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Effect of coffee roasting on in vitro alpha-glucosidase activity: Inhibition and mechanism of action

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Abstract: In vitro α -glucosidase inhibitory activity of unroasted, and medium, dark and very dark roasted Robusta coffee was studied. Coffee extracts significantly inhibited the enzyme activity in a dose-dependent way. The inhibitory activity well correlated with the degree of roast. Coffee components were separated by gel permeation chromatography into low ($1 < MW < 6$ kDa), intermediate ($15 < MW < 60$ kDa) and high ($MW > 100$ kDa) molecular weight fractions, which were analyzed for the α -glucosidase inhibitory capacity. Only fractions obtained from dark and very dark roasted coffee exhibited inhibitory effect. Fractions with the same average molecular weight obtained from coffees with different roasting degree differently inhibited α -glucosidase. This was attributed to compositional changes within each fraction as induced by roasting. Coffee extracts and their fractions revealed a mixed-type to competitive inhibition mechanisms against α -glucosidase, which are consistent with the complex sample composition.

Dear Editor,

I would like to submit the manuscript entitled “*Effect of coffee roasting on in vitro α -glucosidase activity: inhibition and mechanism of action*” by Marilisa Alongi and Monica Anese for consideration for publication in Food Research International.

The manuscript reports on new findings on the potential of coffee with different degree of roast to inhibit *in vitro* α -glucosidase, a key enzyme involved in carbohydrate digestion. Roasted coffee effectively inhibited the enzyme, through a mixed-type to competitive inhibition mechanism. Results suggest that the selection of proper roasting conditions might represent a promising strategy to reduce type 2 diabetes development.

Best regards

Monica Anese

Highlights

Robusta coffee extract inhibited α -glucosidase in a concentration-dependent way

The higher was coffee roasting degree, the higher resulted α -glucosidase inhibition

Low, intermediate, high molecular weight fractions differently inhibited the enzyme

Extracts and fractions inhibited α -glucosidase in a mixed-type to competitive manner

Effect of coffee roasting on *in vitro* α -glucosidase activity: inhibition and mechanism of action

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Abstract

In vitro α -glucosidase inhibitory activity of unroasted, and medium, dark and very dark roasted Robusta coffee was studied. Coffee extracts significantly inhibited the enzyme activity in a dose-dependent way. The inhibitory activity well correlated with the degree of roast. Coffee components were separated by gel permeation chromatography into low ($1 < MW < 6$ kDa), intermediate ($15 < MW < 60$ kDa) and high ($MW > 100$ kDa) molecular weight fractions, which were analyzed for the α -glucosidase inhibitory capacity. Only fractions obtained from dark and very dark roasted coffee exhibited inhibitory effect. Fractions with the same average molecular weight obtained from coffees with different roasting degree differently inhibited α -glucosidase. This was attributed to compositional changes within each fraction as induced by roasting. Coffee extracts and their fractions revealed a mixed-type to competitive inhibition mechanisms against α -glucosidase, which are consistent with the complex sample composition.

Keywords

Robusta coffee; Degree of roast; α -Glucosidase; Inhibitory capacity; Molecular weight; Mechanism of inhibition

Abbreviations

3-CQA, 3-caffeoylquinic acid; 5-CQA, 5-caffeoylquinic acid; CGA, chlorogenic acids; HMW, high molecular weight; I, inhibitor; IMW, intermediate molecular weight; LMW, low molecular weight; S, substrate; TP, total phenols

1. Introduction

Coffee has been consumed over countless centuries and currently belongs to the most marketed food items (Esquivel & Jiménez, 2012). The brew obtained from coffee beans contains a huge number of compounds, which are mainly produced during roasting, upon chemical modification of green coffee composition (Hečimović, Belščak-Cvitanović, Horžić, & Komes, 2011; Moreira, Nunes, Domingues, & Coimbra, 2012). Roasted coffee composition depends on roasting time and temperature, whose choice allows obtaining coffees with different degree of roast. The latter are appreciated for their flavour as well as physiological and psychoactive effects (Dórea & Da Costa, 2005; Hečimović *et al.*, 2011; Yanagimoto Ochi, Lee, & Shibamoto, 2004). Roasting leads to the formation of many bioactive compounds, thus affecting not only coffee sensory properties but also its beneficial health effects. The latter allow defining coffee as a functional food (Dórea & Da Costa, 2005; Hečimović *et al.*, 2011; Ludwig *et al.*, 2014a; Wang, Qian, & Yao, 2013). For instance, the melanoidins developed upon Maillard reaction demonstrated antioxidant, antimicrobial and anti-inflammatory properties (Morales, Somoza, & Fogliano, 2012). Coffee consumption was also associated to a reduced incidence of Parkinson, Alzheimer, cardiovascular diseases and cancer (Baspinar, Eskici, & Ozcelik, 2017; Hu, Bidel, Jousilahti, Antikainen, & Tuomilehto, 2007; Kleemola, Jousilahti, Pietinen, Vartiainen, & Tuomilehto, 2000; Lindsay *et al.*, 2002). Several epidemiological studies highlighted that a moderate and prolonged coffee consumption also contributed to the reduction of type 2 diabetes risk (Van Dam & Hu, 2005; Van Dijk *et al.*, 2009; Lecoultre *et al.*, 2014). This effect was associated with the ability of some compounds contained in coffee to reduce the blood glucose level, thus promoting a hypoglycemic effect (Johnston, Clifford, & Morgan, 2003; Krebs, Parry-Strong, Weatherall, Carroll, & Downie, 2012, Salazar-Martinez *et al.*, 2004; Shearer *et al.*, 2003). Despite some information about the action site of these compounds is available, their mechanism of action has not been clarified yet. In particular, chlorogenic acids (Iwai *et al.*, 2012; Johnston *et al.*, 2003; Lecoultre *et al.*, 2014), caffeic acid and quercetin (Muraš *et al.*, 2012), trigonelline (Van Dijk *et al.*, 2009; Hamden, Bengara, Amri, & Elfeki, 2013), and

Amadori compounds (Ha *et al.*, 2011), can cause the inhibition of α -glucosidase. This enzyme is a hydrolase located on the intestinal cell membrane of the ciliate epithelium and is required to obtain glucose from oligo- and disaccharides (Chiba, 1997). α -Glucosidase plays thus a key role in the final step of carbohydrates digestion and its inhibition currently represents a common therapeutic approach to reduce postprandial hyperglycemia (Goto *et al.*, 2012; Hu, Wang, & Kong, 2013; Kwon, Apostolidis, & Shetty, 2008). The inhibitory effect is obtained by the so-called glycomimetic drugs (i.e. Acarbose, Voglibose, Miglitol) that are able to interact with the enzymatic active site (Akkarachiyasit, Charoenlertkul, Yibchok-Anun, & Adisakwattana, 2010; Dubois, 2014). Nonetheless, these drugs carry undesired side effects, such as weight increase, hypoglycemia and gastrointestinal diseases (Cheng & Fantus, 2005). To avoid these undesired effects, researchers are looking for natural α -glucosidase inhibitors, which could represent a valuable option to artificial drugs (Cheng & Fantus, 2005; Kwon *et al.*, 2008; Ríos, Francini, & Schinella, 2015). According to Ludwig *et al.* (2014a), coffee consumption can reduce by 60% diabetes risk and this value is in the same range as observed with pharmacological approaches. Kim (2015) demonstrated that a β -carboline alkaloid norharman isolated from coffee inhibits α -glucosidase with an uncompetitive mechanism. However, little is known about the inhibitory effect carried out by the whole coffee brew (Singh *et al.*, 2014). The latter consists of a complex mix of compounds, whose interaction may modify the overall effect towards α -glucosidase (Farah, De Paulis, Trugo, & Martin, 2005). Further, different roasting conditions substantially modify coffee composition and this may potentially affect the α -glucosidase inhibitory effect.

Therefore, the aim of this study was to investigate the effect of different roasting degrees on the *in vitro* inhibitory capacity against α -glucosidase, exhibited by coffee extracts and their fractions. Furthermore, the mechanism of inhibition carried out by the extracts and fractions was studied.

2. Materials and methods

2.1. Sample preparation

Coffee samples were prepared by using green coffee beans from the species *Coffea canephora* var. *robusta* Pierre ex Froehn (*Rubiaceae*), from Vietnam. Coffee was roasted in an air circulation oven (TC 40 Thermocenter, Salvis, Reussbühl, Switzerland) at 200 °C during 15, 45 and 60 minutes. After the treatments, samples were immediately removed from the oven and cooled to room temperature. The roasted samples were ground in a mill (Moulinex mod. 505, Paris, France) immediately after the roasting process and sieved through a 35 Mesh sieve. Afterwards, coffee powders were transferred into plastic vessels with pressure lid and stored at -18 °C until analyses were performed. Coffee brews were prepared by solid-liquid extraction with milli-Q deionized water of the ground green and roasted beans. The ratio between coffee powder and water was 1:8 (w/w). The coffee-water mixture was boiled for 5 minutes under stirring at 300 rpm (Yellow line magnetic IKA, Staufen, Germany), cooled, filtered through filter paper and freeze-dried (Laboratory and Pilot Freeze Dryer Mini-fast, mod. 1700, Edwards Alto Vuoto, Milano, Italy).

2.2. Coffee fractions separation

The method of Borrelli, Visconti, Mennella, Anese, and Fogliano (2002) was followed. A HPLC system (LC-10AT VP, Shimadzu Corporation, Kyoto, Japan) equipped with a UV/VIS detector (SPD-10 AT VP, Shimadzu Corporation, Kyoto, Japan) was used. Freeze-dried coffee material was dissolved in milli-Q deionized water (0.4 g/mL), filtered (Whatman 0.45 µm) and loaded onto a Sephadex G-25 gel filtration chromatography column (60 × 1.1 cm i.d.; Pharmacia, Uppsala, Sweden). The injection volume was 20 µL and the mobile phase, delivered at a flow rate of 1.2 mL/min, was milli-Q water. The detection wavelength was 280 nm. Peak integration was performed by Polyview 2000 software (Ver. 5.3, Varian, Texas, USA). In according with Borelli *et al.* (2002) four fractions were collected: fraction I, containing high-molecular-weight (HMW) material, indicated as coffee melanoidins; fractions II and III, containing intermediate-molecular-weight

(IMW) compounds; and fractions IV, containing small compounds such as low-molecular-weight (LMW) phenols. Since fractions II and III both contained IMW compounds, in the present experiment they were mixed together. Thus, three fractions, i.e. high, intermediate and low molecular weight, were obtained from each coffee sample. The coffee fractions were freeze-dried and stored in a desiccator, until analyses were performed.

2.3. Weight loss and total solid content determinations

Sample weight loss upon roasting was calculated as the percentage weight difference between the initial and final weights of the coffee beans. Total solid content was determined by gravimetric method (AOAC, 1995).

2.4. Colour analysis

Color analysis was carried out on ground coffee 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. 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 ($\tan^{-1} b^*/a^*$) (Clydesdale, 1978).

2.5. Phenolic compounds quantification

Chromatographic quantification of chlorogenic acids was performed following the method proposed by Hečimović *et al.* (2011) modified according to Llorach, Tomás-Barberán, and Ferreres (2004). A HPLC pump (LC-10AT VP, Shimadzu Corporation, Kyoto, Japan) equipped with a diode array detector (SPD-10 AT VP, Shimadzu Corporation, Kyoto, Japan) and an inverse phase apolar C18 column (5 μ m, 250 x 4.6 mm, Alltima, Lokeren, Belgium) were used. The injection valve (Rheodyne, Sigma-Aldrich, Milano, Italy) was equipped with a 20 μ L plastic loop and samples were injected using a syringe (SGE LC, 100 μ L, FN). The elution was in gradient mode using a

mixture of 5% formic acid (Sigma-Aldrich, Milano, Italy) in water (solvent A) and methanol (Sigma-Aldrich, Milano, Italy) (solvent B) as mobile phase at a flow rate of 1 mL/min. Gradient was set as follows: solvent A was held at 90% for the first 25 min, decreased to 80% and held at this level for 15 min; then decreased further to 50% and held for 5 min; finally, 90% solvent B was reached and held for 15 min. The detection was conducted at 280, 335 and 350 nm. Quantification was carried out using external standards. Calibration curves were linear ($R^2 > 0.995$) in the 2.0 to 200.0 mg/L concentration interval. Peaks integration was performed by using Polyview 2000 software (Ver. 5.3, Varian, Texas, USA). Total phenolics (TP; i.e., the overall number of phenolic compounds), chlorogenic acids (CGA), 3-caffeoylquinic acid (3-CQA) and 5-caffeoylquinic acid (5-CQA) concentrations were expressed as mg/g_{dm} of the coffee extract.

2.6. α -Glucosidase inhibition assay

The inhibitory activity of coffee extracts and their fractions against α -glucosidase was assessed spectrophotometrically (UV-2501PC, UV-VIS Recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan), following the method of Singh *et al.* (2014) with some modifications. Freeze-dried coffee extracts and fractions were diluted to proper concentrations with a mixture of methanol and deionized water (1:9 v/v). Different aliquots of coffee samples, or 3.33 mg/mL 3-CQA and 5-CQA (Sigma-Aldrich, Milano, Italy) and 0.004 mg/mL Acarbose (Sigma-Aldrich, Milano, Italy) aqueous solutions were introduced in 1 mL capacity cuvettes in the presence of 30 μ L α -glucosidase solution (0.04 mg/mL in 0.1 M phosphate buffer, pH=7, corresponding to 1 U/mL), and phosphate buffer (100 mM, pH 7) to the volume of 900 μ L, and mixed well. After incubation at 37 °C for 10 minutes, the reaction was started by adding 100 μ L of 5 mM 4-nitrophenyl- α -D-glucopyranoside (Sigma-Aldrich, Milano, Italy) solution in 100 mM phosphate buffer (pH 7.0) as substrate. Absorbance was recorded at 405 nm during 15 minutes after every 30 s. Further assays were performed using 10 μ L coffee solution and varying the volume of substrate solution between 0 and 200 μ L. Controls lacking inhibitors were run and defined the control activity in each

experiment. The α -glucosidase inhibition carried out by coffee extracts and fractions was calculated using Equation 1:

$$\text{Percentage of inhibition} = 100 - \left(\frac{k_s}{k_c} \times 100 \right) \quad (1)$$

where k_s and k_c were the kinetic constants in the presence and in the absence of the inhibitor, respectively. The half-maximal inhibitory concentrations (IC_{50}), i.e. the concentration of inhibitor required to produce a 50% inhibition against α -glucosidase, of coffee extracts, coffee fractions, 3-CQA, 5-CQA and Acarbose were assessed by the linear regression of the inhibition percentage versus the inhibitor concentration. The Acarbose equivalent was calculated as $IC_{50 \text{ Acarbose}}/IC_{50 \text{ sample}}$ (Nasu, Miura, & Gomyo, 2005).

2.7. Determination of kinetic parameters of α -glucosidase in the presence and absence of coffee extracts and fractions, and identification of inhibition type

The k_m and V_{max} and apparent k_m (k_{m*}) and V_{max} (V_{max*}) values, respectively in absence and presence of coffee extracts and fractions (hereafter called inhibitors, I), as well as the inhibition type were determined from double reciprocal plots (Lineweaver-Burk). The inhibition constants (k_i and k_i') values were computed using Equation 2 for competitive and Equations 3 and 4 for mixed-type inhibition, respectively (Ahmed, El-Maraghy, Teleb, & Shaheen, 2014).

$$k_i = \frac{k_m(I)}{k_{m*} - k_m} \quad (2)$$

$$k_i = \frac{V_{max} \times k_m \times (I)}{V_{max} k_{m*} - V_{max*} k_m} \quad (3)$$

$$k_i' = \frac{V_{max}(I)}{V_{max} - V_{max*}} \quad (4)$$

2.8. Statistical analysis

Data were reported as mean \pm standard deviation of at least three measurements on two replicated samples. Analysis of variance (ANOVA) was performed with significance level set to $p < 0.05$; the

Bartlett procedure was used to test homogeneity of variances, using R software, version 3.4.3 (The R Foundation for Statistical Computing, 2018). Linear regression analysis was performed by using Microsoft Excel 2016. The goodness of fitting was evaluated based on visual inspection of residual plots and by the calculation of R^2 and p .

3. Results and discussion

3.1. Effect of roasting on the *in vitro* inhibitory activity of coffee extracts against α -glucosidase

Coffees with different degrees of roast were produced by submitting Robusta coffee beans to roasting for increasing lengths of time. Weight loss total solid content and color parameters (i.e. L* and hue angle) changed during roasting (Table 1), in agreement with the literature (Moreira *et al.*, 2012; Opitz *et al.*, 2014; Vargas-Elías, Correa, De Souza, Baptestini, & Melo, 2016). Based on weight loss, roasted coffees were classified as medium, dark and very dark (Clarke, 1987).

Unroasted, medium, dark, and very dark roasted coffee extracts were analyzed for *in vitro* α -glucosidase inhibitory activity (Fig. 1). The coffee extracts differently affected α -glucosidase activity. In particular, the green coffee extract did not exert any inhibitory effect in the whole range of analyzed concentrations (up to 10 mg/mL). Analogously, the medium roasted coffee extract did not show any inhibitory effect at concentrations lower than 2 mg/mL, while a 7% inhibition was recorded at 2.5 mg/mL concentration. Higher coffee extract concentrations were not considered, since absorbance signals were out of the linear range. The inhibitory effect of dark and very dark roasted coffee extracts increased almost linearly with concentration, and was higher for the most intensively roasted sample, reaching a 49% value at 2.5 mg/mL concentration.

Since most functional properties of coffee have been attributed to phenolic content (Iwai *et al.*, 2012; Johnston *et al.*, 2003; Lecoultre *et al.*, 2014), TP, CGA, 3-CQA and 5-CQA were quantified in coffee extracts (Table 1), to understand whether changes in the inhibitory activity could be attributed to modifications occurring during roasting in the phenolic composition. TP content of the coffee extract obtained from unroasted beans fell within the range reported in literature, i.e. 0.2 to

40 mg/g. Such a variability is attributable not only to the species and the variety, but also to the processing conditions of coffee beans (Hečimović *et al.*, 2011; Ludwig *et al.*, 2014b). To this regard, it should be noticed that in agreement with literature findings (Clifford, 1985; Vignoli, Bassoli, Benassi, 2011), TP significantly decreased upon roasting, which produced a nearly 2-fold reduction from the unroasted to the very dark roasted sample. As reported in the literature (Clifford, 1985; Vignoli, Bassoli, & Benassi, 2011), CGA represented the major class of phenolic compounds in coffee, accounting for 85% of the overall TP content in the green coffee extract (Table 1). Upon very dark roasting, CGA were reduced by more than 90%, due to their high susceptibility to thermal degradation (Fujioka & Shibamoto, 2008; Hečimović *et al.*, 2011; Lecoultré *et al.*, 2014). As reported by Farah and Donangelo (2006), roasting conditions would be able to quite completely break down chlorogenic acids to phenolic derivatives, reducing up to 94% the initial CGA content. 3-CQA together with 5-CQA accounted for 55% of CGA in unroasted coffee (Table 1). These caffeoylquinic acids have actually been reported to represent the most abundant CGA in coffee (Farah & Donangelo, 2006; Kim, Keogh, & Clifton, 2016). A 30-fold decrease in 3-CQA concentration was observed in the very dark sample when compared to unroasted coffee, while a 6-fold reduction was found for the 5-CQA, suggesting a different sensitivity to thermal degradation of these compounds, despite they present analogous chemical structures (Dawidowicz & Typek, 2017).

It is noteworthy that no significant relationship between the phenolic content of coffee extracts and the enzyme inhibitory activity ($p>0.05$) was found. Despite the high phenolic content was considered one of the main factors affecting the ability of coffee to reduce diabetes risk (Nieber, 2017), phenols might not be the best candidates influencing the α -glucosidase inhibitory capacity; other compounds, namely melanoidins that are formed during roasting, may play such a role (Ludwig *et al.*, 2014a). A positive linear correlation was actually found between the inhibitory activity and browning development ($p<0.005$; $R^2=0.899$) as well as weight loss ($p<0.05$; $R^2=0.958$).

To obtain a standard indicator for rating the efficacy of coffee extracts in inhibiting α -glucosidase, the IC_{50} (half-maximal inhibitory concentration) of medium, dark and very dark roasted coffee extracts were computed (Table 2). Table 2 also shows the IC_{50} of 3-CQA, 5-CQA and Acarbose solutions as well as the Acarbose equivalents of the enzyme inhibitors. Roasted coffee extracts resulted 2.8- to 3.8-fold, 5.5- to 7.4-fold and 25- to 33-fold less effective in inhibiting α -glucosidase than 3-CQA, 5-CQA and Acarbose, respectively. Thus, in the case of the very dark roasted sample, 7500 mg/die would produce an α -glucosidase inhibitory effect analogous to that carried out by 300 mg/die Acarbose, which is the maximum Acarbose dose recommended to adults (Chiasson *et al.*, 2013). Considering that the very dark coffee extract had a $3.3 \pm 0.2\%$ dry matter, a cup of espresso coffee (25 mL) would provide 750 mg dry matter. This means that 10 cups of espresso coffee would be required to produce an Acarbose-like effect. Nonetheless, studies considering the effect of digestion and absorption of bioactive compounds, as well as validation *in vivo* trials are required to support these speculations.

The complex composition expected in coffee extracts could explain the higher IC_{50} of coffee extracts, when compared to that calculated for 3-CQA and 5-CQA. It is likely that some compounds formed upon roasting do not contribute to the inhibitory effect. Actually, green coffee composition is dominated by carbohydrates (almost 60% dry weight), followed by lipids (8-18% dry weight) and proteins (9-16% dry weight), while phenolic compounds account for 6 to 10% of the dry matter (Ludwig *et al.*, 2014a). As already stated, phenolic compounds are well known to undergo to remarkable changes during roasting and they can be incorporated into melanoidin structures (Borrelli *et al.*, 2002; Moreira *et al.*, 2012). However, the nature and number of phenolic compounds forming melanoidins, the mechanism and the final structure have not been fully clarified yet.

3.2. Effect of roasting on the *in vitro* inhibitory activity of coffee fractions against α -glucosidase

To reduce matrix complexity, medium, dark and very dark roasted coffees were fractionated by gel permeation chromatography and fractions containing LMW, IMW and HMW compounds were analyzed for their α -glucosidase inhibitory activity. Green extract was not fractionated, since it did not show any inhibitory activity in the range of interest (Fig. 1). Table 3 shows the percentage of each fraction obtained from the medium, dark and very dark roasted coffee extracts, which was calculated by integrating peak areas in the gel permeation chromatograms. Only a negligible amount (3%) of HMW compounds (i.e. melanoidins) was found in the medium roasted coffee extract, being these more abundant in the dark roasted sample (12%). Further roasting (i.e. very dark roasting degree) produced a 4% reduction in HMW fraction. Although melanoidins are well known to develop during roasting, prolonged and intensive treatments can induce their fragmentation (Borrelli *et al.*, 2002). This evolution is supported by the observed increase of LMW fraction from 36% in the medium roasted coffee extract to 90% in the very dark roasted one, and the concomitant reduction in the IMW compounds from 61% in the medium roasted coffee extract to 2% in the very dark roasted sample. This reduction can be attributed to the degradation of highly thermosensitive phenolic compounds (Hečimović *et al.*, 2011).

Fractions obtained from each coffee extract were then analyzed for the inhibitory activity against α -glucosidase. Medium roasted coffee fractions did not show any activity in the whole range of analyzed concentrations, up to 10 mg/mL (data not shown), despite a slight inhibition was observed for the correspondent extract (Fig. 1). This suggests a synergistic effect among the compounds contained in the medium roasted coffee, leading to a higher inhibitory activity observed for the extract than for the individual fraction. Fig. 2 shows the α -glucosidase inhibitory activity of dark and very dark roasted coffee fractions as a function of their concentration. HMW, IMW and LMW fractions inhibited α -glucosidase to a different extent. A difference in the inhibitory capacity was also detected within a same fraction, i.e. a same class of compounds (Borrelli *et al.*, 2002), when submitted to different roasting intensities (i.e. dark and very dark). In particular, prolonging roasting

from dark to very dark degree suppressed the inhibitory activity of HMW fraction (Fig. 2a). Analogously, the inhibitory activity of IMW fraction (Fig. 2b) was absent in the very dark roasted sample, whereas it increased exponentially with concentration in the dark processed coffee sample (e.g. 80% inhibition with 1.25 mg/mL). Finally, LMW fraction accounted for lower differences in α -glucosidase inhibitory activity upon roasting between dark and very dark samples than HMW and IMW fractions (Fig. 2c). These results suggest that roasting leads not only to changes in the fraction content of coffee extracts (Table 3), but also to qualitative modifications within the fractions. To get an insight into the CGA composition of coffee fractions, Fig. 3 shows the concentrations of 3-CQA, 5-CQA and other CGA (calculated as the difference between CGA, and 3-CQA and 5-CQA) in the HMW, IMW and LMW fractions of medium, dark and very dark roasted coffee. CGA, 3-CQA and 5-CQA could not be quantified in the HMW fraction of the medium roasted coffee, while only negligible amounts were found in the dark and very dark HMW fractions. This was expected, since the HMW fraction is mainly represented by melanoidins (Borrelli *et al.*, 2002). As observed in the case of coffee extracts (Table 1), the CGA content in the IMW fraction underwent a progressive decrease upon roasting (Fig. 3), that resulted particularly pronounced in the case of 3-CQA (47-fold decrease). Surprisingly, CGA were not quantifiable in the LMW fraction of the medium roasted coffee extract. These compounds can be actually incorporated in melanoidins at the beginning of the roasting process (e.g. medium roasting degree), as reported by Perrone, Farah, and Donangelo (2006). These authors also showed that the melanoidin-CGA complexes are degraded upon further roasting. This could explain the observed increase in 3-CQA, 5-CQA and other CGA concentrations in the LMW fraction upon dark roasting (Fig. 3). Further roasting (i.e. very dark) produced up to 40% decrease in CGA concentration, due to the thermal degradation of these compounds, as observed in the case of coffee extracts (Table 1) and reported in the literature (Fujioka & Shibamoto, 2008; Hećimović *et al.*, 2011).

As in the case of coffee extracts (Table 1 and Fig. 1), no relation ($p>0.05$) was found between the phenolic composition of coffee fractions (Fig. 3) and their α -glucosidase inhibitory effect (Fig. 2).

The latter is probably exerted not only by phenolic compounds or melanoidins, as reported in the literature (Ludwig *et al.*, 2014a; Van Dijk *et al.*, 2009; Johnston *et al.*, 2003; Iwai *et al.*, 2012), but might rely on their interaction as well as on other bioactive compounds, whose structural changes, induced by technological interventions, are well known to affect their functional properties (Esquivel & Jiménez, 2012; Vitaglione, Fogliano, & Pellegrini, 2012).

3.3. Inhibitory mechanisms of roasted coffee extracts and their fractions against α -glucosidase

α -Glucosidase inhibition mechanisms of coffee and coffee components have been little studied (Kim, 2015). In the present research, assays were performed to investigate the inhibition mechanism against α -glucosidase of coffee extracts and fractions. Enzymatic activity data were elaborated to obtain the Michaelis-Menten plot that describes the relation between the reaction rate (v) and the substrate concentration S . As an example, Fig. 4a shows the relationship between the reaction rate and the substrate concentration in the presence of increasing concentrations of the very dark roasted coffee extract, taken as an inhibitor I . As expected, v increased as the substrate concentration increased, approaching a plateau at high concentrations. In our experimental conditions, the evolution of the reaction rate of enzymatic activity as a function of the substrate concentration was comparable in the absence and in the presence of the coffee extract. However, the reaction rate decreased as the inhibitor concentration increased, indicating an inhibitory activity of the coffee sample. Similar effects were found also for the medium and dark roasted coffee extracts as well as for the fractions obtained from coffees with dark and very dark degree of roast (data not shown). To properly describe the inhibition mechanism, V_{max} and k_m or V_{max*} and k_{m*} values, in the absence or in the presence of inhibitor respectively, were computed from the double-reciprocal Lineweaver-Burk plot that describes the relationship between the inverse of the reaction rate ($1/v$) and the inverse of the substrate concentration ($1/S$). Fig. 4b shows the Lineweaver-Burk plot relevant to the very dark roasted coffee extract. Data regression ($p < 0.05$, $R^2 \geq 0.98$) revealed converging lines intercepting on the y-axis and x-axis (Fig. 3b) and provided the reciprocal V_{max} and reciprocal k_m , respectively. The

same procedure was applied to all coffee extracts and fractions that presented inhibitory activity, and results were used to compute the kinetic parameters V_{max} , k_m , V_{max*} , k_{m*} (Table 4). Both coffee extracts (i.e. dark and very dark) presented an increase from k_m to k_{m*} , and a concomitant decrease from V_{max} to V_{max*} (Table 4). The changes in both kinetic parameters can be attributed to a mixed-type inhibition with k_i and k_i' constants (Ahmed *et al.*, 2014). The latter typically occurs when the inhibitor binds to a site other than the active one, inducing enzymatic conformational changes and thus reducing substrate affinity for the active site (Robinson, 2015; Shou *et al.*, 2000). A similar behaviour was observed in the case of the very dark LMW and the dark HMW fractions. These results agree with literature findings, since Ghadyale, Takalikar, Haldavnekar, and Arvindekar (2012) and Hu, Wang, and Kong (2013) found a non-competitive mechanism against α -glucosidase for two different plant extracts, i.e. *Cymbopogon martinii* and *Momordica charantia*, respectively. It should be noted that in mixed-type and non-competitive mechanisms the inhibitor binds to both the free enzyme and enzyme-substrate complex. However, non-competitive inhibitor presents the same affinity towards enzyme and enzyme-substrate complex, while mixed-type inhibitor has different affinities. Table 4 also shows the kinetic parameters of the IMW and LMW fractions of the dark coffee. The latter presented k_{m*} higher than k_m , while V_{max} was not affected, suggesting a competitive inhibition. This mechanism was described in the literature by several authors regarding anthocyanins and other polyphenols (McDougall & Stewart, 2005; Sancho & Pastore, 2012). Interestingly, the competitive mechanism also underlies the activity of synthetic α -glucosidase inhibitors, such as Acarbose (Matsui *et al.*, 2001; Toeller, 1994).

According to the acquired results, roasting affected not only the inhibitory efficacy of coffee fractions (Fig. 2), but also their mechanism (Table 4). Both are expected to depend on the complex composition of coffee matrix, which is known to change upon roasting (Tables 1 and 3, Fig. 3).

358 4. Conclusions

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359 Roasting affected the *in vitro* α -glucosidase inhibitory activity of coffee. The darker the coffee
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360 degree of roast the greater such a property. Gel permeation chromatography of coffee extracts
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361 allowed obtaining low to high molecular weight coffee fractions. Although they were nominally
8
362 representative of phenolic compounds (LMW fraction), phenolic compounds and Maillard reaction
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11
363 intermediates (IMW fraction) and melanoidins (HMW fraction), results showed that increasing
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364 roasting time caused changes in their inhibitory activity against the digestive enzyme. This suggests
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365 compositional changes occurring within each fraction due to the roasting process. Coffee extracts
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366 and their fractions revealed mixed-type to competitive inhibition mechanisms against α -glucosidase,
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21
367 which are consistent with the complexity of samples composition, as well as with literature findings
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24
368 relevant to various vegetable matrices.

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369 Although the results of this study are far to clearing-up the effect of coffee consumption on
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370 diabetes, nevertheless they indicate that coffee negatively affects the *in vitro* activity of one key
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371 enzyme in the carbohydrate digestive process, suggesting the potential of coffee and coffee
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372 derivatives as adjuvants for the treatment of type 2 diabetes. This hypothesis should be verified also
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36
373 considering eventual interactions occurring between food components and the drugs generally used
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374 to treat the disease. Nonetheless, this approach could represent a starting point for the development
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41
375 of integrated strategies to treat and prevent type 2 diabetes. *In vivo* validation of this activity would
43
376 open the possibility to make claims about coffee preventing type 2 diabetes.

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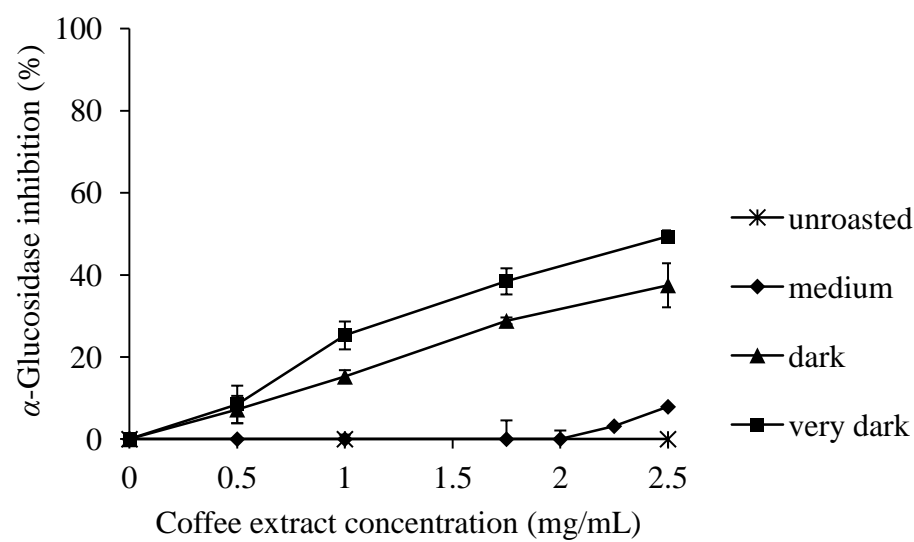


Fig. 1.

Figure

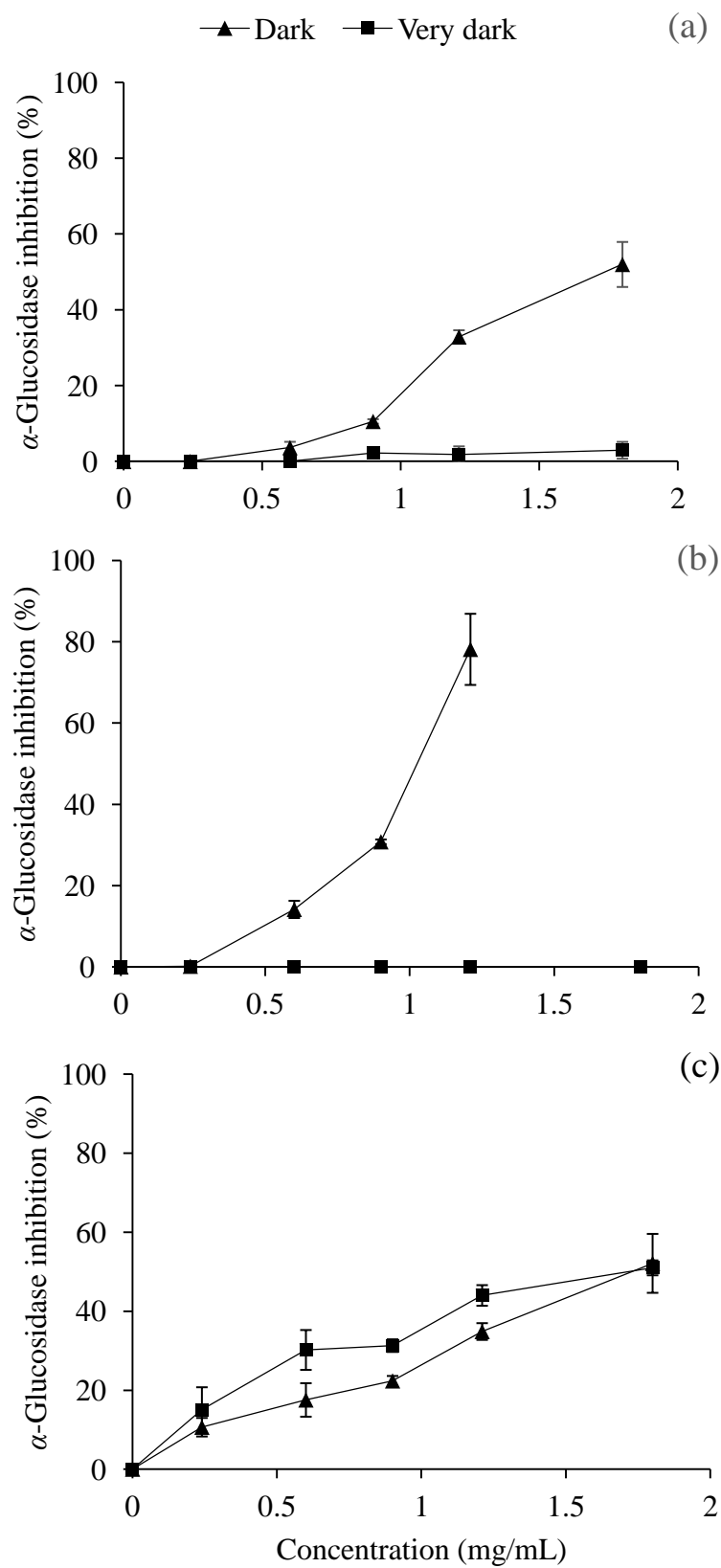


Fig. 2.

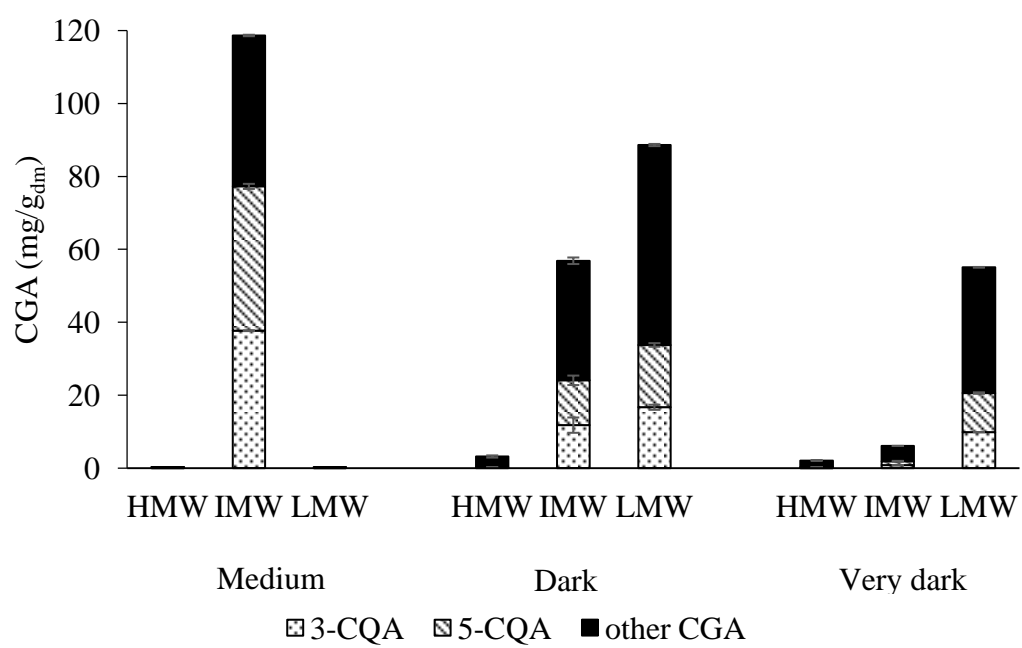


Fig. 3.

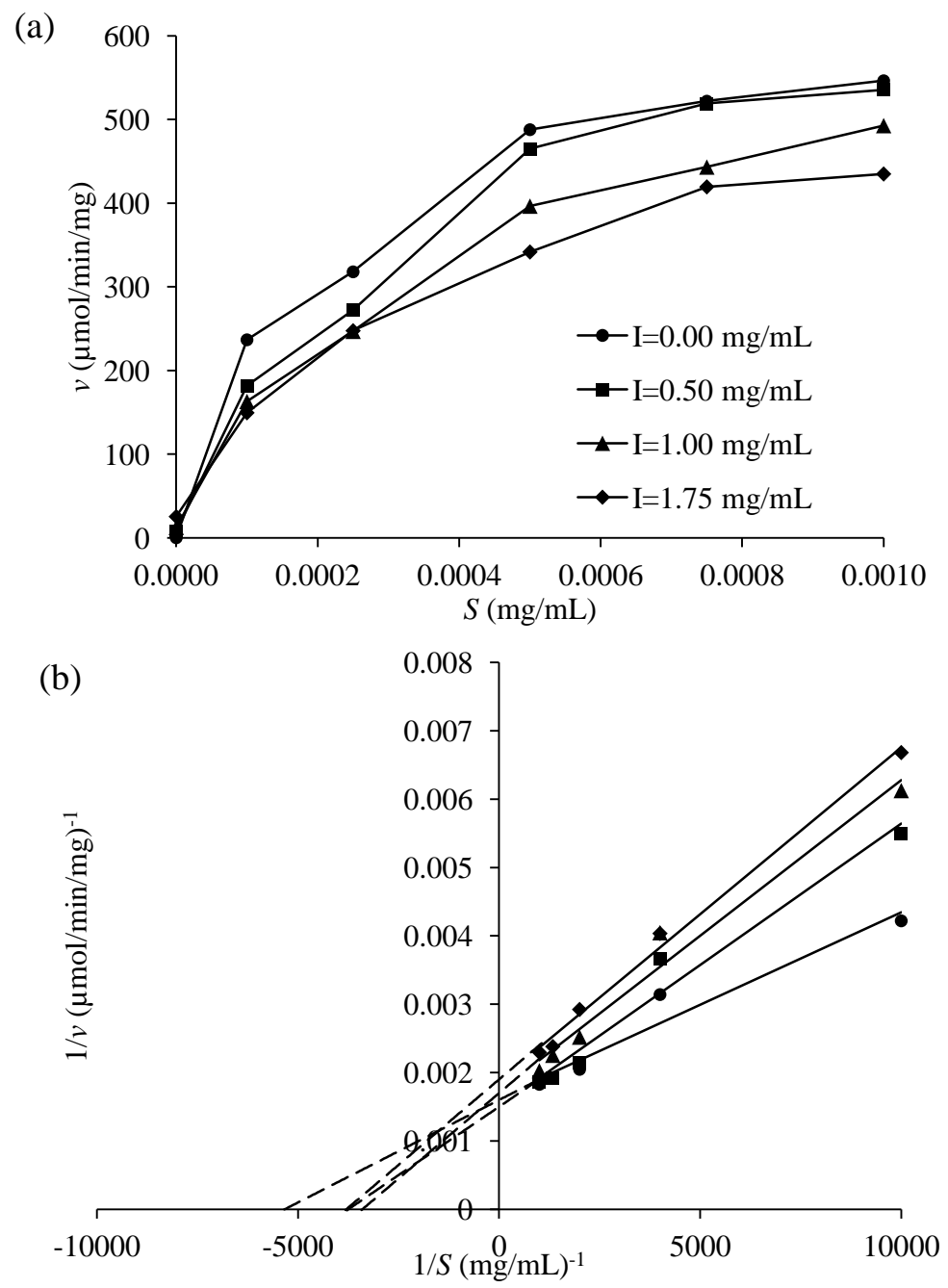


Fig. 4.

Table 1

Weight loss, total solid content and color of ground Robusta coffee beans subjected to increasing lengths of roasting at 200 °C, and total phenolics (TP), chlorogenic acids (CGA), 3-caffeoylquinic acid (3-CQA) and 5-caffeoylquinic acid (5-CQA) contents of the corresponding coffee extracts.

Roasting time (min)	Degree of roast	Ground coffee				Coffee extract			
		Weight loss (%)	Total solid content (%)	L*	Hue angle (tan ⁻¹ b*/a*)	Phenolic compounds			
						TP	CGA	3-CQA	5-CQA
						(mg/g _{dm})	(mg/g _{dm})	(mg/g _{dm})	(mg/g _{dm})
0	Unroasted	-	95.5 ± 0.1 ^b	67 ± 1 ^a	80 ± 1 ^a	16.2 ± 0.2 ^a	13.8 ± 0.6 ^a	6.4 ± 0.3 ^a	1.2 ± 0.0 ^a
15	Medium	9	99.4 ± 0.1 ^a	48 ± 1 ^b	68 ± 0 ^b	15.9 ± 0.1 ^a	10.8 ± 0.3 ^b	3.8 ± 0.1 ^b	1.6 ± 0.0 ^a
45	Dark	12	99.5 ± 0.2 ^a	37 ± 1 ^c	25 ± 6 ^d	11.3 ± 0.2 ^b	2.6 ± 0.3 ^c	0.6 ± 0.1 ^c	0.5 ± 0.0 ^b
60	Very dark	15	99.9 ± 0.1 ^a	25 ± 1 ^d	33 ± 1 ^c	8.4 ± 0.1 ^c	0.9 ± 0.1 ^d	0.2 ± 0.0 ^d	0.2 ± 0.0 ^c

Table 2

*IC*₅₀ (half-maximal inhibitory concentration) and Acarbose equivalents of coffee extracts, 3-caffeoylquinic acid (3-CQA) and 5-caffeoylquinic acid (5-CQA).

Inhibitor	<i>IC</i> ₅₀ (mg/mL)	Acarbose equivalents (<i>IC</i> ₅₀ Acarbose/ <i>IC</i> ₅₀ inhibitor)
Medium roasted coffee extract	2.92	0.03
Dark roasted coffee extract	3.24	0.03
Very dark roasted coffee extract	2.41	0.04
3-CQA	0.86	0.11
5-CQA	0.44	0.21
Acarbose	0.09	1

Table 3

High, intermediate and low molecular weight fractions (HMW, IMW, LMW) content (%) in medium, dark and very dark roasted coffee extracts.

Fraction	Molecular weight (Da)	Composition *	Content (%)		
			Medium	Dark	Very dark
HMW	>100	Melanoidins	3	12	8
IMW	15-60	Intermediate-molecular-weight compounds	61	38	2
LMW	1-6	Phenolic compounds	36	50	90

* according to Borrelli *et al.* (2002)

Table 4
Kinetic parameters of *in vitro* α -glucosidase activity in the absence and in the presence of increasing concentrations of extract or high, intermediate and low molecular weight fractions (HMW, IMW, LMW) of dark or very dark roasted coffee.

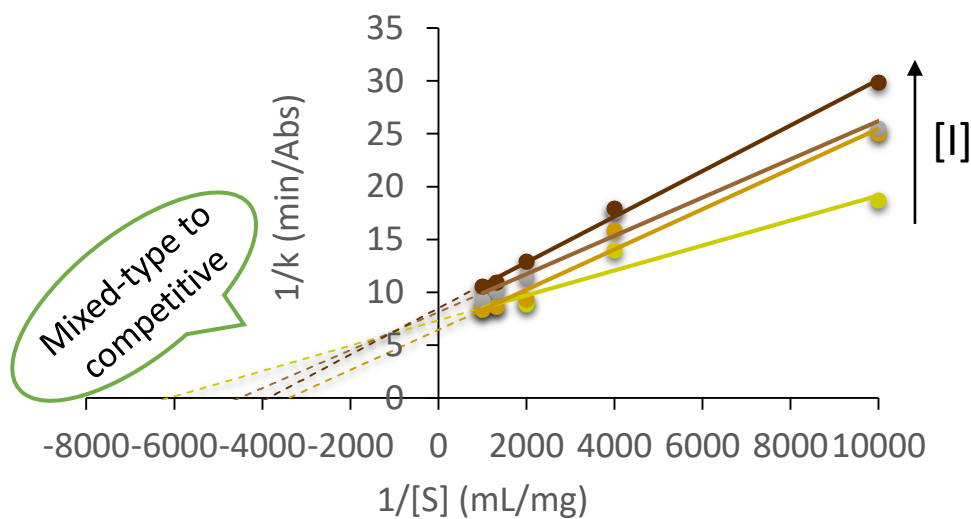
Roasting degree	Sample	I (mg/mL)	V _{max} (μmol/min/mg)	V _{max} * (μmol/min/mg)	k _m (μg/mL)	k _m * (μg/mL)	k _i (μg/mL)	k _i ' (μg/mL)	Type of inhibition
Dark	Extract	0.00	714	-	0.17	-	1,472	12,167	Mixed-type
		0.50	-	667	-	0.21			
		1.00	-	667	-	0.31			
		1.75	-	625	-	0.36			
	HMW	0.00	833	-	0.50	-	1,260	5,460	Mixed-type
		0.24	-	833	-	0.50			
		0.42	-	769	-	0.62			
	IMW	0.00	625	-	0.13	-	206	-	Competitive
		0.12	-	667	-	0.20			
		0.24	-	667	-	0.27			
	LMW	0.00	625	-	0.13	-	360	-	Competitive
		0.12	-	625	-	0.19			
		0.24	-	625	-	0.19			
Very dark	Extract	0.00	625	-	0.19	-	2,063	14,042	Mixed-type
		1.00	-	588	-	0.29			
		1.75	-	526	-	0.26			
	LMW	0.00	1000	-	0.40	-	420	1,120	Mixed-type
		0.42	-	625	-	0.50			

Coffee inhibits α -glucosidase

Inhibitory effect



Inhibition mechanism



Properly selected coffee may help
the prevention of type 2 diabetes