# *IN VITRO* AND *IN VIVO* ASSESSMENT OF THE GLYCEMIC INDEX OF BAKERY PRODUCTS. INFLUENCE OF THE REFORMULATION OF INGREDIENTS.

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## Abstract

*Purpose:* To evaluate if the modification of ingredients of two bakery products, muffins and bread, reduces their glycemic index, by means of *in vitro* and *in vivo* procedures

*Methods*: *In-vitro* and *in-vivo* glycaemic index was evaluated for two types of bread and two types of muffins including one standard product for each category. For the *in-vitro* determination, kinetics of starch digestion method was used. For the *in-vivo* procedure, postprandial glucose measured as IAUC was obtained in a group of eighteen healthy volunteers (ten did the test with muffins and eight with breads).

*Results*: In *in-vitro*, a reduction of the expected glycemic index regarding the control muffin was achieved with the partial substitution of wheat flour by a mixture of resistant starch, dextrin and lentil flour. In breads, with the partial substitution of wheat flour by a mixture of resistant starch and dextrins a decrease in the expected glycemic index was also observed. In *in-vivo*, a reduction of GI was also achieved both in muffin and bread. All the obtained GI were higher in *in-vitro* method.

*Conclusions:* Despite the fact that *in-vitro* overestimate *in-vivo* method, the trend in the reduction of GI seems to be similar in both methods. With the substitution assayed, a reduction of the expected glycemic index and the glycemic index were obtained both in muffins and breads.



## Keywords

Glycemic index, in-vitro, in-vivo, bakery products, starch hydrolysis, blood glucose response.


## Introduction

Type 2 diabetes is one of the commonest lifestyle-related diseases (1). Clinical management of type 2 diabetes requires an adequate selection of carbohydrates. The glycemic index (GI) concept was introduced by Jenkins et al (2) for diabetic patients in 1981 to quantify the glycemic response to carbohydrates in different foods. It is defined as the incremental blood glucose area following the test food, expressed as the percentage of the corresponding area following a carbohydrate equivalent load of a reference product (3). A high-GI food induces a larger area under the glucose curve over the postprandial period when comparing with an equivalent carbohydrate contained in a low-GI food. It was also suggested that reducing the rate of carbohydrate absorption by lowering the GI of the diet could benefit the general population by preventing or delaying the development of diseases that are linked to insulin resistance (4, 5). In large-scale observational studies, diets with the highest average GI were associated with a greater risk of type 2 diabetes (6), coronary heart disease (7) and certain cancers (8, 9). Nowadays there is significant debate about the role of GI for disease prevention or treatment.

It has been argued that the GI values of food intended to form the basis for glycemic control choices, should be based on clinical measurements of the human response to foods. However, since clinical determination is impractical for routine use (10), a promising alternative is *in vitro* digestion. *In vitro* models mimic the physicochemical processes involved in carbohydrate digestion that occur in the upper gastrointestinal tract of humans, and essentially measure carbohydrate hydrolysis as glucose equivalents. They are economical, non-invasive and applicable to large numbers of samples. A universal standardised method has, as yet, not been

adopted from the many available options currently in use (11) and there are large discrepancies about the correlations between *in vitro* and *in vivo* determinations (12, 13). During food product development, *in vitro* measurements of the glycemic potency are recommended (11).

Reducing the glycemic index is of particular interest in frequently-consumed cereal products such as bread and muffins. In general, due to their richness in sugar and white flour, bakery products belong to medium to high glycemic index (GI) categories. Both raw materials and baking processes can therefore influence glycemic response (14). However, the technological role of ingredients in this food group hampers the development of lower GI products. It has been previously showed that it is possible to reduce the GI of bakery products by mixing wheat flour with other types of flours or grains or by adding fibre (15, 16). Resistant starch can also has potential health benefits and functional properties (17). The addition of resistant starch has been studied both in bread and cakes and muffins (18, 19) showing positive effects on texture and slight modifications on sensory characteristics. Legume flours are also interesting candidates to replace wheat flour in bakery products. Legume flours are rich in soluble dietary fibre, with strong interactions between amylose chains (20), leading to lower glycemic indices than cereal or tuber starches. Lentil flour has one of the lowest glycemic indices among pulse flours (21).

The objective of the present study is to evaluate the effects of ingredients reformulation, on reducing the glycemic index of two bakery products. As the GI was determined through *in vitro* and *in vivo* procedures, the agreement of both methods was also assessed.

## Material and methods

### Materials and sample preparation

Two formulae for each product, muffins and breads, were prepared. The most remarkable modifications of ingredients to reduce glycemic index for each sample are shown in Tables 1 (muffins) and 2 (breads).

The control muffin (MC) was based on a commercial recipe. Sample M1 was prepared with a partial replacement of wheat flour by a mixture of 5% resistant starch, 3% dextrins and 7% lentil flour (Los Pisones, Spain). Ingredients were whipped in a mixer (BM11 SAMMIC, Spain). Muffins were baked at 240ºC for 7 minutes.

The control bread (BC) was based on a commercial recipe for long shelf-life bread. B1 was formulated by a mixture of 6% resistant starch (ActiStar 11700, Cargill, France) and 3% dextrins (Nutriose FB06, Roquette, France).

Bread was fermented for 90 minutes at 30ºC and a relative humidity of 80% in a proofer (IVERPAN FC-18/00, SALVA, Spain) and baked for 20 minutes at 180ºC in a conventional industrial oven (LT-4+H Oven, SALVA, Spain).

All the samples were cooled at room temperature for an hour and then wrapped in a plastic film until *in vitro* and *in vivo* analysis sampling.

## The *In vitro* study

### *In vitro* starch hydrolysis

A previously reported *in vitro* method (22) with slight modifications was used. The aim of the *in vitro* starch hydrolysis was to simulate the *in vivo* procedure. Samples were formerly dehydrated and those with more than 5% of fat (muffins) were defatted using Soxtec extraction with petroleum ether (SOXTEC System 2055, Foss, Denmark) (23). The oral phase was simulated by means of mechanical disaggregation through an 8 mm plate (Model MG450, Kenwood, North Ryde, NSW)) of 50 mg food portions. The gastric phase was developed for 1 hour at 40 ºC with 10 ml of HCl-KCl buffer pH=l.5 and pepsin (Sigma P-7000). The intestinal phase was carried out in sodium potassium phosphate buffer 0.05 M pH 6.9 containing pancreatic amylase (Sigma A3176). Samples were then incubated at 37°C in a shaking water bath. 0.2 ml aliquot samples were taken from each tube at 0, 30, 60, 90, 120 and 180 minutes and then immediately analyzed for reducing sugars. This was done using 3, 5-dinitrosalicylic acid method using a glucose standard curve patron. The glucose was converted into starch by multiplying by 0.9. Samples were analyzed in triplicate on two different days, obtaining 6 replicates for each sample. Commercial white bread (WB) was also analyzed as reference product.

A non-linear model established by Goñi et al. (22) was applied to describe the kinetics of starch hydrolysis. The area under the hydrolysis curve (AUC) was calculated using the following equation:

$$AUC = C_{\infty}\,(t_f - t_0) - \left(\frac{C_{\infty}}{k}\right)\left[1 - e^{-k(t_f - t_o)}\right]$$

$C_{\infty}$ corresponds to the concentration at equilibrium ($t_{180}$), $t_f$ is the final time (180 min), $t_0$ is the initial time (0 min) and k is the kinetic constant. The calculated hydrolysis index (calcHI) was obtained by dividing the area under the hydrolysis curve of the sample by the area obtained for white bread (WB). The expected glycemic index (eGI) was calculated using the equation described by Granfeldt et al. (24).

$$eGI_{HI} = 0.862 \times calcHI + 8.198$$

## The *In-vivo* study

The procedures for the determination of the glycemic index (GI) in food products were based on the ISO/DIS 26642 provided by the Spanish Association of Standardisation and Certification (AENOR) (25) and also from the reviewed literature (13, 26, 27).

### Subjects

Between April and June 2009, twenty-two subjects were recruited as volunteers for the *in-vivo* glycemic index study in the city of Zaragoza (Spain). The inclusion criteria were that subjects should be healthy males or females, aged between 18 and 40 years old. The exclusion criteria were the use of medications affecting glucose tolerance (excluding oral contraceptives), the presence of diseases or drugs influencing digestion or absorption of nutrients, major medical or surgical events requiring hospitalisation within the past 3 months, known diabetes mellitus and related conditions and food allergy or intolerance. Each subject gave written informed consent for

the study, and approval was obtained from the Ethical Committee of Clinical Research of Aragón (CEICA). Subject identification numbers were assigned sequentially in the order in which participants were enrolled.

The weights and heights of subjects were obtained using standardised procedures.

Due to the abnormal results obtained in the first oral glucose tolerance test (50g of anhydrous glucose), three subjects were excluded and the final number was nineteen. Ten subjects participated in the test of muffins and nine in the testing of bread. One of the subjects who participated in bread testing was excluded as a result of the analysis of GI of the modified bread 1 because the value obtained for the GI was unusually high (>2SD) (26). Finally, eighteen young healthy subjects (4 males and 14 females) were included for further analysis (27.0 ± 4.63 years; 22.68 ± 2.93 kg/$m^2$ of BMI; 1.74 ± 0.19 $m^2$ of body surface using the Mosteller formula).

**Test meals**

Each subject completed the set of five visits: two days to obtain the IAUC (Incremental Area under the Curve) for the reference food (50g of anhydrous glucose) and three more for testing the products. The test meals consisted of two types of muffins and two types of bread containing 50g of available glycemic carbohydrate, defined as total carbohydrate minus dietary fibre. The final edible amount of each test product was: 110 g of MC, 133g of M1, 113 g of BC and 129 g of B1. Test products were consumed with a maximum of 500 ml of water. Each product was compared with the results obtained from the reference food. Incremental area under the curve was calculated for each test product using the trapezoidal method and compared with the average AUC (n=2) for the reference food in each subject. Any area under the baseline (fasting point) was ignored. Participants were asked to fast 12 hours before the analysis. The GI was determined as the mean ± SE in 10-8 different subjects and was calculated by dividing the AUC obtained after the tested products by the AUC obtained after glucose intake as previously described**.** Therefore, the GI of any one food was based on between 160-128 separate glucose determinations (10-8 subjects, 8 time points, 2 reference food tests and 1 food test). Blood glucose points were determined in a Clinical Analysis Laboratory by electrochemical detection coupled enzyme system (25).

## Data analysis

Data was analyzed using SPSS v.16 for the *in-vivo* method. Results of GI are expressed as means, SD (standard deviation) and SEM (standard error of the mean). The coefficients of variation (CV=100xmean/SD) for the AUC (Area Under the Curve) and for the GI were calculated. The CV of AUC of the two tests with the reference food (anhydrous glucose) to check the intra-individual variation, and the CV of the GI for each product to check the inter-individual variability. A paired t-test was applied to look for differences between the first and the second test with the reference food (anhydrous glucose) in all the subjects and also to know whether obtained differences in GI between products were significant. A T-test was applied to search for differences between weight status regarding the obtained GI. Correlations were performed to search for relations between BMI or body surface area and obtained GI. To assess the agreement between *in vitro* and *in*

*vivo* methods, an adaptation of Bland-Altman scatter plot was used. Instead of using the average value of *in vitro* and *in vivo* measurements, only the *in vivo* values were used, as this is the reference method. Analysis was conducted at the 0.05α level.

## Results

**The *In vitro* study**

Table 3 shows the equilibrium constant (C∞), the kinetic constant (k), the calculated hydrolysis index (calcHI), and the estimated glycemic index (eGI) for muffins and breads. In muffins, partial replacement of wheat flour by a mixture of resistant starch, dextrins and lentil flour (M1) slightly reduced the eGI (68.1± 1.6 vs. 64.5 ±1.8). In breads, partial replacement of wheat flour by a mixture of resistant starch and dextrin (B1) slightly reduced the eGI (82.2 ± 2.9 vs.76.4 ± 2.3). Calculated HI values for muffins and bread samples showed similar trends to estimated glycemic index.

Figure 1 shows the AUCs of digested starch over 3 hours for the test products. The AUCs were significantly lower for the bread and muffins than for white bread. Muffins showed lower AUCs than breads.

**The *In vivo* study**

Obtained GI and AUC were normal distributed. Mean AUC and subsequent GI values for food products with their standard deviations, standard errors and coefficients of variation (CV) are shown in Table 4. The mean within-subject CV (±SEM) for the 2 tests of the reference food were 40.33 ± 9.56% for the subjects who made this study with muffins and 26.76 ± 6.47% for the subjects who tested bread. There were no significant differences between the first and the second measures.

A reduction of GI was obtained for muffins and breads regarding the control products. Control muffin showed a GI of 62.70. With the substitution assayed, a GI of 39.14 was measured. For breads, it was also observed a reduction in GI from 64.39 (BC) to 59.91 (B1).

The CV of GI for muffins was in the range from 69.70% for M1 to 79.01 % for the MC; and for bread, from 31.33 % for B1 to 45.77 % for BC.

When the t-test was applied, no significant differences were noted in the obtained GI values for any of the products, whether muffin or bread, between overweight or non-overweight and male or female subjects (results not shown). There was no correlation between the body mass index or body surface area and the obtained GI for any of the products, regardless of the product.

Bland-Altman figures were used to determine the difference in the obtained GI between the two methods (Fig. 2). It showed a disagreement between them: glycemic indices obtained through *in vitro* analysis were higher than those obtained through *in vivo* analysis for breads and muffins.

## Discussion

Our results show the lack of a strong correlation between the results obtained by means of *in-vitro* and *in-vivo* methods, probably explained by both the characteristics and limitations of both methods and the complex matrix characteristics of the bakery products.

Matrix could influence the starch hydrolysis index. The glycemic effect of foods depend on the food texture and particle size (28), type of starch (the amylose:amylopectin ratio) (29), the physical entrapment of starch molecules within food, food processing and other ingredients, such as sugars, fat, protein, dietary fibre and anti-nutrient content (30-32).

For the determination of *in vitro* GI, procedural analysis was based on starch hydrolysis and the analysis of reducing sugars. *In vitro* determination to evaluate the effect of ingredients substitution on the glycemic index has been previously described by other researchers but there is no standardised methodology for the determination of carbohydrate digestion rate (22, 24, 33). Sample preparation is different in each particular method. Hydrolysis may be performed under unrestricted or restricted (dialysis) conditions. The sample may be broken down by milling or by using subjects to chew the samples. Recent studies concluded that a non-restricted (test tube) mincing method showed good potential as a new *in vitro* starch digestibility method for predicting GI in grain foods (33).

Other source of variation regarding in vitro methods is the use of proteolytic enzymes in combination with amylases in different combinations and concentrations (22, 24, 33). Also the time point from the hydrolysis curve to establish the relationship with in vivo data was different from one method to another being 90 min the time of preference in most studies. However, all methods measured the starch hydrolysis from 0 to 180 minutes.

Besides, the hydrolysis index measured using in vitro methods is then transformed into estimated GI by using different empiric equations.

The values of eGI obtained for control bread (82.2) were similar that the values obtained by other authors (70, 65.31) (33, 34). Muffins have been less studied, although there are studies on bakery products like conventional cakes with similar eGI values (23). Moreover, eGI values were in the range of *in vivo* data (35) for both bread (62 to 95) and muffins (44 to 102).

When a mixture of resistant starch and dextrins was added both to bread and muffins at low percentage, a little reduction of the calcHI and consequently eGI was achieved. Resistant starch is known to exert a similar effect to some dietary fibres by increasing the indigestible carbohydrate portion in small and large intestines (36), thus reducing the rate of dietary carbohydrate absorption. Some authors (15, 29, 36-39) observed a diminution of the starch hydrolysis rate *in vitro* when high contents of resistant starch were added. In summary, according to the results obtained by means of the *in vitro* method, the joint addition of several ingredients with reducing GI properties, added in low percentages, show a decreasing tendency.

Regarding *in vivo* determination, results suggested a GI reduction achieved through the reformulation of every product. In an *in vivo* study in which white kidney bean extract was added in bread in different amounts and formats, significant reductions were not observed in all the studied formulae (40). However, the GI of all the bread in our study are included in the range of values obtained in other studies for white bread as well as their

CV(41). Sample size characteristics could be another important explanation. According to FAO/WHO/ISO standards (25), at least 10 subjects are necessary to develop these tests; in the case of the bread test, only eight subjects were finally used. However, published material highlights other studies which reported a similar sample size (42). Another possible limitation could be gender imbalance, as the difference in our sample is quite considerable (14 females against 4 males); however, material from other studies shows that there are no significant differences between genders (13, 43). Additional subject characteristics did not appear to have a significant effect on mean GI values which have already been described (13) as they were also observed in our study.

Another important issue was the intra- and inter-subject variability obtained in the assessment of the GI of the products *in-vivo*, similar to those previously reported (26, 41). This variability has been largely discussed in existing published material. One of the points mentioned is the use of venous blood instead of capillary blood. Procedures recommended by FAO/WHO (1998) allow variation in blood sampling. However, current recommendations are that capillary blood is preferred in determining GI because the oscillations in venous blood are higher than in capillary blood. Factors like stress, recent exercise, alcohol consumption and smoking habits have been shown to impair glucose tolerance and insulin sensitivity (44); length of fasting time, and previous meal consumption can also influence this variability (45) and that simply advising subjects to avoid certain types of foods is cost-effective (46). In our study, these factors were not controlled. However, there are some studies which concluded that in practice, there is no need for rigorous control of exercise, smoking or diet the day before the test (13, 44).

The fact that we only performed twice the tests with the reference foods (anhydrous glucose) could be considered as a limitation as well. Nevertheless, a recent study suggested that there is no evidence to justify repeating it three times rather than two, because the differences were small and not significant (13, 43). However, it is probable that if we had performed the test 3 times with the reference food, we would have obtained shorter within-person coefficients of variation.

Concerning between-person coefficients of variation, muffins had higher mean CV. One explanation could be that muffins have more proportion of other macronutrients which could potentially reduced the postprandial glycaemic responses (47), although the measurement of GI of the carbohydrate has been shown to be reliable in mixed meals (40, 43).

As an indication of the veracity of this study, standardised strategies were employed to minimise inter- and intra-subject variability, including the restriction of the inclusion criteria to certain age and health status. The glycemic index value determinations were assessed following a protocol that was as consistent as possible among volunteers.
The obtained GI values for the modified muffin and bread were lower than the control products using both *in vitro* and *in vivo* methods.

Despite the fact that the trend toward GI reduction seems to be similar for both *in vitro* and *in vivo* methods, the Bland-Altman plot shows important differences between both approaches. The *in vitro* method overestimated the GI obtained with the *in vivo* method (reference test). This overestimation is lower if the measured GI measured in *in-vivo* is higher. Some researchers have found correlations between the rate of *in vitro* glucose release from starchy foods using pancreatic and brush-border enzymes and the glycemic response *in vivo* (13).

However, associations between *in vivo* and *in vitro* results have not been consistently found (48). Physiological factors (gastrointestinal function, glucose tolerance, the rate of food consumption) and meal factors (physical form, other nutrients) can confound the relationship (Glycemic index and health, 2001). Given the large variation between subjects and between methods in values of glycaemic index for bakery products seems adequate to suggest the need of standardization regarding in vitro methods and the combined use of both in vivo and in vitro analysis. In vitro methodology could be used in a previous screening phase in the development of a product although in vivo method would be necessary to determinate the GI value for the labelling.

Low-GI diets may have implications in the prevention and management of chronic diseases like type 2 diabetes, coronary heart disease or some cancers. Published material suggests that the recommendations of low-fat and high-carbohydrate diets could be implemented with low-GI food choices (4). For this reason, information on glycemic responses of foods can be used in fine-tuning glycemic control.

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## Conflict of interest

The authors declare no conflict of interest in the carrying out of the study and the writing of the manuscript.

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Table 1 Muffins formulations showing major ingredients components and type of substitution (% on flour basis)

| Muffins | | |
|---|---|---|
| Ingredients | MC | M1 |
| Sucrose | 23 | 23 |
| Soft Wheat flour | 29 | 14 |
| Resistant starch | - | 5 |
| Dextrins | - | 3 |
| Lentil Flour | - | 7 |

MC (Control muffin), M1 (Muffin 1)

Table 2 Breads formulations showing major ingredients components and type of substitution (% on flour basis)

| Bread | | |
|---|---|---|
| Ingredients | BC | B1 |
| Forced wheat flour | 36 | 30 |
| Soft wheat flour | 24 | 20 |
| Rye flour | - | - |
| Resistant starch | - | 6 |
| Dextrins | - | 3 |

BC (Control bread), B1 (Bread 1)

Table 3 Concentration at equilibrium ($t_{180}$), Kinetic constant (k), Calculated hydrolysis index (calcHI), and Expected glycemic index (eGI) for all types of muffins and breads.

| **Sample** | **C∞** | **k** | **calcHI** | **eGI** |
|---|---|---|---|---|
| **Mean MC** | 29.8 | 0.25 | 69.5 | 68.1 |
| **SEM** | 0.84 | 0.03 | 1.8 | 1.6 |
| **Mean M1** | 28.5 | 0.13 | 65.3 | 64.5 |
| **SEM** | 0.98 | 0.01 | 2.1 | 1.8 |
| **Mean BC** | 36.4 | 0.35 c | 85.8 | 82.2 |
| **SEM** | 1.44 | 0.01 | 3.4 | 2.9 |
| **Mean B1** | 33.6 | 0.32 b | 79.1 | 76.4 |
| **SEM** | 1.13 | 0.01 | 2.6 | 2.3 |

MC (Control muffin), M1 (Muffin 1), BC (Control bread), B1 (Bread 1), B2
Within each column, mean data with the same letter are not significantly different at the P<0.05 level

Table 4 Glycemic index values and AUC for all types of muffins and breads and for the reference food (based in the results of eight subjects in case of muffins tests and in ten subjects in case of breads tests).

| Muffins | Mean | Standard error | Standard deviation | Coefficient of variation |
|---|---|---|---|---|
| Glucose 1 (AUC[a]) | 170.92 | 27.56 | 87.14 | 50.98 % |
| Glucose 2 (AUC) | 167.74 | 28.70 | 90.76 | 54.11 % |
| MC[b] (AUC) | 89.86 | 14.11 | 44.63 | 49.67 % |
| M1[c] (AUC) | 61.88 | 9.73 | 30.78 | 49.74 % |
| GI[d] MC | 62.70 | 15.67 | 49.54 | 79.01 % |
| GI M1 | 39.14 | 5.76 | 18.22 | 46.55 % |

| Bread | Mean | Standard error | Standard deviation | Coefficient of variation |
|---|---|---|---|---|
| Glucose 1 (AUC) | 122.86 | 25.03 | 75.08 | 61.11 % |
| Glucose 2 (AUC) | 155.11 | 31.58 | 94.75 | 61.09 % |
| BC[e] (AUC) | 89.22 | 22.16 | 66.48 | 74.51 % |
| B1[f] (AUC) | 85.12 | 13.34 | 37.74 | 44.34 % |
| GI BC | 64.39 | 9.82 | 29.47 | 45.77 % |
| GI B1 | 59.91 | 6.64 | 18.77 | 31.33 % |

[a] Area Under the Curve
[b] Control Muffin
[c] Muffin 1
[d] Glycemic index
[e] Control Bread
[f] Bread 1

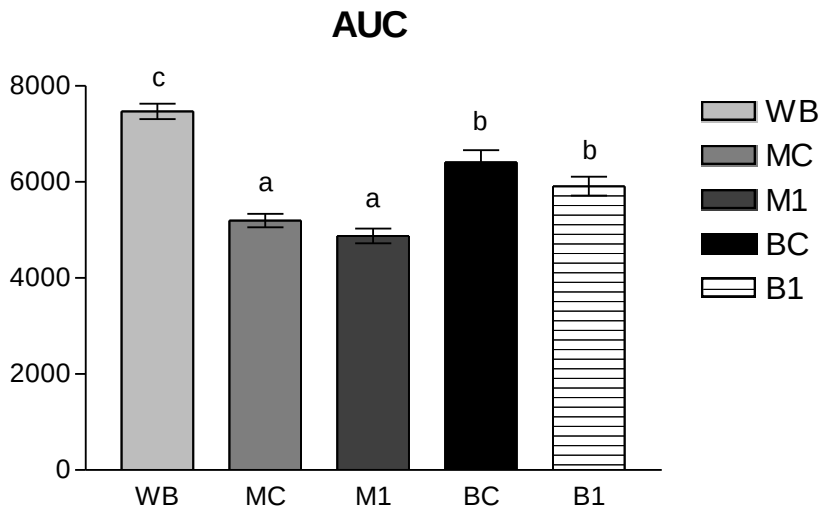
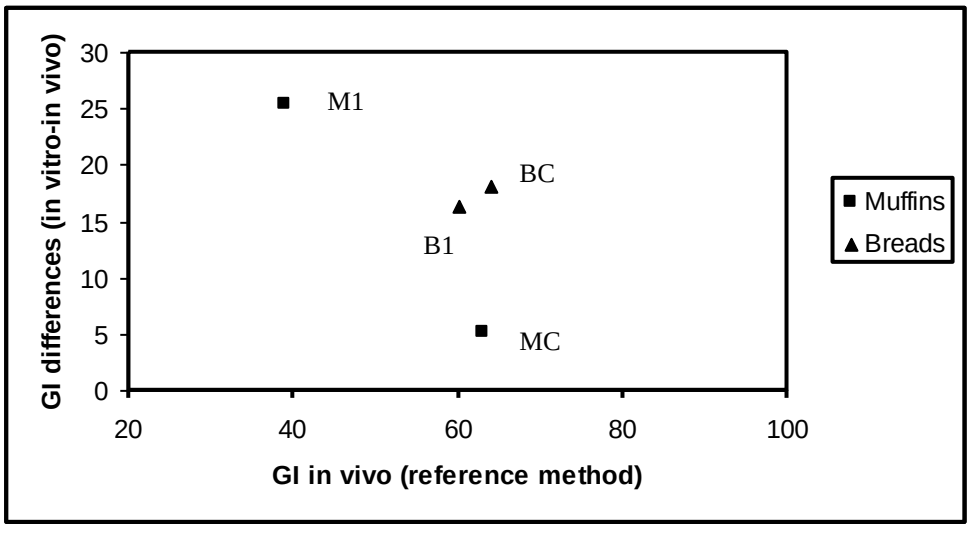



Fig 1. Area Under the Curve for the *in vitro* starch hydrolysis at 180 minutes for all types of muffins and breads. WB (White Bread. Reference product), MC (Control muffin), M1 (Muffin 1), BC (Control bread), B1 (Bread 1).
Data with the same letter are not significantly different at the P<0.05 level.

Fig 2. Difference in GI values obtained from *in vitro* and *in vivo* analysis. Adaptation of Bland-Altman method




MC: Control Muffin
M1: Muffin 1
BC: Control Bread
B1: Bread 1

1