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Title: Effect of pasteurization on in vitro α-glucosidase inhibitory activity of apple juice

Article Type: Research paper

Keywords: Apple juice; α-Glucosidase inhibition; Pasteurization; Type 2 diabetes; Acarbose

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Abstract: The in vitro α-glucosidase inhibitory activity of raw, mildly (F_71.7^5=0.4 min, 5 Log reductions of Cryptosporidium parvum) and intensively (F_90^12=14.8 min, 2 Log reductions of Alicyclobacillus acidoterrestris) pasteurized apple juice was studied. Raw apple juice (23 mgdw/mL) caused 90% alpha-glucosidase inhibition. Analogous results were obtained for the mildly treated sample. The most intense treatment reduced by 35% the alpha-glucosidase inhibition. However, such a decrease was associated with an increase in the phenolic content, suggesting that alpha-glucosidase inhibition might not rely on these compounds, but depend on more complex mechanisms. Apple juice was combined with acarbose to investigate their interaction towards alpha-glucosidase inhibition. A synergistic behavior was observed for concentrations < 2 mg/mL. Increasing the concentration of the combined system (up to 9 mg/mL) produced an antagonistic effect, while a further increase (< 9 mg/mL) allowed approaching an addictive behavior.

Dear Professor Singh,

I would like to submit the revised manuscript entitled "Effect of pasteurization on *in vitro* α-glucosidase inhibitory activity of apple juice" by Marilisa Alongi, Giancarlo Verardo, Andrea Gorassini and Monica Anese for consideration for publication in LWT - Food Science and Technology.

We are glad to hear that the Reviewers found our manuscript worthy of publication unless we answer their questions. We have endeavoured to take into account or to respond to the reviewers' comments as indicated below. We hope that this response is satisfactory, and that the manuscript will be suitable for publication in the LWT- Food Science and Technology.

Best regards

Monica Anese

***Detailed Response to Reviewers**

Answers to Reviewer's comments

(Reviewer text is normal and *Answer text* is in italics, for each numbered item)

Reviewer #1:

1. There were some publications on the inhibitory activity of α-glucosidase by using apple extract or some phenolic compounds from apple, and this may be the potential to reduce the risk of type 2 diabetes. However, apple juice contains high amount of sugars (around 10%), which could increase the risk of type 2 diabetes.

   The ability of apples and their derivatives in facing type 2 diabetes has been reported in the literature, independently from their sugar content (Boyer & Liu, 2004). Nonetheless, the latter should be taken into account when designing foods aimed at reducing type 2 diabetes incidence. These considerations were further implemented in the conclusion (lines 349-350).

2. Line 181-182, the concentration of apple juice is difficult to understand, and dilution of the apple juice should be added.

   Apple juice was tested for the inhibitory activity against α-glucosidase considering increasing concentrations, ranging from 0 to 23 mg_{dw}/mL. Details were added in the text (lines 190-191).

3. Line 262-296, and Table 2. There is a long discussion on the phenolic compounds of the apple juice. However, it seems that this part is not relevant to the aim of this study.

   The discussion of these part was reduced as suggested by the Reviewer (lines 283-286).

4. Line 233-237, "fructose and sucrose in P90 sample were approximately 30 and 60% less concentrated than those found in the control. This reduction can be attributed to the sugars consumption as reagents of the Maillard reaction." Results from similar studies should be cited here to support the discussion.

   References were added as recommended by the Reviewer (lines 244).

5. Other comments, please find in the attached pdf document.

   The text was modified as suggested by the Reviewer in the other comments and changes were highlighted.
   Regarding the phenolic composition, reference for HPLC-DAD-ESI-MS^n analysis was added (line 134). However, no references were specified for SPE purification and the use of calibration curves for the quantification of identified compounds because these are widely applied procedures in the analysis of food products.

Reviewer #2:

1. Line 17-18: Please rewrite the sentence to make it more readable.

The sentence was rewritten.

2. Most referred articles were published years ago. If possible, could you please present more latest research progress related to your study?

More recent research papers relevant to the present study were added as suggested by the Reviewer.

3. Some grammatical errors need to be corrected.

The grammatic has been revised as suggested by the Reviewer.

4. Please justify the interaction study, elucidate the reason why acarbose was chosen in this study, why the investigation of the interaction between apple juice and acarbose was conducted, and any previous studies about juice + acarbose were carried out?

Requested information was added (lines 58-63).

5. Line 86-87: How to determine the location of the coldest point of the sample?

This information was reported in the text (lines 90).

6. Please detail the procedure of color analysis: any container? container dimension? How did you measure the liquid with a colorimeter?

Further details on colorimetric analysis was added (lines 181-182).

7. Table1: Variation of sucrose content in raw juice is fairly big. Please present your apple sampling procedures for juice extraction. Were all apples used in this study purchased at the same time? How many apples were used for each replicate of juice?

More details relevant to apple sampling procedure were added in the text (lines 77-78 and 81-82). We agree with the Reviewer in that the raw sample showed a high variation of sugar content. However, we preferred to keep all the data to compute the mean value.

8. Figure 2: I suggest you using different dash patterns of the lines for different samples.

The figure was modified as suggested by the Reviewer.

Editorial:

1. "v/v" is undefined symbol, change it to "mL: mL"

Changes were made, as indicated by the Editor.

2. Line 121 and other places: The % unit for concentration of acid should be changed to directly measured unit of mL/L, g/100mL or mol/L as appropriate.

Changes were made.

3. Fig 2: The key of symbols should be in the caption's text.

Change were made.

Highlights

Apple juice inhibited α-glucosidase in a concentration-dependent way

Conventional pasteurization did not affect the α-glucosidase inhibitory capacity of apple juice

Severe pasteurization reduced by 35% the apple juice inhibitory capacity

Apple juice-acarbose system played a synergistic effect up to 40% α-glucosidase inhibition

Effect of pasteurization on *in vitro* α-glucosidase inhibitory activity of apple juice

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Abstract

The in vitro α-glucosidase inhibitory activity of raw, mildly ($F_{71.7}^{5}$=0.4 min, 5 Log reductions of *Cryptosporidium parvum*) and intensively (F_{90}^{12}=14.8 min, 2 Log reductions of *Alicyclobacillus acidoterrestris*) pasteurized apple juice was studied. Raw apple juice (23 mg_{dw}/mL) caused 90% α-glucosidase inhibition. Analogous results were obtained for the mildly treated sample. The most intense treatment reduced by 35% the α-glucosidase inhibition. However, such a decrease was associated with an increase in the phenolic content, suggesting that α-glucosidase inhibition might not rely on these compounds, but depend on more complex mechanisms. Apple juice was combined with acarbose to investigate their interaction towards α-glucosidase inhibition. A synergistic behavior was observed for concentrations < 2 mg/mL. Increasing the concentration of the combined system (up to 9 mg/mL) produced an antagonistic effect, while a further increase (< 9 mg/mL) allowed approaching an addictive behavior.

Keywords: Apple juice; *α*-Glucosidase inhibition; Pasteurization; Type 2 diabetes; Acarbose

1. Introduction

Apple consumption is known to reduce the risk of chronic diseases, such as cancer, cardiovascular diseases and type 2 diabetes (Boyer & Liu, 2004; Guo, Yang, Tang, Jiang, & Li, 2017). The protective effect has mainly been attributed to polyphenols, and in particular to the chemical families of flavones (e.g. luteolin, apigenin), flavonols (e.g. quercetin, kaempferol), flavanols (e.g. catechin, epicatechin), hydroxycinnamic acids (e.g. chlorogenic acid) and anthocyanidins (Boyer & Liu, 2004; Hanhineva *et al.*, 2010; Shoji *et al.*, 2017). Apple phytochemicals affect carbohydrate metabolism and glucose homeostasis at different sites (Hanhineva *et al.*, 2010). Individual phenolic compounds (e.g. catechol, catechin, chlorogenic, ferulic and caffeic acid) extracted from apple reduced intestinal glucose uptake through SGLT1 transporter inhibition (Schulze *et al.*, 2014). Some phenolic compounds also inhibited the enzyme α-glucosidase, which plays a key role during carbohydrates digestion (Agustinah, Sarkar, Woods, & Shetty, 2016; Tadera, 2006). Bortolotto and Piangiolino (2013) reported that an apple extract inhibited the activity of α-amylase and α-glucosidase by 70% and 90%, respectively. Despite these studies showed the potential of apples in facing the risk of type 2 diabetes, the relationship between the whole fruit intake and the reduced diabetes risk has not been fully elucidated yet. Most effects have actually been demonstrated on simplified systems obtained upon extraction of bioactive compounds from the original matrix (Williamson, 2013).

Apple juice is the most consumed apple derivative since more than 20% of freshly harvested apples are consumed as juice (Schulze *et al.*, 2014). Apple juice production implies several technological interventions, among which are skin and pomace removal, enzymatic depectinization, and pasteurization. These technological treatments, which are intended to improve the stability of fruit and vegetable derivatives, significantly affect the phenolic content of the final product (Van Buren, De Vos, & Pilnik, 1976; Schulze *et al.*, 2014) and thus its potential health benefits. To our knowledge, no data regarding the effect of pasteurization on the ability of apple juice to inhibit α-

glucosidase are available. Therefore, the aim of the present study was to investigate the effect of pasteurization on the *in vitro* inhibitory activity of apple juice against α-glucosidase. Apple juice was subjected to a conventional thermal treatment to obtain 5 Log reductions of *Cryptosporidium parvum* (FDA, 2004), or to a more intense pasteurization to achieve 2 Log reductions of *Alicyclobacillus acidoterrestris* (Silva & Gibbs, 2001). Further, since the drugs currently used to treat type 2 diabetes often carry undesired side effects (Kumar & Sinha, 2012), for the first time the interaction between apple juice and acarbose was studied. Acarbose was chosen because it is widely used as a therapy for type 2 diabetes. The purpose was to understand whether the combination of juice and acarbose might allow drug dosage reduction while keeping the efficacy against α-glucosidase.

2. Materials and methods

2.1.Chemicals and materials

Methanol (MeOH), formic acid (HCOOH), fructose, glucose, sucrose, (+)-catechin, (–)-epicatechin, chlorogenic acid, phloridzin, phloretin, 3-hydroxycinnamic acid (internal standard; I.S.), α-glucosidase, 4-nitrophenyl-α-D-glucopyranoside, and acarbose were purchased from Sigma-Aldrich (Milan, Italy). Quercetin-3-*O*-galactoside, procyanidin B2, and epigallocatechin gallate were obtained from ExtraSynthese (Lyon, France). Quercetin-3-*O*-arabinoside and quercetin-3-*O*-rhamnoside were purchased from Carbosynth (Berkshire, UK). Milli-Q grade water was produced by Elgastat UHQ-PS system (ELGA, High Wycombe Bucks, UK).

Solid phase extraction (SPE) columns ISOLUTE C18, 1 g, 6 mL were from Biotage (Milan, Italy).

2.2.Sample preparation

A 10 kg batch of apples (Malus domestica Borkh., cv. Golden Delicious) were purchased at the local market and maintained at 7 °C until use. Apples were washed, wiped and the juice was

extracted (Ariston Hotpoint Slow Juicer, Fabriano, Italy) at 4 °C to minimize enzymatic browning. The juice was centrifuged at 5000 *g* for 5 min at 4 °C (Beckman Avanti J-25 Beckman Instruments Inc., Palo Alto, CA, USA) and filtered through filter paper. Approximately 1.5 kg of apples were used for each replicate. Ten mL aliquots of apple juice were poured into 20 mL capacity glass vials (Vetrotecnica, Padova, Italy), which were closed with screw caps and kept refrigerated. Samples were subjected to technological treatments within 10 min after preparation.

2.3.Pasteurization

Thermal treatments were performed in a silicone oil bath (Haake Phoenix B5, Thermo Electron Co., Karlsruhe, Germany). Samples were pasteurized by applying two different time-temperature combinations. A copper-constantan thermocouple probe (Ellab, Denmark), whose tip (2.0 mm) was placed in the coldest point of the sample (i.e. at two-thirds of depth in glass vials), measured temperature changes of apple juice during pasteurization. The thermal effect *F* (min) was computed using Equation 1 (Ball, 1923):

$$F = \int_0^t 10^{(T-T_{ref})/z} \cdot dt \quad (1)$$

where T_{ref} is the reference temperature, *T* is the actual temperature of the treatment (°C), *t* is the time (min). The first treatment ($P_{71.7}$) provided a sterilizing equivalent to 0.4 min at 71.7 °C and aimed at achieving 5 Log reductions of *Cryptosporidium parvum* ($D_{71.7}$=3 s and z=5 °C) (FDA, 2004). The second treatment (P_{90}) provided a sterilizing equivalent to 14.8 min at 90 °C and aimed at reducing by 2 Log *Alicyclobacillus acidoterrestris* (D_{90}=7.4 min and z=12 °C) (Silva & Gibbs, 2001). After treatment, the samples were rapidly cooled in a spray of water until they reached a temperature of approximately 30 °C. Apple juice not subjected to heat treatment was taken as a control.

2.4.Total solid content and pH

The total solid content was measured by a gravimetric method (AOAC Official Method 925.009 (1995). pH was measured by a pHmeter (HANNA Instruments, pH 301, Padova, Italy).

2.5. Sugar content

The method by Englyst, Englyst, Hudson, Cole, & Cummings (1999) was followed, upon slight changes. Apple juice was mixed with methanol (1:5, mL: mL), left at room temperature for 1 h and centrifuged at 4000 *g* for 10 min at 4 °C (Beckman Avanti tm J-25, Beckman Instruments Inc., Palo Alto, CA, USA). The supernatant was analyzed using an HPLC pump (LC-10AT VP, Shimadzu, Japan) equipped with a refractive index detector (RID-10A, Shimadzu, Japan). An inverse phase apolar C18 column (5 µm, 250 x 4.6 mm) was used (Grace Davison Discovery Sciences, Alltima, Lokeren Belgium). The injection valve (Rheodyne, Sigma-Aldrich, Milano, Italia) was equipped with a 20 µL plastic loop and samples were injected using a syringe (SGE LC, 100 µL, FN). The mobile phase was represented by acetonitrile and deionized water (70:30, mL: mL) and 1.3 mL/min flow rate was applied. Quantitative analysis of sugars was carried out by comparing the sugar peak area with the results of calibration lines obtained by injecting fructose, glucose, and sucrose standard solutions serially diluted. Calibration lines were linear ($R^2 > 0.995$) in the 1.0 to 250.0 g/L concentration interval.

2.6.Phenolic composition

2.6.1. SPE purification

Ten µL 3-hydroxycinnamic acid (50 µg/mL) methanolic solution as internal standard and 1 mL juice was diluted with 2 mL deionized water and loaded on a C18 SPE column previously conditioned with 5 mL of 2 mL/L formic acid in methanol and 5 mL of 20 mL/L formic acid in water.

After loading, the column was washed with 10 mL of 20 mL/L formic acid in water and the phenolic fraction was eluted with 5 mL methanol. The solvent was removed and the residue was

properly diluted with H_2O/MeOH (9:1, mL: mL). The solution was transferred to an autosampler vial for the HPLC-DAD-ESI-MS/MS analysis.

2.6.2. HPLC-DAD-ESI-MS^n analysis

The method by Kahle, Kraus, & Richling (2005) was followed, with slight changes. Chromatographic analysis was performed with a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) equipped with a thermostated autosampler and a column oven. The UPLC system was coupled with a diode array detector and an electrospray ionization mass detector (HPLC-DAD-ESI-MS^n) in parallel by splitting the mobile phase 1:1.

Negative-ion ESI mass spectra were obtained with a Finnigan LXQ linear trap mass spectrometer (Thermo Scientific, San Jose, CA, USA). The typical ESI source conditions were transfer line capillary at 275 °C; ion spray voltage at 3.30 kV; sheath, auxiliary and sweep gas (N_2) flow rates at 10, 5 and 0 arbitrary units, respectively. Helium was used as the collision damping gas in the ion trap set at a pressure of 0.13 Pa. ESI-MS^n spectra were obtained by collision-induced dissociation (CID) experiments after isolation of the appropriate precursor ions in the ion trap (isolation width 1.2 *m/z* unit), and subjecting them to the following typical conditions: normalized collision energy between 20% and 30%, selected to preserve a signal of the precursor ion in the order of 5%; 0.25 activation Q and 30 ms activation time.

The chromatographic separation was performed with a column Synergi Hydro, 4 mm, 250 x 2.0 mm (Phenomenex, Italy), thermostated at 30 °C. Elution was carried out at 0.3 mL/min flow rate, using as mobile phase 2 mL/L formic acid in methanol (A) and 2 mL/L formic acid in water (B) with the following gradient: 0-6 min 10% A, 20 min 40% A, 40 min 40% A, 46 min 100% A, 52 min 100% A, 54 min 10% A, 54-60 min 10% A. The injection volume was 20 µL. The acquisition was carried out in full scan (*m/z* 50 - 1500) and in full scan MS^2 (*m/z* 50 - 600) selecting the precursor ion $[M-H]^-$ at *m/z* 289.1 for (+)-catechin and (-)-epicatechin, *m/z* 577.2 for procyanidin B2, *m/z* 457.1 for epigallocatechin gallate, *m/z* 353.1 for chlorogenic acid, *m/z* 163.0 for 3-hydroxycinnamic acid

(I.S.), *m/z* 463.1 for quercetin galactoside, *m/z* 435.2 for phloridzin, *m/z* 433.1 for quercetin xyloside and quercetin arabinoside, *m/z* 447.1 for quercetin rhamnoside and *m/z* 273.1 for phloretin, respectively. Phloretin-xyloglucoside ($[M-H]^-$ *m/z* 567.2; MS^2: *m/z* 273) and 4-*p*-coumaroylquinic acid ($[M-H]^-$ *m/z* 337.1; MS^2: *m/z* 173, 163, 155) were tentatively characterized, by comparison of their fragmentation pattern with those available in the literature (Sommella *et al.*, 2015).

The quantitative analysis was carried out using an Ultimate 3000 RS Diode Array detector (Thermo Scientific, San Jose, CA, USA) controlled by Chromeleon software (version 6.80). Spectral data from all peaks were accumulated in the range 200-400 nm and chromatograms were recorded at 280 nm for (+)-catechin, (-)-epicatechin, procyanidin B2, epigallocatechin gallate, 3-hydroxycinnamic acid (I.S.), phloretin-xiloglucoside, phloridzin, phloretin, 314 nm for 4-*p*-coumaroylquinic acid, 328 nm for chlorogenic acid, 258 nm for quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside and quercetin-3-*O*-rhamnoside, respectively.

2.6.3. Calibration curves and quantification

A stock solution of (+)-catechin, (–)-epicatechin, procyanidin B2, epigallocatechin gallate, chlorogenic acid, phloridzin, phloretin, quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-rhamnoside in H_2O/MeOH (9:1, mL: mL) was serially diluted with the same solvent to prepare 7-point calibration curves in the range 12-3000 ng/mL with a constant concentration of the I.S. (500 ng/mL). The R^2 coefficients for the calibration curves were > 0.99. When standards were unavailable, the quantification of the analyte was carried out using the calibration curve of available standard presenting similar chemical structure.

2.7.Color analysis

Color analysis was carried out using a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300 measuring head. The instrument was standardized against a white tile before measurement. Approximately 20 mL juice was poured in a plastic sample

container (50 mm diameter) and the instrument measuring head was precisely placed 5 mm below the liquid surface. Color was expressed in CIE units as L* (lightness/darkness), a* (redness/greenness) and b* (yellowness/blueness). The parameters a* and b* were used to compute the hue angle (arctan b*/a*) (Clydesdale, 1978).

2.8. α-Glucosidase inhibition assay

The *α*-glucosidase inhibitory activity was determined spectrophotometrically (UV-2501PC, UV-Vis recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) as previously described (Alongi & Anese, 2018). The inhibitory activity (%) against *α*-glucosidase was plotted *vs* apple juice concentration, which was tested in the range 0-23 mg_{dw}/mL. A logarithmic model was used to fit data so that IC_{50} was calculated as the concentration of apple juice required to produce a 50% inhibition against *α*-glucosidase. The same evaluation was applied to acarbose solutions, with increasing concentration in the range 0.02 – 0.80 mg/mL, as a standard indicator for rating the efficacy of juice samples in inhibiting *α*-glucosidase. The acarbose equivalent was calculated as $IC_{50\ acarbose}/IC_{50\ sample}$ (Nasu, Miura, & Gomyo, 2005).

2.9. Study of apple juice and acarbose interactions

The method proposed by Chou and Talalay (1984) was used, with some modifications, to investigate apple juice and acarbose interaction towards *α*-glucosidase inhibition. The inhibitory activity (%) against *α*-glucosidase, namely effect *x*, carried out by $P_{71.7}$ apple juice or acarbose was represented as a function of apple juice or acarbose concentration, respectively.

Combined systems of juice and acarbose with a proportionally increasing concentration of both juice and acarbose were obtained according to Equation 2:

$$Fm_n \times \left[(D_m)_j + (D_m)_a\right] = (C_n)_{j,a} \quad (2)$$

where Fm_n represents a multiplicative factor, $(D_m)_j$ and $(D_m)_a$ are the doses of juice and acarbose able to produce a 50% α-glucosidase inhibition (i.e. IC_{50}) and $(C_n)_{j,\,a}$ is the total concentration of juice and acarbose in the combined system. Six combined systems were obtained by substituting six different multiplicative factors (i.e. 0.25; 0.5; 1.5; 2; 2.5; 3.5) in Equation 2. The combined systems were tested for their ability to inhibit α-glucosidase and the inhibition percentage was plotted against the concentration. The sum of juice and acarbose doses $(D_x)_{j,\,a}$ corresponding to an effect x was thus determined, and the relevant single doses of juice and acarbose were calculated by Equations 3 and 4, respectively:

$$(D)_j = (D_x)_{j,a} \times \frac{(D_m)_j}{(D_m)_a + (D_m)_j} \quad (3)$$

$$(D)_a = (D_x)_{j,a} \times \frac{(D_m)_a}{(D_m)_a + (D_m)_j} \quad (4)$$

The Combination Index (CI) was finally calculated by Equation 5:

$$CI = \frac{(D)_j}{(D_x)_j} + \frac{(D)_a}{(D_x)_a} \quad (5)$$

where $(D)_j$ represents the dose of juice in the combined system producing an effect x, $(D)_a$ represents the dose of acarbose in the combined system producing the same effect x, $(D_x)_j$, is the dose of juice alone producing the effect x and $(D_x)_a$ is the dose of acarbose alone producing the effect x.

2.10. Statistical analysis

Results are averages of at least three measurements carried out on two replicated samples and are reported as means ± standard error ($n \geq 6$). Statistical analysis was performed using R (version 3.2.3, The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance, one-way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p<0.05$). Best fitting analysis was carried out

using Table Curve 2D (version 4.0, SPSS Inc., Chicago, IL, USA) and the goodness of fitting was evaluated based on statistical parameters of fitting (R^2, p and standard error SE).

3. Results and discussion

3.1.Effect of pasteurization on some chemical and physical properties of apple juice

Apple juice with average total solid content and pH of 13.7 ± 2.1 g/100 mL and 3.7 ± 0.1, respectively, were subjected to two different pasteurization processes providing sterilizing effects equivalent to $F_{71.7}^{5}$=0.4 min and F_{90}^{12}=14.8 min. As expected, total solid content and pH did not change upon heat treatment. Table 1 shows sugar concentration and color parameters of untreated and pasteurized apple juices.

Fructose was the most abundant sugar in apple juice samples, followed by sucrose and glucose. Raw and $P_{71.7}$ samples did not significantly differ for sugar content, while fructose and sucrose in P_{90} sample were approximately 30 and 60% less concentrated than those found in the control. This reduction can be attributed to the sugars consumption as reagents of the Maillard reaction (Rivas *et al.*, 2006; Garza, Ibarz, Pagán, & Giner, 1999). On the contrary, glucose concentration did not change significantly, probably due to the heat-induced release of aglycone compounds, and thus of glucose, that might counterbalance the Maillard reaction effect (Neveu *et al.*, 2010).

Pasteurization processes, especially the most intense one, caused an increase in L* and hue angle values, which are indices of sample bleaching. Such an increase may be due to hydrolysis of polymeric aggregates formed upon polyphenoloxidase activity and consequently to the formation of low molecular weight, soluble compounds (McKenzie & Beveridge, 1988).

3.2.Effect of pasteurization on α-glucosidase inhibitory activity of apple juice

Fig. 1 shows α-glucosidase inhibitory activity as a function of the total solid concentration of untreated apple juice. Apple juice significantly inhibited the enzyme activity in a dose-dependent way, in agreement with literature data (Nasu *et al.*, 2005; He, Yang, Zhang, Ma, & Ma, 2014;

256 Ankolekar *et al*., 2012; Adyanthaya, Kwon, Apostolidis, & Shetty, 2009). The enzymatic activity
257 was suppressed by almost 85% when apple juice concentration was 23 mg_{dw}/mL.
258 Fig. 1 also shows α-glucosidase inhibitory activities of the pasteurized apple juices as a function of
259 total solid concentration. No significant changes in the α-glucosidase inhibitory activity of the $P_{71.7}$
260 apple juice were found as respect to the untreated sample. On the contrary, the most intense
261 pasteurization was responsible for approximately 35% decrease in the α-glucosidase inhibitory
262 activity of P_{90} apple juice.
263 The potential of apple derivatives in reducing the postprandial rise in blood glucose has already
264 been reported (Adyanthaya *et al*., 2009; Ankolekar *et al*., 2012; Nasu *et al*., 2005; Shoji *et al*.,
265 2017). Several authors (Boyer and Liu, 2004; Guo *et al*., 2017) described an inverse relationship
266 between apple consumption and risk of type 2 diabetes. The α-glucosidase inhibitory effect carried
267 out by apple juice was attributed to naturally present phenolic compounds (Williamson, 2013;
268 Schmidt, Lauridsen, Dragsted, Nielsen, & Staerk, 2012). Based on these considerations, the latter
269 were quantified in apple juice submitted or not to pasteurization (Table 2), to understand whether
270 thermal processing-induced changes in phenolic compound profile, potentially affecting the α-
271 glucosidase inhibitory capacity of apple juice.
272 As reported in Table 2, the number of phenolic compounds differed in apple juice submitted or not
273 to pasteurization. The total phenolic content increased concomitantly with the intensity of thermal
274 treatment, in agreement with literature findings (Agcam, Akyildiz, & Evrendilek, 2014; Gerard &
275 Roberts, 2004). In particular, 2-fold and 6-fold increases in total phenolic content were found when
276 the mildest and most intense pasteurization treatments were applied, respectively. Among phenolic
277 compounds, flavonols and hydroxycinnamic acids represented the major classes, accounting for
278 nearly 90% of the overall content in untreated juice. Chlorogenic acid were the most abundant
279 compound and its concentration markedly increased upon the most intense treatment, accounting
280 for 50% of the total phenolic content. Other authors reported this compound to be the most

281 abundant phenol in apple derivatives and observed an increase in its concentration upon thermal
282 treatment (Keenan, Brunton, Butler, Wouters, & Gormley, 2011).
283 The increase in phenolic compounds upon pasteurization could be attributed to thermal induced
284 hydrolysis of the most heat-labile compounds (such as epigallocatechin gallate and procyanidin
285 polymers) resulting in the release of monomers and dimers, among which are (-)-epicatechin and
286 procyanidin B2 (De Paepe *et al*., 2014).
287 The increase in phenolic compounds can be also caused by thermal inactivation of
288 polyphenoloxidase. This enzyme is well known to induce phenolic compound polymerization in
289 vegetable matrices, producing complex dark-colored pigments (Oszmianski, Wolniak, Wojdyło, &
290 Wawer, 2008). By inactivating polyphenoloxidase, pasteurization can thus prevent phenolic
291 compounds from being involved in browning reactions (Ioannou, Hafsa, Hamdi, Charbonnel, &
292 Ghoul, 2012).
293 Overall, acquired data indicate that the reduction in the inhibitory activity (Fig. 1) corresponded to
294 an increase in total phenolic content (Table 2). On the contrary, Adyanthaya *et al*. (2009) found that
295 an increase or decrease in α-glucosidase inhibition corresponded to a similar rise and fall in the
296 concentration of phenolic compounds. However, it is noteworthy that literature data refer to
297 unprocessed juice. The results of the present study suggest the existence of more complex
298 mechanisms underlying α-glucosidase inhibition. In particular, the decrease in the inhibitory
299 activity could be attributed to the thermally induced degradation of bioactive molecules other than
300 phenolic compounds (Shori, 2015).
301
302 *3.3.Apple juice-acarbose interactive capability of inhibiting the α-glucosidase activity*
303 The α-glucosidase inhibitory capacity of apple juice was compared to that carried out by acarbose
304 (Table 3). Table 3 reports the IC_{50} of untreated, $P_{71.7}$, P_{90} apple juices, and acarbose solution, as well
305 as the acarbose equivalents of the enzyme inhibitors.

As expected, acarbose was more effective in inhibiting α-glucosidase than apple juice (Table 3). Raw and $P_{71.7}$ apple juices were 100-fold less effective than acarbose in inhibiting the enzyme. However, considering their dry matter (137 mg/mL) and the suggested daily intake of acarbose (300 mg/d), 220 mL/d of juice would provide an acarbose like effect. This dose should be increased to 547 mL/d when the most intensely treated juice (P_{90}) is considered since the latter was 250-fold less effective than acarbose in inhibiting α-glucosidase. These results demonstrated the efficacy of apple juice in inhibiting α-glucosidase *in vitro*, considering ordinarily consumed amounts of juice, as reported in the literature (Nasu *et al*., 2005).

The combined system of apple juice and acarbose was also analyzed, to understand if apple juice may play a role in enhancing the inhibitory effect carried out by acarbose. The interaction of acarbose with conventionally pasteurized apple juice (i.e. $P_{71.7}$) was investigated. Fig. 2 shows the α-glucosidase inhibition produced by apple juice, acarbose and apple juice-acarbose combined system.

It can be noticed that the combined system of apple juice and acarbose was more effective than apple juice alone in inhibiting the enzyme. Better to understand the interactive behavior of apple juice and acarbose, the combination index (CI) was computed and plotted as a function of the inhibition percentage against α-glucosidase (Fig. 3). To this purpose, six combined systems were prepared by multiplying six multiplicative factors (i.e. 0.25; 0.5; 1.5; 2; 2.5; 3.5) to the IC_{50} (i.e. the concentration required to cause 50% α-glucosidase inhibition) of apple juice and acarbose.

As already mentioned, the CI provides an indication of the interaction between apple juice and acarbose in the overall enzyme inhibition range. In particular, CI > 1 indicates antagonistic effect; CI < 1 means synergic effect and CI = 1 stands for additive effect. As reported in Fig. 3, apple juice and acarbose played a synergistic effect up to 40% inhibition, which corresponded to a concentration of apple juice-acarbose combined system near to 2 mg/mL (Fig. 2). An antagonistic behavior between apple juice and acarbose was found when the inhibition was between 40 and

80%, corresponding to a concentration of the combined system ranging from 2 to 9 mg/mL. However, it is noteworthy that, when inhibition percentages were higher than 70%, the CI progressively decreased, approximating the additive effect when 90% inhibition was approached. These results highlight that different proportions of acarbose and apple juice may differently affect the inhibitory activity against α-glucosidase.

Conclusions

The results of the present study draw attention to the ability of apple juice in inhibiting α-glucosidase that is one of the key enzymes involved in carbohydrates digestion. Acquired data demonstrated that conventional pasteurization, which is aimed to kill *Cryptosporidium parvum* oocysts, did not significantly affect the physical and chemical properties of apple juice, nor its inhibiting ability against α-glucosidase. By contrast, severe pasteurization, aimed to destroy *Alicyclobacillus acidoterrestris* spores, caused up to 35% loss of the inhibiting capacity of apple juice. This suggests that food functionality relies upon an adequate choice of processing parameters. Moreover, results showed that the apple juice-acarbose combined system played a synergistic effect up to 40% α-glucosidase inhibition, whereas higher concentrations led to an antagonistic behavior. Obtained results may represent a starting point to further investigate the potential effect of apple juice in bolstering the efficacy of conventional drugs used for the treatment of type 2 diabetes. Nonetheless, it should be considered that apple juice is an important source of sugars. The latter should be taken into account when designing foods aimed at reducing type 2 diabetes incidence. Thus, further studies may consider apple juice derivatives with low sugar concentration to be exploited for their antidiabetic effect.

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450

Captions for figures

Fig. 1. α-Glucosidase inhibitory activity of apple juice not pasteurized (Raw) or subjected to pasteurization providing sterilizing effects equivalent to $F_{71.7}^{5}$=0.4 min ($P_{71.7}$) and F_{90}^{12}=14.8 min (P_{90}), as a function of total solid concentration. Data fitting: lines, estimates; symbols, experimental data (NP; P71.7; P90).

Fig. 2. α-Glucosidase inhibitory activity of apple juice, acarbose and the apple juice-acarbose combined system as a function of total solid concentration. Data fitting: lines, estimates; symbols, experimental data (Apple juice + Acarbose; Acarbose; Apple juice).

Fig. 3. Combination index (CI) relevant to apple juice-acarbose combined system as a function of the α-glucosidase inhibition percentage. CI > 1: antagonistic effect; CI < 1: synergic effect; CI = 1: additive effect.

Table 1

Sugar concentration and color parameters of apple juice not pasteurized (Raw) or subjected to pasteurization providing sterilizing effects equivalent to $F_{71.7}^{5}$=0.4 min ($P_{71.7}$) or F_{90}^{12}=14.8 min (P_{90}).

Sample	Sucrose (g/L)	Fructose (g/L)	Glucose (g/L)	L*	Hue angle (arctan b*/a*)
Raw	20 ± 12^{ab}	159 ± 35^{ab}	7 ± 1^{a}	51 ± 2^{b}	70 ± 1^{b}
$P_{71.7}$	33 ± 1^{a}	201 ± 1^{a}	5 ± 1^{a}	50 ± 1^{b}	71 ± 1^{b}
P_{90}	8 ± 1^{b}	109 ± 1^{b}	5 ± 1^{a}	59 ± 1^{a}	87 ± 1^{a}

Means in the same column indicated by a common letter (a-c) are not significantly different ($p>0.05$).

Table 2

Phenolic compound concentrations of apple juice not pasteurized (Raw) or subjected to pasteurization providing sterilizing effects equivalent to $F_{71.7}^{5}$=0.4 min ($P_{71.7}$ sample) and F_{90}^{12}=14.8 min (P_{90} sample).

	Raw (mg/L)	**$P_{71.7}$ (mg/L)**	**P_{90} (mg/L)**
Chlorogenic acid	1.12 ± 0.02	3.85 ± 0.01	16.26 ± 0.15
p-Cumaroylquinic acid	0.89 ± 0.04	2.28 ± 0.03	4.35 ± 0.14
∑ Hydroxycinnamic acids	**2.01**	**6.13**	**20.61**
Phloretin	n.d.	n.d.	n.d.
Phloretin xyloglucoside	0.27 ± 0.02	0.69 ± 0.01	2.30 ± 0.02
Phloridzin	0.13 ± 0.00	0.38 ± 0.01	1.18 ± 0.01
∑ Dihydrocalcone derivatives	**0.40**	**1.06**	**3.47**
Epigallocatechin gallate	0.21 ± 0.01	0.25 ± 0.01	n.d.
(+) Catechin	n.d.	n.d.	n.d.
Procyanidin B2	n.d.	n.d.	0.20 ± 0.00
(-) Epicatchin	n.d.	0.10 ± 0.01	3.79 ± 0.13
∑ Flavanols	**0.21**	**0.36**	**3.99**
Quercetin-3-*O*-galactoside	0.85 ± 0.02	1.18 ± 0.03	1.42 ± 0.06
Quercetin-3-*O*-hexoside	0.08 ± 0.01	0.87 ± 0.00	0.97 ± 0.04
Quercetin-3-*O*-xyloside	0.12 ± 0.00	0.18 ± 0.00	0.23 ± 0.00
Quercetin-3-*O*-arabinoside	n.d.	n.d.	n.d.
Quercetin-3-*O*-rhamnoside	1.45 ± 0.02	1.55 ± 0.01	1.65 ± 0.02
Others quercetin-pentoside	0.21 ± 0.01	0.28 ± 0.01	0.31 ± 0.01
∑ Flavonols	**2.72**	**4.06**	**4.59**
Total phenolic compounds	**5.33**	**11.61**	**32.66**

n.d.: not detectable

Table 3.

IC_{50} (half-maximal inhibitory concentration) and acarbose equivalents of apple juice not pasteurized (Raw) or subjected to pasteurization providing sterilizing effects equivalent to $F_{71.7}^{5}$=0.4 min ($P_{71.7}$ sample) and F_{90}^{12}=14.8 min (P_{90} sample).

Inhibitor	IC_{50} (mg/mL)	Acarbose equivalents ($IC_{50\ acarbose}/IC_{50\ inhibitor}$)
Raw	6.24	0.014
$P_{71.7}$	6.08	0.014
P_{90}	18.41	0.004
Acarbose	0.09	1

Figure

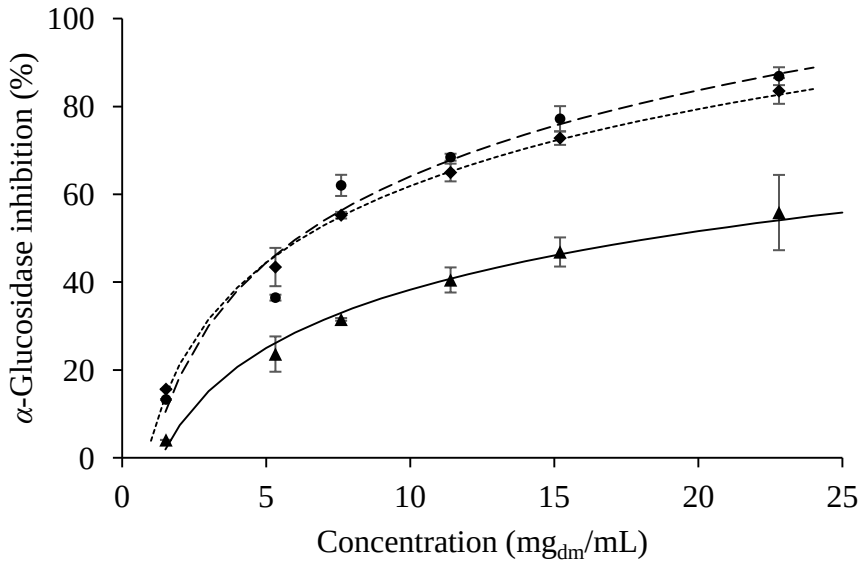
100
80
60
40
20
0
α-Glucosidase inhibition (%)
0
5
10
15
20
25
Concentration (mg$_{dm}$/mL)

Fig. 1.

Figure

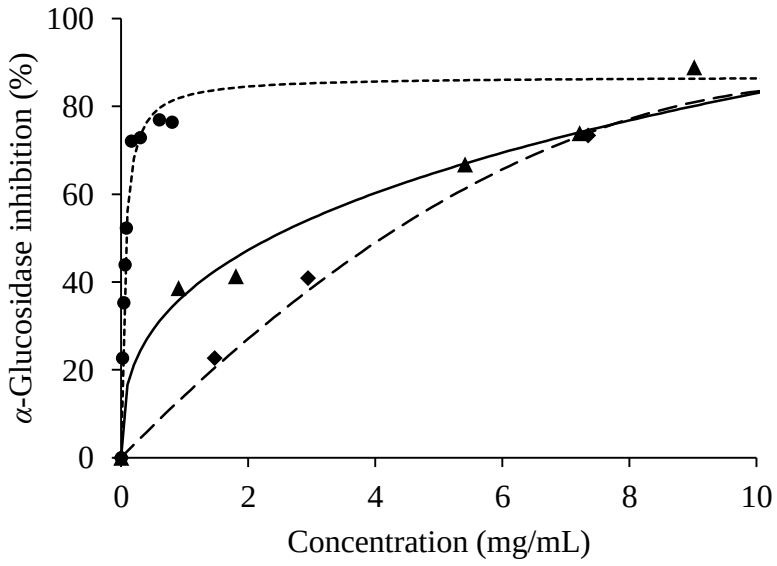
100
80
60
40
20
0
α-Glucosidase inhibition (%)
0
2
4
6
8
10
Concentration (mg/mL)

Fig. 2.

Figure

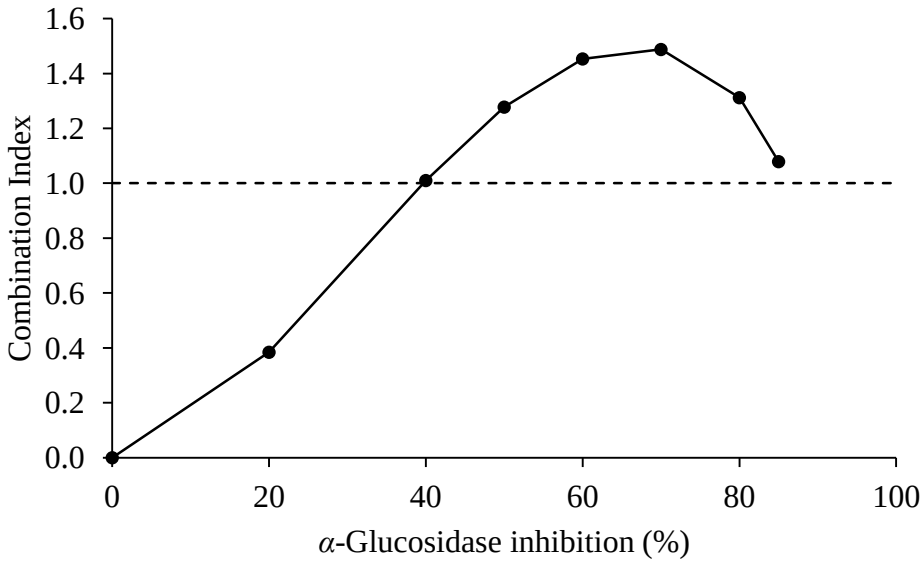
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Combination Index
0
20
40
60
80
100
α-Glucosidase inhibition (%)

Fig. 3.