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In vitro digestion kinetics and bioaccessibility of starch in cereal food products

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ABSTRACT

The study of starch digestion in cereal-based products is essential since the extent and rate of hydrolysis affects the glycemic index associated with several food-related diseases. Besides, a unique matrix is usually studied and many variations of *in vitro* techniques (e.g. enzymes, pH, food:enzymes ratio) are selected, then comparison of results became difficult. The recently published INFOGEST *in vitro* static method with international consensus (Minekus et al., 2014) was applied to several cereal-based products to address whether it is suitable for analysis of starch hydrolysis kinetics. Bread, pasta and cookies were selected taking into account the analysis of different cereal matrixes, including gluten-free products. White bread presented the highest *in vitro* starch hydrolysis (87%) showing significant differences compared to gluten-free bread (76.5%). Refined flour sheeted pasta (72.6%), whole-wheat extruded pasta (92.0%) and gluten-free pasta (54.3%) showed differences in the extent and rate of hydrolysis. Cookie samples presented the lowest starch hydrolysis (~45%). The starch availability was estimated by the dializability method, which measures the maltose equivalents dialyzed after simulating digestion. Starch dializability was 35%, 25% and 15% on average for bread, pasta and cookie samples respectively, with positive correlation with rapidly digested starch. The tested *in vitro* method allowed discriminating the effect of different processing techniques, product types and formulation of the three most common cereal-food matrixes in starch digestion.

1. Introduction

The major carbohydrate in human diet is starch, which generates increase of glucose in blood after digestion of a starchy food. As a consequence, starch hydrolysis within the food matrix has been the focus of many research studies since the digestion rate of starch in food determines glycemic response, which has been associated with a range of illnesses linked to diet (Björck and Liljeberg Elmståhl, 2003; Norton et al., 2015). On the other hand, the extent of starch digestion is related to the production of short-chain fatty acids in the colon generated by indigestible fraction (Ashwar et al., 2016). Considerable differences in the extent and rate of starch hydrolysis are produced through the ingestion of different cereal-based products containing identical amounts of starch. The fact that such differences are found evidences variation in the physical state of starch, food matrix, formulation, processing or even the presence or absence of gluten (Bhattarai et al., 2016; Singh et al., 2013). So that, the chemical and physical characteristics of food influence the degree of digestion and the changes taking place during each step of the process. As a consequence much research focused on pasta, bread and cookie aspects that influence starch digestion has been generated (Foschia et al., 2015; Gao et al., 2015; Villemejane et al., 2016). However, only a few consider a unique technique to evaluate different food matrixes, which could facilitate comparison of results. Therefore, most reports could not be compared, even when using the same enzymes (mainly α-amylase, pepsin and pancreatin). Additionally, it is important to characterize starch hydrolysis kinetics using *in vitro* digestion techniques that not only have to be easy to use to investigate nutritional or functional attributes (screening tool), but also be able to address differences between cereal-food matrixes such as compact structure, presence of dietary fiber or lipids or the absence of gluten.

Cereal food researchers need an *in vitro* method reaching international consensus so that it could be used for analysis of starch hydrolysis and allow discriminating differences in product structure, presence or absence of lipids, dietary fiber, gluten or specific

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proteins, and also in the processing technique used to make a specific product. This will facilitate comparison of results and establish the key factor of cereal products that affects glucose metabolism.

In order to find a harmonization of currently used digestion models, an open international network of institutes undertaking multidisciplinary basic research on food digestion has been developed with the name of COST FA1005 Action - "Improving health properties of food by sharing our knowledge on the digestive process (INFOGEST). As a result, a standardized static *in vitro* digestion method was published in 2014 (Minekus et al., 2014) which stated the relevant parameters required to improve studies about digestibility. This protocol needs to be tested in a variety of applications to determine its use and limitations.

As a result, we propose the application of INFOGEST static *in vitro* digestion method in order to test whether it is suitable for the analysis of starch hydrolysis kinetics of the three most widely consumed cereal-based food as tool to understand the differences in glycemic response.

2. Experimental

2.1. Materials

Digestive enzymes: amylase from porcine pancreas (A3176), pepsin from porcine gastric mucosa (P7000), pancreatin from porcine pancreas (P7545), and bile salts (B8756) were purchased from Sigma-Aldrich (Buenos Aires, Argentina). The chemicals used in this study were of analytical grade and the ingredients for food formulation were of food grade.

2.2. Preparation of cereal food samples

The cereal-based foods used for *in vitro* digestion were white bread, gluten-free bread, sheeted pasta, extruded whole-grain spaghetti, whole-wheat cookies and peach cookies. Each product was made twice as follows:

2.2.1. White bread

The ingredients used (g/100 g flour) were 60 g/100 g of water, 1 g/100 g of instant dry yeast, 1.8 g/100 g of salt and 0.01 g/100 g of ascorbic acid according to Steffolani et al. (2015).

2.2.2. Gluten-free bread

The gluten-free bread formulation consisted of 45 g of rice flour, 45 g of cassava starch, 10 g of soy flour, 2 g of salt, 2 g of shortening, 3 g of instant dry yeast and 65 g of water according to Sciarini et al. (2012).

2.2.3. Sheeted pasta

The white wheat sheeted pasta formulation was prepared with 50 g of bread wheat flour, 0.5 g of salt and 20 g of water according to Bustos et al. (2011b). The dried pasta was cooked for 14 min prior to analysis.

2.2.4. Whole-grain extruded spaghetti

The whole-wheat extruded pasta formulation consisted of 50 g of whole-grain wheat flour, 0.5 g of salt and 20 g of water. The dough was mixed for 3 min and shaped through the die to obtain spaghetti (diameter of 2.1 ± 0.2 mm) in a pasta extruder (ATMA, Argentina). The pasta strands were dried according to Bustos et al. (2011b) and cooked for 13 min prior to analysis.

2.2.5. Gluten-free pasta

The gluten-free extruded pasta formulation consisted of 50 g of sorghum flour, 1.25 g of guar gum, 5.5 g of albumin, 2.85 g of dry egg, 0.45 g of pre-gelatinized maize starch, 0.5 g of salt and 20 g of water. The dough was mixed for 3 min and shaped through the die to obtain spaghetti (diameter of 2.1 ± 0.2 mm) in a pasta extruder (ATMA, Argentina) according to Palavecino et al. (2017). The pasta strands were cooked for 13 min prior to analysis.

2.2.6. Whole sweet cookies

The cookie made from whole-grain flour was formulated as follows: 60 g of whole-grain wheat flour, 36 g of sugar, 27 g of shortening, 3 g of milk powder, 0.7 g of sodium bicarbonate, 0.6 g of salt and 12 g of water. The cookies were prepared according to León et al. (1996).

2.2.7. Peach cookies

The sweet peach cookie formulation was as follows: 20 g of wheat flour, 13.5 g of sugar, 7.5 g of inulin (Orafti HP, Beneo-Orafti Latin America, Brazil), 2.5 g of polydextrose (Granotec, Argentina), 4.5 g of shortening, 3.6 g of maize oil, 7 g of commercial peach pulp, 1.2 g of milk powder, 0.25 g of sodium bicarbonate, 0.2 g of salt and 3 g of water. The cookies were prepared according to Serial et al. (2016).

2.3. Methods

2.3.1. In vitro digestion

In vitro digestion of the cereal-based food samples was performed according to the proposal by INFOGEST's scientists for a static method (Minekus et al., 2014) with modifications to evaluate dialyzability of starch.

Simulated digestion fluids were prepared according to Minekus et al. (2014) with the following final concentration of salts in the digestion mixture:

Simulated salivary fluid (SSF): 15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H_2O)_6$, 0.75 mM $CaCl_2(H_2O)$, 75 U/ml amylase from porcine pancreas; the pH was adjusted to 7.0 with HCl 6M.

Simulated stomach fluid (SGF): 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2(H_2O)_6$, 0.075 mM $CaCl_2(H_2O)$, and 2000 U/ml of pepsin from porcine pancreas; the pH was adjusted to 3 with HCl 6M.

Simulated duodenal fluid (SDF): 6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, 0.3 mM $CaCl_2(H_2O)$, 3 mg/ml of pancreatin from porcine pancreas (8xUSP), and 8 mg/ml of bile salts; the pH was adjusted to 7.0 with HCl 6M.

Briefly, the ratio used in the model was 50/50 w/v for: food/Simulated Salivary Fluid (SSF); oral content/Simulated gastric fluid (SGF) and gastric content/Simulated intestinal fluid (SDF). Five grams of food as eaten were mixed with 5 ml of SSF (containing α-amylase); the simulated chewing was performed for 1 min by disrupting sample with a Teflon pestle (Cole Parmer, EW-44468-18, USA) and incubated for 2 min at 37 °C with orbital agitation. After oral digestion, 10 ml of SGF (containing pepsin) were added to the sample and the pH was adjusted to 3.0; the samples were then incubated for 2 h at 37 °C with orbital agitation. Finally, the intestinal phase was performed by incorporating 20 ml of SDF (containing pancreatin and bile salts); the pH was adjusted to 7.0 and incubated for an additional 3 h with orbital agitation. Aliquots of 1 ml were withdrawn at time 0, after the salivary step, at 60 and 120 min of the gastric step and at 10, 30, 90 and 180 min of the intestinal step to monitor the hydrolysis degree of starch. The *in vitro* digestion method was performed in duplicate in each product batch.

2.3.2. Monitoring starch hydrolysis during in vitro digestion and kinetic analysis

Starch hydrolysis was monitored by the analysis of reducing sugar content in each aliquot using the 3,5− dinitrosalicylic acid (DNS) method. Two non-linear models (Eqs. (1) and (2)) were applied to describe separately oral-gastric and intestinal digestion for starch hydrolysis. Parameter estimation was carried out using the SIGMA PLOT software, version 12. The rate of starch digestion was expressed as the percentage of total starch present in sample hydrolyzed at different times.

$$\text{Oral} - \text{gastric } \textit{in vitro} \text{ digestion}: \; C_g = C_{g\infty} \times \left(1 - e^{-k_g t}\right) \quad (1)$$

$$\text{Intestinal } \textit{in vitro} \text{ digestion}: \; C_i = C_0 + C_{i\infty} \times \left(1 - e^{-k_i t}\right) \quad (2)$$

where C is the percentage of starch hydrolyzed at time t during digestion, C_∞ is the percentage of starch hydrolyzed at time ∞, K is the kinetic constant and C_0 is the percentage of starch hydrolyzed at the beginning of the intestinal phase. Parameters from oral-gastric digestion are identified as "g" and from the intestinal phase as "i".

Starch classifications based on the rate of hydrolysis were also determined: rapidly digestible (digested within 20 min) starch (RDS), slowly digestible (digested between 20 and 180 min) starch (SDS) and resistant (undigested after 180 min) starch (RS).

2.3.3. Starch dialyzability

For dialyzability determinations, the assay was conducted as explained in section 2.2.3, until the gastric step. Then, for the intestinal phase, the method was performed with a dialysis tube. Briefly, after the gastric stage the SDF (containing pancreatin and bile salts) was added. Immediately, a cellulose dialysis tube (molecular mass cut-off value 10,000–12,000 Da) filled with 25 ml of $NaHCO_3$ equivalent to the titrable acidity (previously measured) was placed in the flasks containing stomach digest and SDF solution. Incubation was continued for 3 h with orbital agitation. The $NaHCO_3$ inside the dialysis bag diffuses into the incubation medium and allows gradual adjustment of pH and, because the dialysis bag contains the correct amount of $NaHCO_3$, overshoot of pH can be avoided. Finally, aliquots were withdrawn from the inside (dialyzable) of the dialysis tube for starch hydrolysis determination (described above).

Dialyzability involves the hydrolyzed starch that passes thought the dialysis tube, calculated as follows:

$$\text{Dialyzability}(\%) = \frac{N_{DD}}{N_{SC}} \; x \; 100 \quad (3)$$

where N_{DD} is the *in vitro* digested starch inside the dialysis bag and N_{SC} is the starch content in the sample.

All determinations were performed in quadruplicate.

2.3.4. Statistical analysis

Each product was prepared twice and then analyzed by the *in vitro* digestion method in duplicate each. The results were expressed as the mean of replications ± SD. An analysis of variance was performed and the data were compared by the test of Di Rienzo, Guzmán y Casanoves (DGC) (Di Rienzo et al., 2002). This method uses multivariate analysis of conglomerates in a matrix obtained from the sample mean. This allowed the samples to be grouped according to descending levels of preference (A, B and C) and with a degree of significance of $p < 0.05$. Pearson's correlation analysis was also performed. All analyses were performed using the Infostat Statistical Software (Facultad de Ciencias Agropecuarias, UNC, Argentina).

2.4. Results and discussion

2.4.1. Starch in vitro digestion of cereal-based food samples

2.4.1.1. Oral in vitro digestion. White bread and cookie simulated chewing lasted 27 s and pasta chewing 20 s in agreement with Hoebler et al. (1998). Pasta simulated chewing minimally affected food shape, mainly reducing strand length. On the other hand, gluten-free bread chewing was mimicked for 35 s due to the compact structure of the product that delays hydration. White bread and cookie simulated chewing led to the disruption of the food matrix, thus starch released from the protein network became susceptible to enzyme attack. These results agree with those published by Bornhorst & Singh (Bornhorst and Singh, 2012) who reported that chewing duration and bites varied slightly with food hardness and volume of ingested food. Ten seconds were enough for cookie simulated chewing, as reported by Chen (2015), who found that dry brittle solid foods have a high breakage function and hence few chewing cycles are needed. Consequently, differences between food matrixes and shapes generated differences in the hydrolysis degree during oral digestion, clearly conditioning the entire digestion process, as shown in Table 1.

White and gluten-free bread presented significant differences, the latter being less hydrolyzed than white bread ($P < 0.05$). This result may be attributed to the compact structure of gluten-free bread that delays hydration and hydrolysis of starch, as some authors had already reported (Gao et al., 2015; Parada and Aguilera, 2007). In this regard, this also explains that sheeted pasta presented the lowest hydrolysis degree during oral digestion simulation, due to the compact structure of the product that allows only a minimal attack of starch by α amylase to produce maltose (Heneen and Brismar, 2003; Kim et al., 2008; Zou et al., 2015). The change from bread wheat flour to whole-grain flour or the use of gluten-free ingredients to produce pasta generated a more susceptible product for starch hydrolysis probably due to the more porous structure (Petitot et al., 2009), even when the sheeted process was changed for extrusion ($P < 0.05$). Gluten-free pasta made from sorghum flour presented a significantly higher degree of starch hydrolysis compared to the other pasta products, which is associated with the lack of protein matrix that produces a more porous structure, more accessible to α amylase at this phase (Marti and Pagani, 2013). Cookies hydrated very quickly and lost their structure, which may lead to high levels of starch hydrolysis as shown in Table 1.

2.4.1.2. Gastric and intestinal in vitro digestion. Fig. 1 shows the experimental data obtained for starch hydrolyzed at oral-gastric and intestinal phases during *in vitro* digestion. The gastric

Table 1
In vitro oral digestion of starch in cereal-based food samples.

Cereal food sample	Starch hydrolysis (g/100 g starch)
White bread	5.1 ± 0.1^{d}
Gluten-free bread	2.4 ± 0.2^{b}
Sheeted pasta	1.4 ± 0.3^{a}
Whole-grain pasta	3.5 ± 0.2^{c}
Gluten-free pasta	5.2 ± 0.2^{d}
Peach cookies	9.8 ± 0.1^{f}
Whole cookies	5.5 ± 0.3^{e}

*Different letters in the columns indicate significant difference $p < 0.05$.

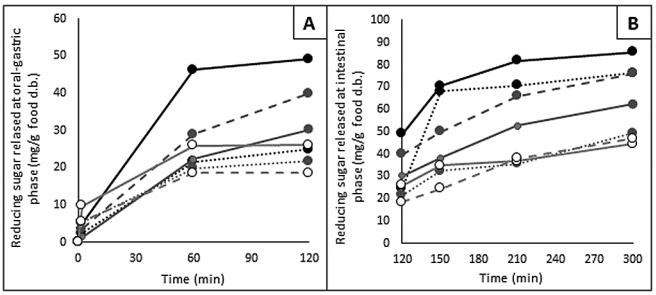

Fig. 1. ***In vitro*** **digestion of starch during oral-gastric (A) and intestinal (B) phases of cereal food samples tested**.
(—●— white bread; ···●··· gluten-free bread; —●—sheeted pasta; –●– whole-grain pasta; ···●··· gluten-free pasta; —○—peach cookie; –○– whole-grain cookie).

(including the oral step) and intestinal phases were analyzed separately. Table 2 shows the adjusted parameters obtained from Eqs. (1) and (2) (R^2 values were above 0.98 in all cases). Total starch hydrolysis was calculated as the sum of the adjusted equilibrium percentages of hydrolysis during the gastric ($C_{g\infty}$) and intestinal ($C_{i\infty}$) steps.

The first observation to be made is that amylase continues active to a certain extent at pH > 2 as reported previously (Bhattarai et al., 2016; Hoebler et al., 1998) and, as a result, the major contribution was found during oral and gastric phases (for up to 60 min) releasing, as reducing sugars, about 43% and 21% of the starch in bread and gluten-free bread, respectively. For pasta products until 60 min of gastric digestion, about 22%, 29% and 18% of the starch in sheeted, whole-grain and gluten-free pastas were released as reducing sugars, respectively. Finally, 18% and 26% of starch was hydrolyzed at the same conditions for whole-grain and peach cookies, respectively. These findings are well related to those of the distinct glycemic index reported for these cereal-based products.

The role of salivary amylase in gastric digestion has been largely ignored considering that amylase is inactivated in the stomach at low pH and thus has an insignificant role on starch hydrolysis at this stage. Present results indicate that amylase activity in the stomach conditions made a significant contribution to starch hydrolysis. Butterworth et al. (2011) and Bhattarai et al. (2016) stated that the activity of amylase can be protected from inactivation by proteins, starch and oligosaccharides released from it during gastric digestion. It can also be expected to reach the intestinal conditions to continue hydrolyzing starch, despite the fact that, as far as we know, there is no research that characterizes *in vitro* starch hydrolysis kinetics under gastric conditions or even research that evaluates different patterns generated by several cereal food matrixes.

Considering intestinal digestion and since conditions became optimal for amylase action, the rate of digestion increased and, around 60 min of incubation, the curve tended to reach a constant value (Fig. 1). At this phase, the kinetic constants found were different for each product tested; breads, pastas and cookies samples being around 0.130, 0.022 and 0.033 min^{-1} on average (Table 2).

In particular, white bread was digested to a higher extent and more rapidly than gluten-free bread during the gastric step (Fig. 1). The starch hydrolyzed until that phase was ~52% lower for gluten-free bread than for its gluten counterpart ($P < 0.05$), although the difference was inverse during the intestinal step (Table 2). These results are related to the increase in the kinetic constant observed in the intestinal step compared to that in the gastric one, which was higher in the gluten-free bread, reaching a total hydrolysis of 76.5% (Table 2). These observations are in agreement with various reports which indicated that gluten-free bread showed an increased kinetics on starch hydrolysis during intestinal digestion (Berti et al., 2004; Matos Segura and Rosell, 2011). It should be noted that the low degree of starch hydrolysis of gluten-free bread during oral digestion related to the delay in hydration due to compact structure already reported (Sciarini et al., 2012). This fact could explain the lower gastric phase kinetic constant compared to white bread since hydrocolloids in GFB hydrate quickly and generated viscosity, so that, starch hydration and hydrolysis are slowed, leading to lower percentage of starch digested compared to white bread during gastric *in vitro* digestion, as was reported by others (Fardet et al., 2006; Pellegrini and Agostoni, 2015; Sciarini et al., 2017). When the intestinal phase began (after 2 h of digestion) the hydration of the remaining food structure (lacking gluten matrix) is probably complete and accessible for amylase, leading to an increase in starch hydrolysis rate.

Pasta products showed significant differences between refined

Table 2
Adjusted parameters obtained with kinetic equations for starch hydrolysis during *in vitro* oral-gastric and intestinal phases*.

Sample	Starch hydrolyzed at oral-gastric phase (%) ($C_{g\infty}$ *1)	Kinetic constant at oral-gastric phase (min^{-1}) (K_g *1)	Initial starch concentration at intestinal phase (%) (C_0 *2)	Starch hydrolyzed at intestinal phase (%) ($C^2_{i\infty}$)	Kinetic constant at intestinal phase (min^{-1}) (K_i *2)	Total starch hydrolysis (g/100 g of starch)
White bread	49.1 ± 0.2^{f}	0.050 ± 0.003^{c}	49.5 ± 0.2	37.9 ± 0.7^{d}	0.074 ± 0.006^{b}	87.0^{f}
Gluten-free bread	25.4 ± 0.5^{c}	0.034 ± 0.005^{b}	25.1 ± 0.3	51.1 ± 0.5^{f}	0.183 ± 0.024^{c}	76.5^{e}
Sheeted pasta	34.7 ± 1.5^{d}	0.018 ± 0.003^{a}	30.2 ± 0.6	37.8 ± 0.4^{d}	0.027 ± 0.002^{a}	72.6^{d}
Whole-grain pasta	46.0 ± 1.6^{e}	0.017 ± 0.002^{a}	40.9 ± 0.5	46.1 ± 1.4^{e}	0.022 ± 0.002^{a}	92.0^{g}
Gluten-free pasta	20.8 ± 0.3^{b}	0.144 ± 0.007^{d}	23.9 ± 0.9	33.2 ± 0.5^{c}	0.017 ± 0.002^{a}	54.3^{c}
Peach cookie	26.0 ± 1.2^{c}	0.237 ± 0.016^{f}	27.2 ± 0.8	18.2 ± 0.9^{a}	0.036 ± 0.004^{a}	43.6^{a}
Whole cookie	18.6 ± 0.6^{a}	0.178 ± 0.012^{e}	17.9 ± 0.3	31.9 ± 0.5^{b}	0.029 ± 0.001^{a}	50.7^{b}

Note: *1Parameters of the kinetic equation $C = C_\infty (1 - e^{-Kt})$. *2Parameters of the kinetic equation $C = C_0 + C_\infty (1 - e^{-Kt})$.
Parameter C_0 has no statistical analysis because it is equivalent to $C_{g\infty}$. Values are presented to demonstrate the accurate adjustment of both equations.*Different letters in the columns indicate significant difference $p < 0.05$.

wheat flour, whole-grain flour and gluten-free types (Fig. 1); the latter showing the lower starch hydrolysis ($P < 0.05$). Whole-grain pasta presented the highest starch hydrolysis (92.0%) due to the porous structure characterizing whole-grain products. However, the kinetic constant of the gastric and intestinal phases during whole-grain pasta digestion had no significant differences compared to that shown in the sheeted pasta (Table 2), in agreement with Kristensen et al. (2010). Gluten-free pasta showed the lowest starch digestion between pasta products, which seems to be in conflict with the idea that the glycemic response of carbohydrate-rich pasta may be increased by the removal of gluten as the strengthened network of gluten traps the starch material, limiting its swelling and hydrolysis (Bustos et al., 2011a; Colonna et al., 1990; Kim et al., 2008). In addition to this, there are only a few studies of gluten-free pasta digestibility with some controversial results. For example, the increased starch hydrolysis due to gluten absence was reported by Berti et al. (2004) who demonstrated that glycemic index of gluten-free pasta was higher that their counterpart. On the other hand, Parker et al. (2000) exposed opposite results by demonstrating gluten-free pasta presented comparable glycemic responses to wheat pasta. Recently, Scazzina et al. (2015) reported the glycemic index of commercial gluten-free products with different formulations that leads to low and medium glycemic indexes could be suitable for celiac patients without adversely influencing their postprandial blood glucose levels. That means, glycemic response of gluten-free pasta products is highly influenced by the addition of additives, the types of starch included and product format (Aravind et al., 2012; Dhital et al., 2015; Pellegrini and Agostoni, 2015).

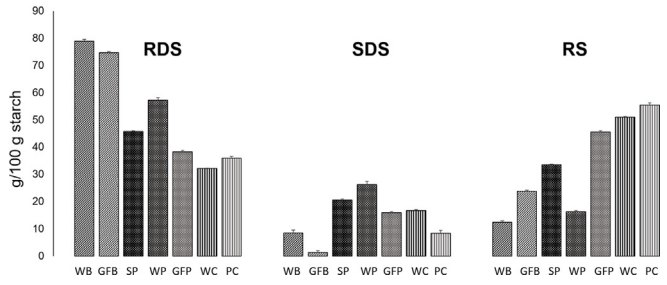

Fig. 2. Rapidly (RDS), slowly (SDS) and resistant digestible starch (RS) fractions in the cereal food samples tested*.
WB: White bread, GFB: gluten-free bread, SP: sheeted pasta, WP: whole-grain pasta, GFP: gluten-free pasta, WC: whole grain cookie and PC: peach cookie.

Both cookies analyzed showed the minimum percentage of starch hydrolysis with a high kinetic constant during gastric digestion, the highest being for the peach cookie ($P < 0.05$). As for the intestinal step, the kinetic constants were markedly lower than those for the gastric one. In addition, it is known that pectin from fruits and wheat brans retards starch hydrolysis during intestinal phase affecting glycemic index, by increasing the tortuosity and viscosity of the environment (food matrix) serving as a barrier to digestive enzyme action (Bharath Kumar and Prabhasankar, 2014) in agreement with that observed in cookie analysis.

Considering physiological effects and according to the rate of digestion, starch has been classified in three fractions: rapidly digested starch, slowly digested starch and resistant starch. As a result, from starch hydrolysis curves, we calculated the fraction digested up to 20 min, between 20 and 120 min, the fraction that remains undigested (resistant starch) and the area under the curve (Figs. 2 and 3).

Fig. 2 shows the difference in starch fractions related to its digestibility in the three cereal product matrixes tested. It is known that the disintegration kinetics of food affects the degree of starch hydrolysis during gastric and intestinal phases, which could also account for the fact that the highest percentage of rapidly hydrolyzed starch was observed in the products with a porous structure like white bread and whole pasta. Gluten-free bread showed a lower RDS content than white bread ($P < 0.05$), which could be attributed to the high rate of intestinal hydrolysis once the structure was hydrated. Resistant starch (RS) values were maximum for

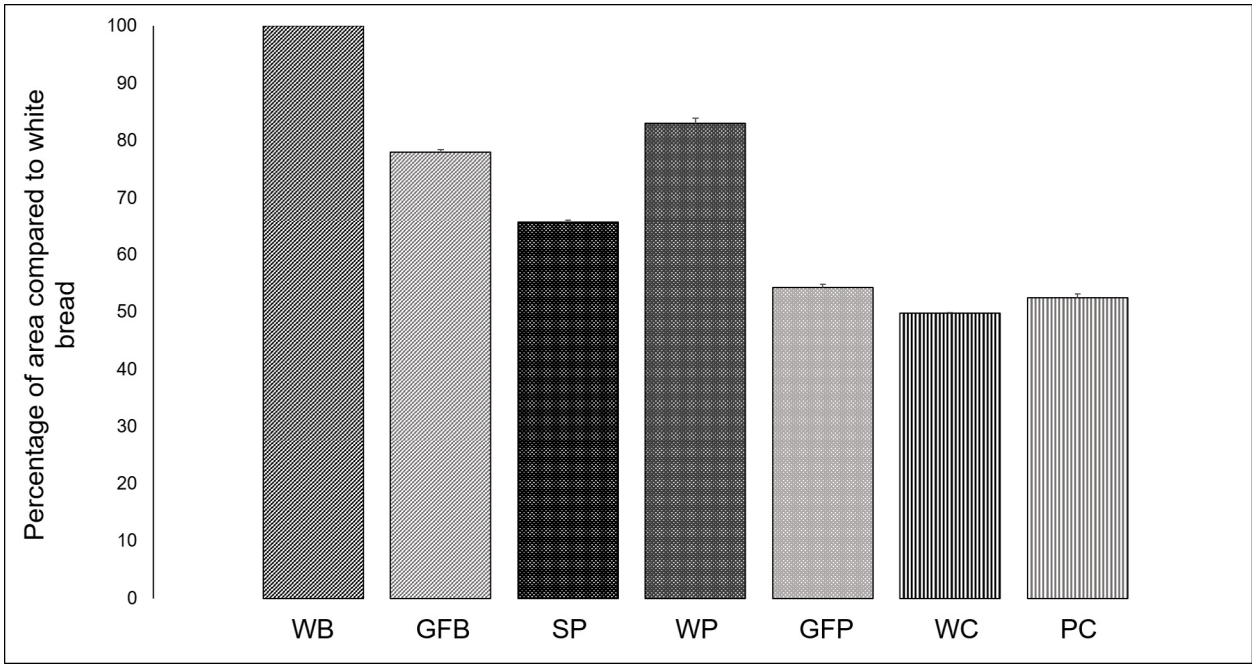

Fig. 3. Relative area under the starch hydrolysis curve considering white bread as 100%.
WB: White bread, GFB: gluten-free bread, SP: sheeted pasta, WP: whole-grain pasta, GFP: gluten-free pasta, WC: whole grain cookie and PC: peach cookie.

gluten-free pasta and cookies according to the low starch *in vitro* digestion observed and the presence of hydrocolloids and lipids (Bharath Kumar and Prabhasankar, 2014) (see Table 2).

Starch in bread was rapidly digested and only less than 20% remains undigested, while in pasta the compact structure delays starch hydrolysis (Bornhorst and Singh, 2012; Zou et al., 2015) and around 20% of starch is slowly digested with a considerable percentage of resistant fraction (except for whole-grain pasta). Cookies presented intermediate levels of rapidly digested starch and a high level of resistant one due to the high levels of sugar and lipids that prevents starch gelatinization, which is the fraction susceptible to enzyme hydrolysis (Agama-Acevedo et al., 2012). In addition, the whole cookie showed higher SDS value and lower RDS and RS in relation to peach cookie, which could be probably ascribed to soluble/insoluble fiber rate in each cookie type (Sozer et al., 2014).

In order to complete the analysis of *in vitro* digestion of starch in cereal matrixes, the area under the starch hydrolysis curve was determined since it could be easily related to the *in vivo* glycemic response of each food being compared with a reference food. Fig. 3 shows the relative area under the starch hydrolysis curve after the mimic digestion of each cereal food product selected in relation to white bread. As described, the delay in starch hydrolysis observed in gluten-free bread that leads to a decrease in rapidly digestible starch and increase of resistant fraction was enough to lower the maltose response *in vitro* around 25%. Pasta product analysis showed that the incorporation of insoluble fiber generates many disruptions in structure becoming easily accessible to enzymes and the decrease in relative curve did not reach 20% compared to that seen in white bread. In addition, the high protein content and hydrocolloids in gluten-free pasta lead to a decrease of almost 50% in relative area. Finally, cookies showed the lowest values according to the low starch hydrolysis determined. It should be noted that cookies also have high sugar and lipid content in formulation, which must be considered when evaluating nutrition quality, despite the fact that whole-grain cookies slightly differ from peach cookie, since bran has a different effect to that shown in pasta products.

2.4.2. Starch bioaccessibility and dialyzability

The rate at which macronutrients are released from food through the disruption of the matrix and the action of enzymes on starch is therefore an important determinant of carbohydrate entry to the portal vein. Most of *in vitro* techniques applied to cereal foods do not include the analysis of dialyzability of starch metabolites. This is probably due to the fact that many authors assume that sugars released from starch hydrolysis are immediately available, without considering that the matrix and other ingredients could affect availability calculates by dialyzability (Parada and Aguilera, 2007).

Fig. 4 shows the fraction of starch that passes throughout the dialysis tube: the dialyzable starch. Additionally, Pearson's correlation analysis was performed between total starch hydrolysis (Table 2) that corresponds to available starch (dialyzable) (Fig. 4) and RDS, SDS and RS fractions (Fig. 3). As a result, rapidly digested starch presented two positive correlations, with available starch (R^2 = 0.89, <0.0001) and with dialyzable starch (R^2 = 0.95, <0.0001). No significant correlation was found for SDS or RS fraction.

Starch dialyzability showed that during the 3-h intestinal digestion of white bread, 38.4% of starch was available as a maltose equivalent, which was significantly higher than its gluten-free counterpart ($P < 0.05$), and in agreement with RDS results. This observation agrees with that reported by other authors who argue that this difference may be associated to the hydrocolloids and proteins included in the gluten-free formulation, slowing down transit through the dialysis tube (Berti et al., 2004). The starch dialyzability in pasta products was significantly different ($P < 0.05$). Whole-grain pasta presented a ~22% higher starch hydrolysis (available) than that in sheeted pasta (Table 2). Yet, the metabolites resulting from starch hydrolysis generated a similar dialyzable quantity (Fig. 4). That means that insoluble fiber from bran fraction in the WP could retard the diffusion of those metabolites (e.g. maltose) (Bornhorst and Singh, 2014). On the other hand, incorporation of peach pulp and inulin in the cookie recipe increased soluble fiber which could lead to decreased bioaccessibility

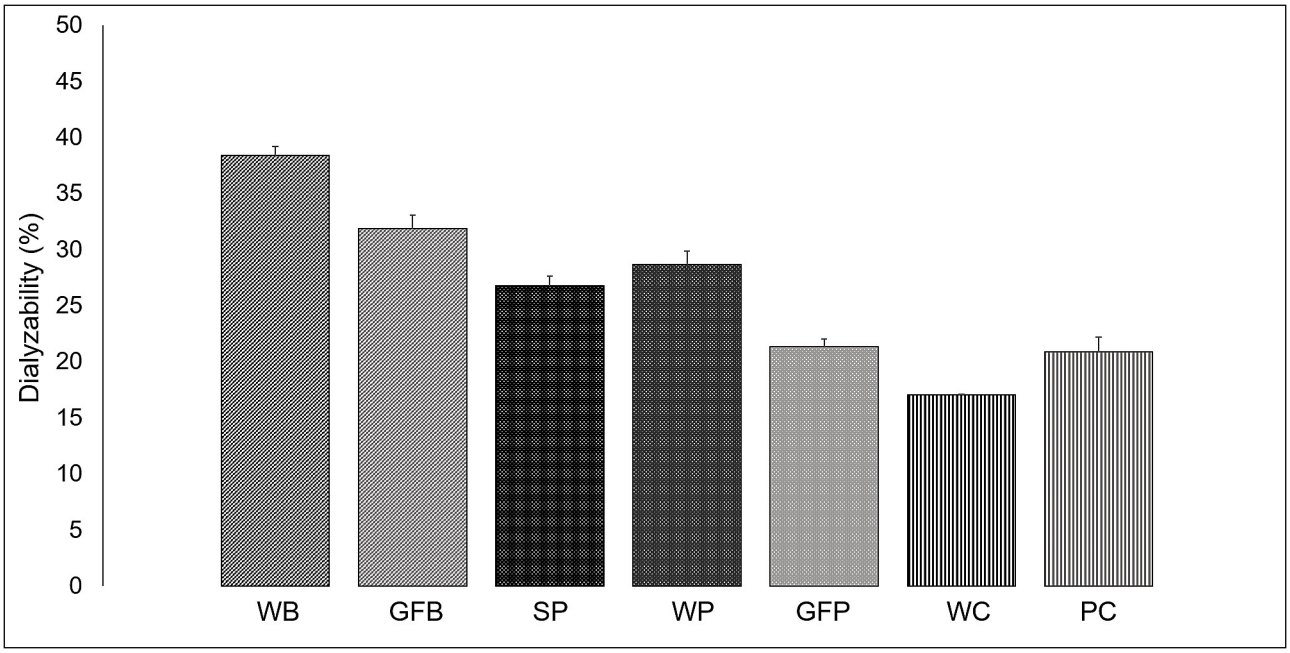

Fig. 4. Starch dialyzability in the cereal food samples tested*.
WB: White bread, GFB: gluten-free bread, SP: sheeted pasta, WP: whole-grain pasta, GFP: gluten-free pasta, WC: whole-grain cookie and PC: peach cookie.

(Table 2), compared to whole-grain cookie, but to an increased dialyzability (Fig. 4), also in agreement with higher RDS values observed for peach cookies as compared with those for whole cookie.

These results show that RDS was probably the most important factor determining dialyzability, meaning that ingredients and process applied to cereal-based foods that disturb the matrix of each product and affect the extent and rate of starch hydrolysis could determine the glycemic response. Despite that, the technique used in this research involves an easy way to screen ingredients effects on the digestion of cereal food products.

2.5. Conclusion

It is well known that *in vitro* methods are unable to reproduce all the conditions found in *in vivo* digestion, hence, their application allows comparison of preliminary results prior to advance into *in vivo* studies, despite being extensively used due to low cost, simplicity, low time-consuming and lack of ethical implications for animal or human testing. Until now, the INFOGEST proposed method needed to be tested in different food matrixes to address specific applications and its ability to reflect differences between cereal products that allow estimation of glycemic response. The application of this method with minor modifications led to three major conclusions. First, throughout the slightly modified INFOGEST protocol applied to cereal products it was possible to study *in vitro* starch digestion kinetics and analyze the effect of processing, food structure, additives and presence or absence of gluten in the three most widely consumed cereal products (bread, pasta and cookies). Second, amylase used at the oral phase in the beginning of the *in vitro* digestion process is still active during gastric conditions, making an important contribution to the study of glycemic responses in products by considering the role of enzymes in each digestion step. Third, the modifications of the original method allowed the analysis of dialyzability of starch that correlated with rapidly digested starch. Exploring the extent and kinetics of carbohydrate digestion is essential for nutrition labeling in cereal food, product development and nutrition research, thus using a single *in vitro* technique is remarkably important.

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