record the masses. The recovery rate of acrylamide spiked to the samples ranged between 100 and 106 %. The relative standard deviations (RSDs) of the analysis were < 10 % for all the determinations. Precision and repeatability of the analytical method were evaluated by analysing different samples on the same day by similar operators (repeatability) and on different days by different operators (precision). The limit of detection (LOD) and limit of quantification (LOQ) were calculated by injecting lower concentrations of standards. A concentration determined as a signal-to-noise ratio of 3 was assigned to LOD (4.5 µg/kg), and that determined as a signal-to-noise ratio of 10 was assigned to LOQ (15 µg/kg).

To avoid quantifying possible interferences commonly found in complex matrices that could lead to an overestimation of acrylamide levels (Desmarchelier et al., 2022), a previous set up of the analytical method for acrylamide determination was carried out, as it is described in González-Mulero et al., 2022. This aspect is especially important in samples coming from *in vitro* gastrointestinal digestion and fermentation due to the enzyme and microorganism actions.

## 2.6. Acrylamide exposure assessment

Dietary exposure to acrylamide from the four snacks analysed was estimated combining the acrylamide content for each sample with the data of total per capita consumption for these kind of products by the Spanish population according to the 2020 report of the Ministry of Agriculture, Food and Environment (MAPA, 2021). Considering the age of the volunteers and the weight data reported in different studies of the literature (Fernández et al., 2011; AESAN, 2020; Eglitis, 2020; NCD RisC, 2020), average body weights (bw) of 23, 48 and 70 kg were used to estimate the daily acrylamide exposure for children, adolescents and adults, respectively. Results were expressed as µg/kg bw/day (Equation (1)).

$$\text{Daily exposure } (\mu\text{g/kg bw/day}) = \frac{\text{Daily food consumption (g/day)} \times \text{acrylamide concentration in food } (\mu\text{g/kg})}{\text{Body weight (kg)}}$$

The acrylamide exposure through the consumption of a normal portion size of these snacks was also estimated, considering an individual bag of chips of 50 g.

## 2.7. Statistical analysis

Statistical analyses were performed using Statgraphics Centurion XVI.I. A one-way ANOVA test followed by Tukey HSD test were used to identify the overall significance of differences between the initial acrylamide content, after the *in vitro* digestion and after the *in vitro* fermentation of the four snacks. Homogeneity of variances was determined using Levene's test. All statistical parameters were evaluated assuming a level of significance of  $p < 0.05$ .

### 3. RESULTS AND DISCUSSION

#### 3.1. Acrylamide content in the snack samples

The acrylamide content in the snack samples is shown in Table 2. Potato chips exhibited the lowest concentration (524 µg/kg) with a value below the benchmark level of 750 µg/kg established in the European Regulation (EC, 2017). It is in accordance with the acrylamide levels described for the industrial potato chip sector in the latest years (Mesías et al., 2020; Powers et al., 2021). In contrast, significantly higher levels were observed in the veggie chips, being 947 µg/kg, 1279 µg/kg and 2342 µg/kg for beetroot, carrot and sweet potato chips, respectively. Results are in line, even below in some cases, with levels reported in the literature for carrot (2893 µg/kg / 700–3090 µg/kg), beetroot (1267 µg/kg / 77–1560 µg/kg) and sweet potato chips (1110 µg/kg / 100–2780 µg/kg) (Nguyen et al., 2022; Oellig et al., 2022). The high acrylamide content in veggie chips corroborates the need of monitoring the presence of acrylamide in these foods (EC, 2019). The higher acrylamide content in veggie chips could be partly explained by the presence of acrylamide precursors in the raw product. Asparagine content has been reported to range between 3.8 and 5.1 mmol/kg in carrot, 6.9–21.7 mmol/kg in beetroot, and 28.7–105.2 mmol/kg in sweet potato (Elmore et al., 2019) and reducing sugar concentrations from 640 to 1000 mmol/kg for carrot, from 920 to 1080 mmol/kg for beetroot and from 610 to 2730 mmol/kg for sweet potato (Bavec et al., 2010; Baranski et al., 2012; Laurie et al., 2013; Elmore et al., 2015). The highest figures of asparagine and reducing sugars reported for sweet potato explain the greatest acrylamide level found in this veggie chip. In addition, the variability in the composition of the raw material, which is influenced by crop cultivars, soil composition, climatic conditions, harvest season and agronomic practices (FDE, 2019), together with the heat treatments applied, could justify the differences in the acrylamide levels for the different snacks.

Considering the acrylamide content in the different snacks and the consumption of this type of products by the Spanish population (MAPA, 2021), the daily dietary acrylamide exposure was estimated in different age groups. Due to the lack of specific consumption data, the category of “potato crisps and other salty snacks” was taken as a reference for all the products (1.09 kg/person/year). Despite being an overestimation, the objective of this calculation was to compare the acrylamide exposure from each snack, assuming that all the consumption corresponded to a specific vegetable product. Due to the lower body weight of children, acrylamide exposure from snacks would be higher in this age group, ranging between 0.068 and 0.304 µg/kg bw/day, followed by adolescents (0.033–0.146 µg/kg bw/day) and adults (0.022–0.100 µg/kg bw/day). The high acrylamide levels found in the snacks involve that a high consumption of these products, especially in children and adolescents, should be considered in the estimation of the risk associated with the exposure to acrylamide. However, a more realistic approach should not only contemplate the exposure to acrylamide through food consumption, but also its bioaccessibility, estimating then the real risk associated with the potentially toxic effects of this contaminant on humans (Belhadj Benziane et al., 2019).

#### 3.2. Acrylamide after the *in vitro* digestion process

The behaviour of the acrylamide in the different snacks during the digestion process was evaluated by using the internationally standardised *in vitro* digestion protocol INFOGEST. This protocol involves a three-phase static assay and is based on human *in vivo* data, considering physiological enzyme activities and salt concentrations. After the digestion process, acrylamide levels were determined not only in bioaccessible fractions (acrylamide available to be absorbed after the gastrointestinal digestion) but also in non-bioaccessible (non-soluble) fractions. This fact is a novelty of the present research since previous studies have estimated the acrylamide bioaccessibility evaluating only the soluble fraction but disregarding the non-bioaccessible one (Hamzalioglu & Gökmen, 2015; Sansano et al. 2017; Badoud et al., 2020). Considering the acrylamide content in both soluble and non-soluble portions, it was possible to estimate the total recovery compared to the initial acrylamide levels and the magnitude of the increment or reduction during the digestive process.

Taking into account the acrylamide level in the snacks, acrylamide content in the portions subjected to the digestive process was calculated (Table 2, Fig. 3). After the digestion, both bioaccessible and non-bioaccessible fractions were analysed. Very similar behaviour was observed in all the samples, acrylamide being distributed mainly in the bioaccessible fraction and in a lower extent in the non-bioaccessible fraction (Fig. 3). Expressed in percentages, acrylamide in the bioaccessible fraction was practically the same in all veggie chips (range: 59.7–60.4 %) but significantly higher in potato chips (71.7 %). Non-bioaccessible fractions were very close (range: 22.8–28.4 %) and in this case, the highest percentage was observed in beetroot chips (28.4 %), with significant differences compared to potato and sweet potato chips. The recovery rate ranged between 84.2 and 94.5 %, potato chips showing again the highest values (Table 2).

As previously reported, acrylamide content in the bioaccessible fraction after the *in vitro* digestion has shown to be highly influenced by the food matrix (Hamzalioglu & Gökmen, 2015; Sansano et al. 2017; Badoud et al., 2020; González-Mulero et al., 2022). Components such as starch, proteins and amino acids as well as fibre are important constraints in acrylamide bioaccessibility. The gastrointestinal digestion compromises the food integrity as vegetable cells are broken down, releasing starch, proteins and lipids that could be hydrolysed into

smaller molecules and interact with the acrylamide (Pérez-Burillo et al., 2021). High levels of sugars and lipids have been reported to prevent starch gelatinisation, which is the fraction susceptible to enzyme hydrolysis (Agama-Acevedo et al., 2012), leading then to a lower digestibility of the matrix and probably involving a reduced acrylamide bioaccessibility. In contrast, an increase of the starch hydrolysis is associated with the lack of protein matrix, which produces a more porous structure with a more accessibility to the enzymes for the digestion process (Marti & Pagani, 2013).

In the present study, fried snacks exhibited similar fat content (27.8–32.9 g/100 g), whereas the sugar content showed the highest variability of up to 30-fold (Table 1). In this case, potato and sweet potatoes presented the lowest levels and beetroot and carrot chips the highest, which could explain the higher acrylamide percentage in non-soluble fraction for these last samples. Although differences in the protein content were also minor (3.0–6.3 g/100 g) as compared to sugars (Table 1), variations in the amino acid composition for these food matrices cannot be discarded. According to USDA, (2019a, b, c, d), lysine, histidine, methionine and cysteine contents for potatoes, sweet potatoes, beetroots and carrots, respectively, are as follows: lysine (0.114, 0.066, 0.058, 0.101 g/100 g), histidine (0.041, 0.031, 0.021, 0.040 g/100 g), methionine (0.030, 0.029, 0.018, 0.020 g/100 g) and cysteine (0.024, 0.022, 0.019, 0.083 g/100 g). It is known that amino groups of certain free amino acids such as lysine and histidine, N-terminal amino acid residues of proteins, and -SH groups of cysteine or methionine could lead to the formation of nucleophilic molecules after the protein digestion (Friedman, 2003; Hidalgo et al., 2010; Zamora et al., 2010). Acrylamide is an electrophilic compound with an  $\alpha$ ,  $\beta$ -unsaturated structure and an amide group (Kocadagli & Gökmen, 2016), making it prone to react with those released nucleophile compounds at the pH conditions of the intestinal digestion through the Michael reaction (Hidalgo et al., 2010). Due to this fact, a possible interaction between acrylamide and nucleophilic compounds leading to the formation of acrylamide adducts may not be discarded, affecting in different proportion to different snacks and consequently to the acrylamide recovery.

Another aspect to consider is a possible effect of the fibre content in the food matrix. Fibre has been suggested to adsorb acrylamide in the gastrointestinal tract (Woo et al., 2007) and to reduce the bioaccessibility of the contaminant in the final stages of the digestion process (Vázquez-Sánchez et al., 2018). This fact could explain the higher non-bioaccessible fraction in beetroot and carrot chips, both with the highest fibre content (12.6 and 13.6 g/100 g, respectively), compared with the other snacks (4.3 g/100 g in potato, 9.3 g/100 g in sweet potato chips) (Table 1).

In summary, after the *in vitro* digestion, acrylamide bioaccessibility was close to 72 % in the potato chips and around 60 % in the other snacks, possibly being affected by the composition of the food matrix. Similar

or even higher percentages have been reported by Hamzalıoğlu & Gökmen (2015) in the acrylamide bioaccessible fraction of fried potatoes digested, ranging from 78.2 to 96.8 % in the duodenal phase.

### 3.3. *In vitro* fermentation

After the digestion, a simulation of the fermentation step carried on by the gut microbiota was developed. Following the information reported by Pérez-Burillo et al. (2021), the complete undigested residue (insoluble non-bioaccessible portion) obtained after the *in vitro* digestion together with the 10 % of the total soluble fraction is used as fermentation substrate for the colonic microbiota. As only 0.5 g of the non-soluble fraction was subjected to the fermentation step in the experimental conditions, the proportional amount of the soluble fraction was calculated and added to the samples to be submitted to the fermentation process.

It is known that the human microbiota composition is associated with the host subject, which means that each individual seems to harbour a specific core (a subset of bacteria that is preserved throughout time). This core consists on specific phylotypes distributed within anaerobic and ecosystem-specific genera that include: *Allistipes*, *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Blautia*, *Dorea* and *Ruminococcus* (Rajilic-Stojanovic et al., 2013). In addition to individual variations, the proportions of bacteria which constitute the human microbiome usually vary throughout life. Hopkins et al. (2002) observed that the faecal microbiota of children was bacteriologically less complex with a predomination of *Bifidobacterium* and *Enterobacteriaceae*, and while growing age, these bacteria decreased and *Bacteroides* species increased. Although from the two years old the microflora in infants is stabilised reaching the 'adult-like' colonic microbiota, the diet fluctuations, the physiological variations that occur in the body decreasing acid secretion by the gastric mucosa and enhancing the permeability of mucosal membranes in the gut (Hopkins et al., 2002), along with the use of antibiotics, during the childhood, adolescence and adulthood, could change this microflora. Taking these facts into account, the present study was designed to analyse possible differences between three populations, children, adolescents and adults, recruiting four volunteers per group to donate faecal material. Faeces from those four donors were pooled to embrace the maximum types of bacterial strains, reducing the interindividual variability and reinforcing the results. Acrylamide released after *in vitro* fermentation of non-bioaccessible fractions of the snack samples is shown in Table 3. Once the *in vitro* fermentation was finished, beside a soluble portion, an insoluble pellet was obtained. After analytical determination, acrylamide was not detected in that pellet, which might be due to several reasons: i) our analytical methodology for acrylamide determination did not detect such negligible amount (below our detection limit); ii) acrylamide was not present in this pellet due to its release in the accessible fraction after fermentation; iii) acrylamide present in non-accessible fraction after fermentation was combined with nucleophilic compounds of the food matrix via Michael addition (such as the amino acids from the peptides or also the fibre components), then it was not detectable as free acrylamide. Therefore, only levels of the contaminant in the soluble portion (released at colonic level) is included in the Table 3. Released acrylamide after fermentation ranged between 43.03 and 71.89 % for different veggie chips and populations. In the case of potato chips, acrylamide levels subjected to the fermentation step were very low and after this process, concentrations were below the limit of detection. Consequently, no information is included for this sample and results cannot be discussed in detail.

As described in the case of the acrylamide bioaccessibility after *in vitro* digestion, the food matrix could also affect the release of acrylamide during the fermentation process, mainly due to the differences in the protein and fibre content. In addition, possible variations in the microbiota of the different population groups could also condition the release of acrylamide from the non-bioaccessible fraction. The most remarkable effect in the colon phase could be the hydrolysis of remaining proteins and peptides due to the proteolytic activity of the enzymes of the microbiota, promoting then the Michael addition of amino acids to acrylamide. Although the protein content was very similar between all these veggie snacks, the amino acid profile has been shown to be significantly different in these vegetables, especially regarding to lysine, histidine, methionine and cysteine



content (USDA, 2019a, b, c, d). This fact would also lead to different formation rates of Michael adducts at colonic level, explaining the lower detection of acrylamide after the *in vitro* fermentation in potato chips, similarly to results reported by Hamzalioglu & Gökmen (2015) in a multistep enzymatic digestion process. Probably, the fibre content had a deeper impact in the release of acrylamide during fermentation than other components, since some microorganisms such as Bifidobacterium and Bacteroides are able to degrade undigested fibre at colon (Sun et al., 2021) and then release the trapped contaminant. The higher presence of fibre in the portion submitted to fermentation in the case of carrot and beetroot chips could suppose a greater substrate for the degradation by specific microbiota compared to the other snacks, perhaps leading to higher release of acrylamide after the fermentation step. However, this fact did not happen in the present study neither in the children nor the adolescent populations, which exhibited their maximum percentage of acrylamide released after fermentation of sweet potato (71.89 and 63.42 %, respectively) compared to the beetroot (65.22 and 43.03 %, respectively) and carrot (59.52 and 50.15 %, respectively). In the KOALA Birth Cohort Study developed in Dutch children, Zhong et al. (2019) stated that the higher levels of Bifidobacterium present in the microbiota of 6 to 9 years old kids compared to adults could lead to increased fibre fermentation. If this happened in our study it would explain the higher acrylamide release observed in our younger population. Additionally, we observed that the adult group also showed good ability to degrade these food matrices (58.82 and 60.91 % for sweet potato and beetroot, respectively), and almost better than children in the case of the carrot (55.75 %) (Table 3).

Other aspect to consider is the influence of the food matrix on the microbiota profile. The high sugar content in the non-bioaccessible fraction of these vegetables could provide high availability of glucose, used as substrate for the growing of lactic acid bacteria and certain Firmicutes, whose cell walls contain proteins and peptidoglycan able to bind little amounts of acrylamide (Shen et al., 2019; Albedwawi et al., 2021) and thus causing its removal through the faeces, together with the bacteria (dos Reis et al., 2017). These low amounts could be undetectable in this study with our methods and equipment.

In summary, different profiles of bacterial species in each population group together with the food matrix composition, may influence the acrylamide release during the colonic phase. Our data suggest that acrylamide released from the non-bioaccessible fraction of sweet potato during the fermentative process was significantly favoured in the children (71.89 %) compared to adolescents (63.42 %) and adults (58.82 %). Unmodified values of acrylamide release were observed for beetroot chips in the three population groups. On the contrary, although without significant differences, children microbiota exhibited a worse efficiency to release acrylamide from the non-bioaccessible fraction of carrot than adults (43.03 % < 50.15 % < 55.75 % for children, adolescents and adults, respectively).

### **3.4. Total amounts of absorbable acrylamide after the *in vitro* digestion and fermentation**

The total percentages of acrylamide potentially available to be absorbed are depicted in Fig. 4. These data include the acrylamide available after *in vitro* digestion and colonic fermentation. It is important to bear in mind that bioaccessible acrylamide values after *in vitro* digestion of snacks are the same for all age groups (solid bars), children, adolescents and adults, since food was only digested once and then fermented with the faecal material from each one of the populations considered. For the representation, the percentage of bioaccessible acrylamide after *in vitro* digestion of each snack (subtracting the 10 % that reaches the colon and goes through fermentation) was added to the percentage of acrylamide released after the colonic fermentation in the presence of faeces from the three population groups (excepting potato chips since the amount was not detectable). Globally, the lowest total available acrylamide was shown in potato chips and the highest in beetroot and sweet potato chips when fermented with faeces from the children group, while the rest of the foods and fermentation groups did not show significant differences. Therefore, considering the global

gastrointestinal transit (digestion + fermentation) of sweet potato and beetroot, the youngest population seemed to be more prone to an increased acrylamide absorption from these veggie chips.

Taking these results and the consumption data by the Spanish population previously mentioned (MAPA, 2021), the daily acrylamide potentially absorbed after the digestion and fermentation step of each snack was estimated. After the digestion process, bioaccessible acrylamide would be 1.1 µg/day in the potato chips, 4.2 µg/day in sweet potato chips, 1.7 µg/day in beetroot chips and 2.3 µg/day in carrot chips. In the same way, after the fermentation step, the acrylamide available to be absorbed from sweet potato chips would range between 1.2 and 1.5 µg/day for children, adolescents and adults. Levels of acrylamide released at colon would be almost equal in beetroot and carrot chips (ranges: 0.5–0.6 µg/day for children, 0.6 µg/day for adolescents and 0.6–0.7 µg/day for adults).

As a realistic example, the consumption of an individual commercialised snack bag (50 g) was considered to estimate the acrylamide potentially absorbable after digestion and colonic fermentation (Table 4), exhibiting a range between 18.8 and 88.7 µg per bag depending on the population. The highest acrylamide intake through a bag of sweet potato chips compared with the rest of the snacks would lead to increased bioaccessible and released contaminant after fermentation (25.3, 22.4 and 20.8 µg per bag for children, adolescents and adults), involving a potentially greater acrylamide absorption (mean value for the three populations: 86.2 µg per bag). A bag of beetroot or carrot chips would provide similar *in vivo* amounts of the compound (mean value for the three populations: 35.5 µg and 45.2 µg per bag, respectively), whereas the same portion of the traditional potato chips would suppose the lowest levels of potentially absorbable acrylamide in the organism (18.8 µg per bag). These data should be available for consumers and producers so that they could make healthier decisions when buying/designing snacks, taking into account not only nutritional criteria but also the risk associated with the exposure of this processing contaminant.

#### 4. Conclusions

In this study, the acrylamide bioaccessibility after *in vitro* digestion and its release after colonic fermentation of potato and veggie chips was assessed. The aim was to evaluate the influence of the food matrix and the microbiota from three age stages of the population (childhood, adolescence and adulthood) on the potentially absorbable acrylamide.

Acrylamide bioaccessibility of potato and veggie chips after *in vitro* digestion ranged from 59.7 to 71.7 % and the non-bioaccessible fraction between 22.8 and 28.4 %. After *in vitro* fermentation, the acrylamide percentage in the soluble fraction was between 43.03 and 71.89 %, suggesting that acrylamide present in the non-bioaccessible fractions could be released by the microbial action during the colon phase. The highest values were observed in sweet potato chips fermented with faecal samples from children, indicating that the specific microbiota profile in each population group together with the food matrix composition may influence the acrylamide levels potentially absorbable after the whole gastrointestinal digestive process. Results confirm the importance of considering not only the acrylamide exposure from foods, but also its availability during the digestion and colonic fermentation processes. This fact should be considered in the estimation of the real risk associated with the potentially toxic effects of this contaminant on humans.

#### Formatting of funding sources

This work is part of the R&D project ACRINTAKE (RTI2018-094402-B-I00), financed by MCIN/AEI/ <https://doi.org/10.13039/501100011033/> and Fondo Europeo de Desarrollo Regional (“FEDER”) A way to make Europe.

#### CRedit authorship contribution statement

L. González-Mulero: Data curation, Formal analysis, Methodology, Software, Writing – original draft. M. Mesías: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. F.J. Morales: Conceptualization, Investigation, Resources, Software, Supervision, Visualization, Writing – review & editing. B. Navajas-Porras: J.A. Rufián-Henares, C. Delgado-Andrade: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

### **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### **Data availability**

The authors do not have permission to share data

### **Acknowledgements**

The authors wish to thank Ms. I. Alvarez for her technical assistance. We also thank faecal donors for their selfless participation.

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## FIGURES AND TABLES

**Table 1.** Nutritional composition of snacks according to the information provided by the manufacturer. Data are expressed as g/100 g of sample

<b>Snack</b>	<b>Total fat</b>	<b>Total Carbohydrate</b>	<b>Sugars</b>	<b>Dietary Fibre</b>	<b>Protein</b>
<b>Potato chips</b>	32.9	50.4	1.1	4.3	6.3
<b>Sweet potato chips</b>	32.8	55.4	11.3	9.3	3.0
<b>Beetroot chips</b>	27.8	57.4	37.5	12.6	4.2
<b>Carrot chips</b>	29.2	48.4	38.5	13.6	3.6



**Table 2.** Acrylamide content in the analysed snacks and distribution in bioaccessible and non-bioaccessible fractions after the *in vitro* enzymatic digestion process. Results are represented as mean  $\pm$  standard deviation. Different letters in the same column involve significant differences ( $p < 0.05$ ).

Snack samples	Acrylamide content in snacks ( $\mu\text{g}/\text{kg}$ )	Acrylamide in the portion for digestion ( $\mu\text{g}$ )	Acrylamide distribution (%)		
			Bioaccessible fraction	Non-bioaccessible fraction	Recovery rate
Potato chips	524 $\pm$ 32a	6.3 $\pm$ 0.4a	71.7 $\pm$ 1.6b	22.8 $\pm$ 0.8a	94.5 $\pm$ 0.8b
Sweet potato chips	2342 $\pm$ 148d	28.1 $\pm$ 1.8d	60.1 $\pm$ 0.6a	24.1 $\pm$ 0.2a	84.2 $\pm$ 0.8a
Beetroot chips	947 $\pm$ 12b	11.4 $\pm$ 0.1b	59.7 $\pm$ 0.1a	28.4 $\pm$ 0.7b	88.0 $\pm$ 0.7ab
Carrot chips	1279 $\pm$ 59c	15.3 $\pm$ 0.7c	60.4 $\pm$ 2.0a	26.8 $\pm$ 1.7ab	87.1 $\pm$ 3.6ab

**Table 3.** Acrylamide levels in the soluble fraction after the *in vitro* fermentation of digested snack samples.

Snack	Acrylamide before <i>in vitro</i> fermentation (ng)	Acrylamide released after <i>in vitro</i> fermentation					
		Children		Adolescents		Adults	
		(ng)	(%)*	(ng)	(%)*	(ng)	(%)*
Potato	34 ± 4	n.d.	-	n.d.	-	n.d.	-
Sweet potato	119 ± 6c	86 ± 4b	71.89 ± 3.40B	76 ± 3ab	63.42 ± 2.17A	70 ± 2a	58.82 ± 1.42A
Beetroot	53 ± 1b	34 ± 2a	65.22 ± 4.11B	31 ± 3a	59.52 ± 4.77A	32 ± 3a	60.91 ± 6.38A
Carrot	78 ± 1b	33 ± 1a	43.03 ± 0.43A	39 ± 6a	50.15 ± 7.27A	43 ± 3a	55.75 ± 3.75A

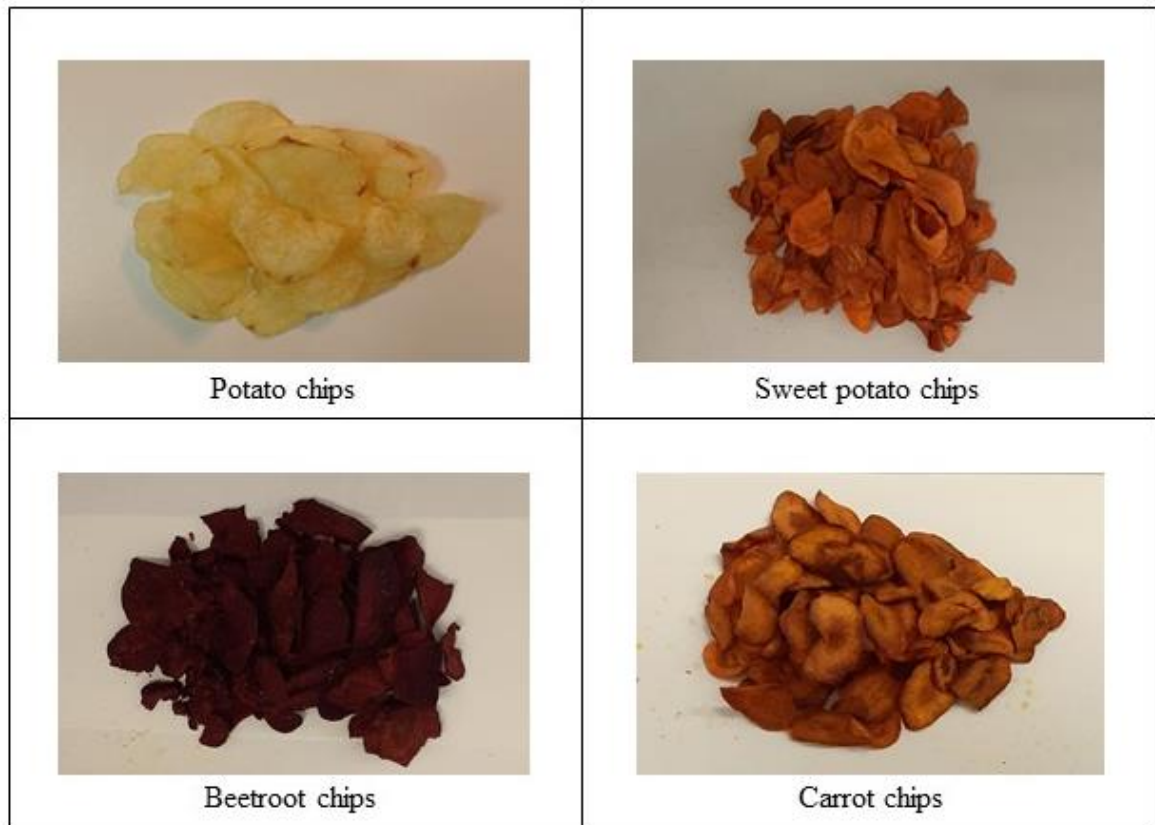
\* Initial acrylamide before *in vitro* fermentation was taken as 100%. Results are mean ± standard deviation. Different lowercase letters mean significant differences ( $p < 0.05$ ) between the acrylamide content before and after *in vitro* fermentation by using faeces from different population groups (children, adolescents and adults). Different capital letters mean significant differences ( $p < 0.05$ ) in percentages of accessible acrylamide after *in vitro* fermentation between different vegetable chips in each population group.

**Table 4.** Estimation of the acrylamide potentially absorbable after the digestion and colonic fermentation through the consumption of an individual commercial snack bag (50 g).

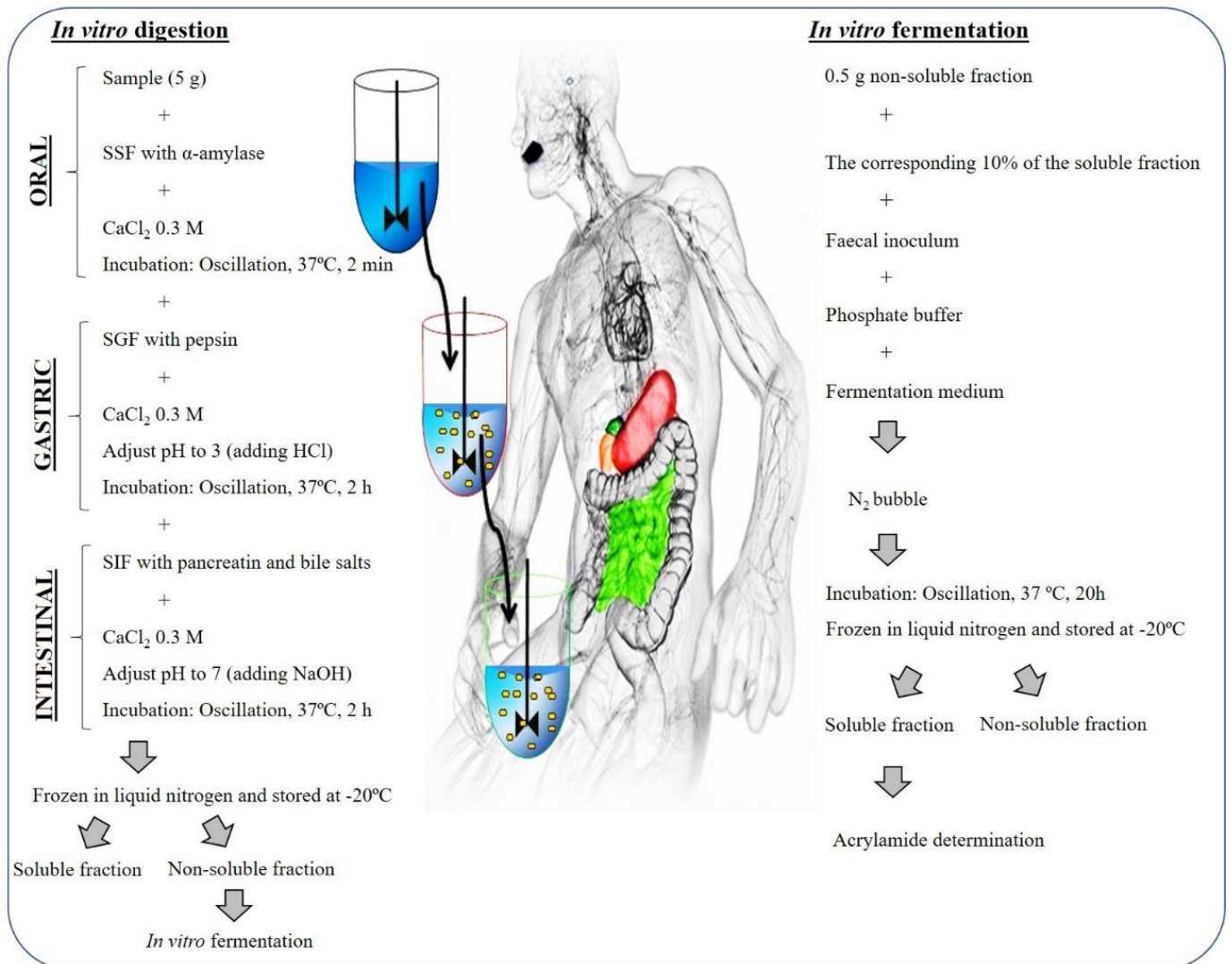
Snack	Ingested acrylamide (µg)	Bioaccessible after digestion (µg)	Released after fermentation (µg)*		Total acrylamide potentially absorbable (µg)*	
Potato	26.2	18.8	n.d.		18.8	
Sweet potato	117.1	70.4	22.9 ± 2.3	(20.8 – 25.4)	86.2 ± 2.3	(84.1 – 88.7)
Beetroot	47.4	28.3	10.1 ± 0.5	(9.7 – 10.6)	35.5 ± 0.5	(35.1 – 36.1)
Carrot	63.9	38.6	10.4 ± 1.4	(9.0 – 11.7)	45.2 ± 1.3	(43.8 – 46.4)

\* Mean value ± sd and range pooling data from children, adolescents and adults

**Fig. 1.** Snack samples (potato, sweet potato, beetroot, and carrot chips) evaluated in the present study.

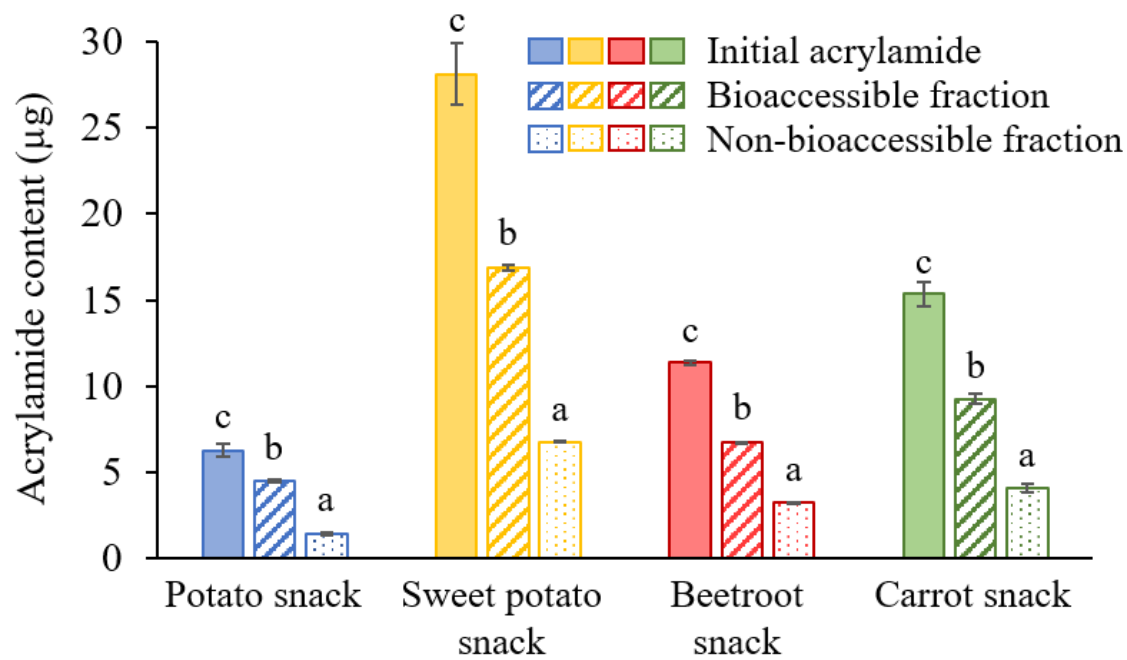


**Fig. 2.** Schematic description of the methodology used for the *in vitro* digestion and fermentation processes (adapted from Egger et al. (2016) and Pérez-Burillo et al. (2021)).



SSF: simulated salivary fluid; SGF: simulated gastric fluid; SIF: Simulated intestinal fluid.

**Fig. 3.** Acrylamide content before and after the *in vitro* enzymatic digestion of the four vegetable snacks including bioaccessible and non-bioaccessible fractions. Different letters represent significant differences ( $p < 0.05$ ) in each snack.



**Fig 4.** Total percentage of acrylamide potentially absorbable after *in vitro* digestion and fermentation of snacks. Solid bars represent the percentage of bioaccessible acrylamide after the *in vitro* digestion subtracting the 10 % that will reach the colon together with the non-bioaccessible fraction. Dashed bars represent the percentage of released acrylamide after the *in vitro* fermentation corresponding to the three age groups tested (dots for children, squares for adolescents and stripes for adults). Different letters mean significant differences ( $p < 0.05$ ) between the total potentially absorbed acrylamide percentages obtained for each population comparing the four types of vegetable chips.

