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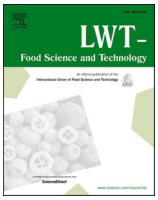

A tetraazahydroxypyridinone derivative as inhibitor of apple juice enzymatic browning and oxidation

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ABSTRACT

Enzymatic browning in fruits and vegetables can produce undesirable colour changes and adversely affect the taste, flavour, and nutritional value. This fact poses a challenge to the food industry to apply appropriate inhibitors to control enzymatic browning to maintain food quality. Accordingly, this study aims to evaluate the effect of small mazamacrocyclic compounds modified with a hydroxypyridinone similar to kojic acid on enzymatic browning, total polyphenols and antioxidant activity in apple juice. The results showed how these compounds interact with the tyrosinase enzyme in a complex interaction inhibiting its activity. The hydroxypyridinone attached to the macrocycle (**I1**) was crucial to induce the greatest inhibition, being the most powerful inhibitor. The kinetic studies of **I1** reveal mixed-type inhibition over tyrosinase with an IC_{50} of 0.30 mM, which was much higher than the calculated IC_{50} for **I2** and **I3**. Furthermore, **I1** at a concentration of 2.25 mM, significantly reduced the enzymatic browning in fresh apple juice by more than 50% after 1 h under stirring. Also, it completely stops the decrease in the total phenolic content and delays loss of antioxidant capacity during the first 30 min.

1. Introduction

Enzymatic browning is induced by mechanical and physical stresses taking place during post-harvest processing and storage in a wide range of products including fruits (e.g., apple, bananas and grapes), vegetables (e.g., potatoes, mushrooms and lettuce) and even seafood (e.g., shrimps, spiny lobsters and crabs) (Aksoy, 2020). It generates not only colour changes, but also adverse effects on taste, flavour, and nutritional value losing polyphenols and antioxidants, among others.

Enzymatic browning is one of the main obstacles in the industrialization of some fruit/vegetables products (Es-Safi, Eronique Cheynier, & Moutounet, 2003). Polyphenol oxidase (PPO), which is primarily responsible for the enzymatic browning, is a copper-containing enzyme belonging to the oxidoreductase family. The Enzyme Commission (EC), according to its substrate specificity, has classified them in EC1.14.18.1 (cresolase or tyrosinase) and EC1.10.3.1 (diphenol oxidase or catechol oxidase) (Derardja, Pretzler, Kampatsikas, Barkat, & Rompel, 2019; Moon, Kwon, Lee, & Kim, 2020). In enzymatic browning, PPO catalyses the oxidation of monophenols into o-quinones which is followed by a non-enzymatic polymerization. The polymerization of the quinones gives rise to reddish-brownish pigments called melanonids, which are the reason for colour change (Kanteev, Goldfeder, & Fishman, 2015).

Apples (*Malus domestica*) are the third most cultivated fruit worldwide, with a production of around 87 million tons in 2019 (FAOSTAT, 2019), with a significant part of total production meant for apple juice (e.g. in the U.S. IN 2018; it was approximately 12% of the total) (Bortolini et al., 2020). Apple juices are highly susceptible to this deteriorative mechanism since apples contain both active PPO and high polyphenol content (Bondonno, Bondonno, Ward, Hodgson, & Croft, 2017; Martino et al., 2019).

Currently, there is an increasing trend to consume fresher and minimally processed foods in a way that preserves the sensory and nutritional properties of raw foods. Colour changes derived from enzymatic browning cause rejection by the consumers, leading to high amounts of food waste and economic losses for the industry. In response, the food industry has developed different methods for the prevention of

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the enzymatic browning (Muñoz-Pina, Ros-Lis, Argüelles, Martínez-Máñez, & Andrés, 2020). Thermal treatments are the most widely used physical method in the food industry, especially in beverages production. However, it has major drawbacks since it affects the nutritional quality of food, affecting thermosensitive nutrients such as vitamins, carotenoids, and anthocyanins. Other technologies have been explored as an alternative to thermal processing for fruits and vegetables, and especially for apple juice, such as ultra-high-pressure homogenization, high-pressure carbon dioxide, or cold plasma (Tinello & Lante, 2018). Nonetheless, its application in the industry is still scarce as they have some limitations such as negative effects on the nutritional quality of products or high cost.

Chemical inhibitors offer an interesting alternative due to their lower cost, manageability, and minimal alteration of the bioactive compounds. Among all the possibilities, only sulphites or acidifiers are a real alternative to thermal treatments in the food industry. In the case of acidifiers, such as citric acid and ascorbic acid, they inactivate the PPO by lowering the pH value while acting as a chelator or reducing agent (Zhou et al., 2020). On the other hand, reducing agents like sulphites and their derivatives act as irreversible inhibitors of PPO. Nevertheless, the taste alteration and the allergies in the population (to sulphites, for example) have restricted their use (Iyengar & McEvily, 1992).

Therefore, the challenge for the food industry is to apply appropriate inhibitors to control enzymatic browning maintaining the quality and extending shelf life while meeting current consumer needs. Inhibitors of synthetic origin have been reported to inhibit the PPO. Synthetic polyphenols like deoxybenzoins or synthetic isoflavones, thiosemicarbazones, or chalcones have shown effective ppo inhibition. in fact, in some cases, they have shown greater inhibiting power than their natural analogues (Zolghadri et al., 2019).

Polyamines like chitosan, a polymer containing several organic groups, has been studied in food science for its potential (Huang, Huang, Huang, & Chen, 2007) and also has demonstrated its effectiveness as inhibitor of enzymatic browning (Sapers, 1992). Furthermore, macrocyclic polyamines have also shown great PPO inhibition depending on the number of amino groups and chemical structure, and additionally are very easy to functionalise or branch with different chemical groups (Muñoz-Pina, Ros-Lis, Delgado-Pinar, et al., 2020). On the other hand, kojic acid is very well known as a powerful inhibitor that acts as a reducing agent and as a non-competitive inhibitor, becoming a positive reference when testing new PPO inhibitors (Zolghadri et al., 2019). Based on these key features, therefore, we hypothesize that combining a polyamine with a functional group like kojic acid could be an interesting approach for the development of a new family of PPO inhibitors. Thus, a PPO inhibitor that includes a tetraazamacrocyle modified with a hydroxypyridinone similar to kojic acid has been prepared (**I1**) and its effect on enzymatic browning, total polyphenols and the antioxidant activity of apple juice has been studied.

2. Materials and methods

2.1. Chemicals

Commercial tyrosinase enzyme (2687 U/mg), dopamine hydrochloride, kojic acid, 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) and Folin Ciocalteu reagent (2N) were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). Sodium bisphosphate, disodium phosphate and sodium carbonate were acquired from Scharlau (Sharlab S.L., Spain) and anhydrous sodium acetate from Panreac AppliChem (Panreac AppliChem, Barcelona, Spain). Lastly, methanol (99.9%) was purchased from Honeywell (Honeywell, France). The apple juices used in the tests were obtained in the laboratory by liquefying apples (Golden Delicious variety) obtained in a local store.

The inhibitors **I1**, **I2**, and **I3** were synthesised according to known procedures: Verdejo et al., 2007 and López-Martínez et al., 2016.

2.2. Study of enzyme-inhibitor interaction over tyrosinase

The effect of the inhibitors (0.67 mM) over commercial PPO (tyrosinase from mushroom) (93.75 U) was tested in presence of dopamine (2.5 mM) at different pH (4.5, 5.5 and 6.5) in a phosphate-acetate buffer of 10 mM (Muñoz-Pina, Ros-Lis, Delgado-Pinar, et al., 2020). For each pH, a sample in absence of the inhibitor was used as a control. The dopamine oxidation reaction was followed spectrophotometrically at 420 nm by a DU-730 Life Science UV/Vis spectrophotometer (Beckman Coulter, USA). The assays were done in triplicate at 24 °C, measurings the absorbance each 10 s for 5 min. From the absorption-time curves, the initial speed of the reaction was determined from the slope of the linear stretch of the absorption-time curves. The different slopes were used to compare the inhibitory effect of the tested compounds against the control (see equation (1)).

$$PPO\ inhibition\ (\%) = \left(\frac{V_{0i}}{V_{00}} * 100 \right) \quad (1)$$

Where V_{o0} is the control initial rate and V_{oi} is the initial rate obtained for the different samples.

2.3. Enzyme kinetics in model system

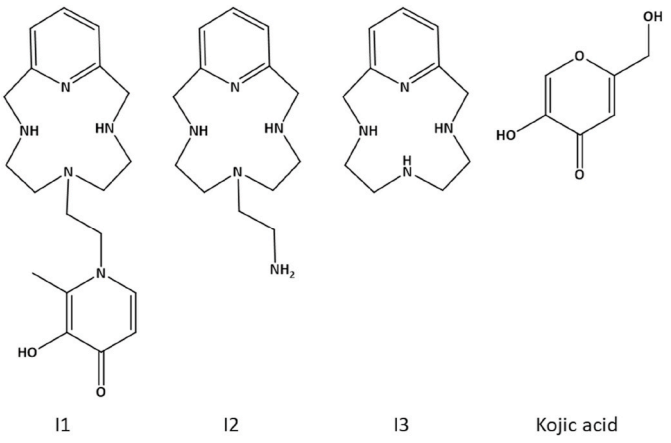
To determine the inhibition type for the different compounds, a model system was designed where commercial PPO (tyrosinase from mushroom) was reacted with the inhibitor at pH (5.5) using phosphate-acetate buffer 10 mM. A one millilitre solution of varying concentrations of dopamine with a final concentration of 0.08 mM–2 mM was prepared. Then, 0.50 mL of inhibitor/enzyme mixture was subsequently added. The final concentration of enzyme in the assay was 94 U and two inhibitor concentrations were tested (0.66 and 1.5 mM). Enzyme activity was determined by the formation of dopachrome following the increase in absorbance at 420 nm for 5 min ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) in a Spectrophotometer Beckman Coulter DU-730 Life Science UV/Vis (Beckman Coulter, USA). (Vermeer, Higgins, Roman, & Doorn, 2013). Since the enzymatic reaction of polyphenol oxidase follows Michaelis−Menten kinetics (Espín et al., 2000), the type of inhibition was determined by calculating K_m and v_{max} constants using the classical method of Lineweaver-Burk representation as a linearization method (Doran, 1998; Fan, Ding, Zhang, Hu, & Gong, 2019).

2.4. Inhibitory effect of enzymatic browning over apple juice

Apple juice from cv. Golden Delicious obtained in the laboratory was selected to verify the effect of the best inhibitor on real samples. The inhibitory effect was measured following the procedure reported by Muñoz-Pina, Ros-Lis, Argüelles, et al., 2020 with slight modifications. Briefly, 2 mL of an aliquot of the cloudy juice were put in contact with 1 mL of an I1 solution, to get a final concentration of 1.12 mM and 2.25 mM. These blends were kept under agitation (400 rpm) for 60 min to accelerate the oxidation process and the colour change was measured with a spectrophotometer Minolta CM-3600 d (Konica Minolta, Japan) at 0, 5, 10, 20, 30, and 60 min. CIE L*a*b* (CIELAB) colour space which comprises L* (lightness), a* (red to green) and b* (yellow to blue) coordinates were obtained using a D65 illuminant and 10° observer as a reference system. The total colour difference (ΔE*) between the juice at time 0 and treated samples was calculated following equation (2)

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (2)$$

The colour difference between the initial colour and the samples can be classified as not noticeable (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5–3.0), well visible (3.0–6.0) and great (6.0–12.0), depending on the value of ΔE_{ab}^* (Xiang et al., 2020).



Scheme 1. Molecular structures of the inhibitors **I1**, **I2**, **I3** and kojic acid.

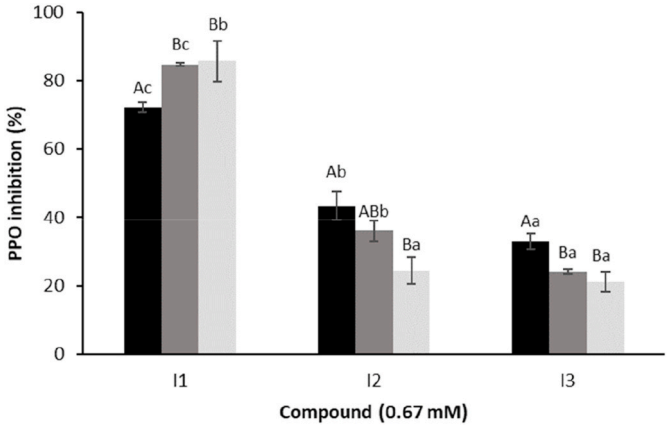


Fig. 1. Polyphenol oxidase (94 U) inhibition in presence of the three different aza-macrocyclic inhibitors (0.67 mM) using dopamine as substrate (2.5 mM) at different pH: 4.5 (black), 5.5 (grey) and 6.5 (light grey). A–C: different letters indicate significant differences in PPO inhibition between pH. a–c: different letters indicate significant differences between inhibitors ($p < 0.05$).

2.5. Determination of total phenolic content (TPC) and antioxidant activity

Total phenolic content was determined using the Folin-Ciocalteu method (Waterhouse, 2002; Aranibar et al., 2018). Briefly, 10 μL of juice sample (see section 2.4) was diluted into 1.58 mL of deionized water and then mixed well with 100 μL of the Folin-Ciocalteu. After reacting for 3 min, 300 μL of Na_2CO_3 (20% w/v) was added to the mixture and left in the dark for 60 min before measuring the absorbance at 765 nm. A blank with the inhibitors was also measured and subtracted if present. The calibration curve was prepared with standard solutions of gallic acid (from 0 to 500 mg/L) by following the same method. The results were expressed as gallic acid equivalent (GAE) (mg GAE/L).

The antioxidant activity of the juices by the DPPH method was determined following the method described by Wu et al. (Wu et al., 2020) with slight modifications. A sample of 10 μL of juice (see section 2.4) was added to 1 mL of methanolic solution of DPPH (100 μM). Then, the mixture was kept in the dark for 30 min and absorbance was measured at 517 nm using a UV–Vis spectrophotometer (Thermo scientific Helios-zeta, USA). The results were determined based on the standard calibration curve from 0 to 200 mg/L of Trolox. The data were expressed as Trolox equivalent (mg Trolox/L). All assays were done in triplicate.

2.6. Software and data analysis

Species distribution curves were calculated with the Hyss2009 software of Hyperquad using the protonation constants published in López-Martínez et al., 2016. Data are reported as mean ± standard deviation. Statgraphics Centurion XVII software was used to perform the analysis of variance (One-Way ANOVA) and Multiple Range Tests by the LSD procedure (least significant difference) of the Fisher test to identify homogeneous groups. A confidence level of 95% (p-value < 0.05) was used.

3. Results and discussion

3.1. Impact of pH on inhibitor-PPO interaction

As noted above, aza-macrocyclic compounds have proved their efficiency in binding PPO via supramolecular interactions. The central cavity can be branched and/or functionalized to produce a wide variety of new compounds with different chemical properties enhancing their supramolecular interactions. 6-[2′-(3′-hydroxy-2″-methyl-4′-pyridinone)ethyl]-3,6,9-triaza-1(2,6)-pyridinacyclodecaphane (**I1**) was synthesised as a model by a combination of a hydroxy pyridinone group (similar to kojic acid) with a tetraazamacrocycle. To better understand the influence of the chemical structure on the inhibitor activity, also the related compounds 3,6,9-triaza-1(2,6)-pyridinacyclodecaphane (**I3**) and 2-(3,6,9-triaza-1(2,6)-pyridinacyclodecaphane-6-yl)ethan-1-amine (**I2**) were synthesised. (see Scheme 1).

As a first approach, to analyse their inhibitory power, a preliminary screening was carried out in the pH range where PPO is active (4.5, 5.5, 6.5). The pHs were selected considering that the behaviour of polyamines is pH-dependent and most of the fruits that show enzymatic browning present a pH in this range.

In general terms, all the tested substances reduce the initial reaction rate, which can be translated as a partial inhibition of the enzymatic activity. The degree of inhibition varies strongly among the assays with

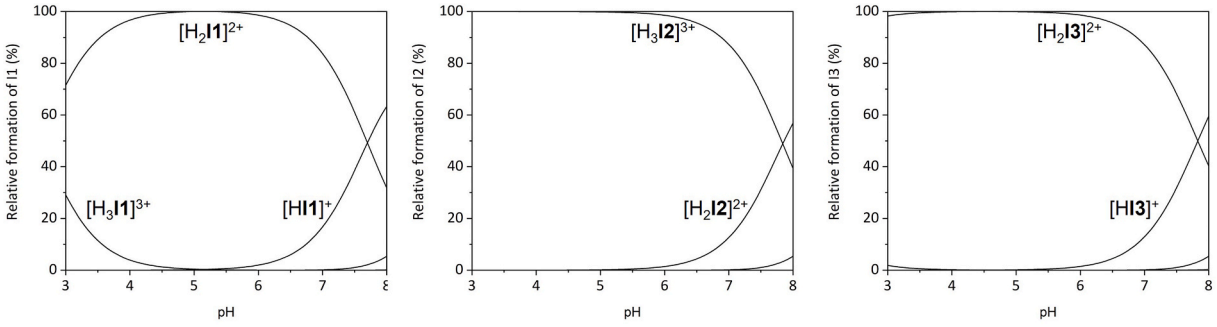


Fig. 2. Species distribution curves calculated for the aqueous solution of the inhibitor. Percentage represents concentrations relative to the total amount of inhibitor at an initial value of 0.67 mM.

Table 1
Kinetic parameters and type of inhibition of the enzyme tyrosinase (94 U) in presence of the inhibitors at different concentrations.

Compound (mM)		K_M[a](mM)			v_{max} [b] (mM min^{-1})			k_{cat}[c](min^{-1})			Catalytic efficiency (mM^{-1} min^{-1})			IC_{50} (mM)	Inhibition type
Control		1.52*	±	0.09	0.259	±	0.014	1350	±	80	888	±	108		
I1	0.67	$1.02^{+\S}$	±	0.07	0.064^{+}	±	0.003	329	±	14	320	±	40	0.30 ± 0.05 (0.18 mg/mL)	Mixed type
	1.50	0.33	±	0.03	0.0157	±	0.0008	81	±	4	250	±	30		
I2	0.67	$1.35^{*+¥}$	±	0.19	0.151*	±	0.008	780	±	40	577	±	108	0.79 ± 0.06 (0.45 mg/mL)	Mixed type
	1.50	$0.77^{\S}$	±	0.08	0.035^{+}	±	0.003	178	±	16	230	±	50		
I3	0.67	1.85*	±	0.08	0.222	±	0.008	1140	±	50	620	±	50	1.82 ± 0.16 (0.81 mg/mL)	Non-competitive
	1.50	1.7*¥	±	0.2	0.151*	±	0.019	784	±	108	460	±	140		

The enzyme (tyrosinase from mushroom) was constant for all assays (94U) [a] Michaelis-Menten constant. [b] Reaction rate. [c] Catalytic constant, $k_{cat} = v_{max}/[E]$. [d] Catalytic efficiency, calculated by k_{cat}/K_M. *+§¥ There are no statistically significant differences for $p < 0.05$ between rows for K_M or v_{max}. **I1** refers to the ligand 6-[2′-(3′-hydroxy-2″-methyl-4′-pyridinone)ethyl]-3,6,9-triaza-1(2,6)-pyridinacyclodecaphane; **I2** concern to the compound 2-(3,6,9-triaza-1(2,6)-pyridinacyclodecaphane-6-yl)ethan-1-amine and **I3** is the compounds 3,6,9-triaza-1(2,6)-pyridinacyclodecaphane.

a decrease in the initial PPO rate from 20 to 85% depending on the pH and the inhibitor (Fig. 1). Initially, the unfunctionalized macrocycle (**I3**) reduces the PPO activity but to a lesser extent than the other inhibitors. The effect decreases as the pH increases reaching 20% at pH 6.5. Similar behaviour is reported by **I2**, however, the extra alkyl amino chain enhances its inhibitory power, especially at pH 4.5 showing a 40% of inhibition. Finally, it is noticeable how the presence of the 3-hydroxy-4-pyridinone functional group (similar to kojic acid) in **I1** increases the inhibition, inducing the greatest inhibition among the inhibitors. At pH 5.5 and 6.5, it reaches an almost complete PPO inhibition (85%) but at low pH, the inhibition slightly decreases (72%).

Taking into account that the tested inhibitors can be present as diverse species depending on the pH, the distribution diagram of species at the studied pH range can be found in Fig. 2 (Verdejo et al., 2007). **I1**, **I2** and **I3** share the tetraaza macrocycle unit that contains two protonated amino groups at slightly acidic pH because the third protonation needs very acidic conditions due to electrostatic repulsions with the other cationic groups. The 3-hydroxypyridinone unit is able to lose one proton at basic pH, thus it is neutral under the pH of study. In the 4.5–6.5 pH range, the main species are $[H_2\mathbf{I1}]^{2+}$, $[H_3\mathbf{I2}]^{3+}$ and $[H_2\mathbf{I3}]^{2+}$.

Since the isoelectric point of PPO is placed around pH 4.5–5 (Y. Fan & Flurkey, 2004) certain repulsion between the PPO and the positive charges could be expected in particular at pH 4.5. For **I2** and **I3** the percentage of PPO inhibition increases as the pH moves from 6.5 to 4.5, and **I2**, with an additional positive charge (+3) offers a slightly stronger degree of inhibition than I3. On the contrary, at pH 6.5 a lower concentration of the partially protonated compounds $[H\mathbf{I1}]^{+}$, $[H_2\mathbf{I2}]^{2+}$ and $[H\mathbf{I3}]^{+}$ is present, which could explain the slight reduction of inhibition found for **I2** and **I3** at this pH. These results suggest that the molecule interacts with the enzyme through complex interactions. In accordance with the behaviour observed for similar systems based on cyclic polyamines, an increase in the number of nitrogens improves the inhibition activity (Muñoz-Pina, Ros-Lis, Delgado-Pinar, et al., 2020). In any case, the most remarkable observation is that the pyrone group present in **I1** quadruples the inhibitory activity (an 85% in comparison with the 21% of **I3** at pH 6.5). However, the macrocyclic cavity, although to a lesser degree compared to the pyrone, also exhibits an inhibitory effect.

Taking into account these results, and in order to get a deeper insight, the pH 5.5 was selected for further testing as PPO still had high activity (around 85%) (Munjal & Sawhney, 2002) but was close to or similar to the pH found in some fruit juices like banana or pear (Bora, Handique, & Sit, 2017; Saeeduddin et al., 2017).

3.2. Inhibition mechanisms of aza-macrocyclic compounds

The classical method of the Lineweaver-Burk plot was used to determine the kinetic parameters to identify the inhibition mechanism of each inhibitor (see Table 1).

The kinetics parameters for the control were, in the case of Michaelis-Menten constant, K_m = 1.52 mM and 0.26 mM min^{-1} for v_{max}. As expected, the v_{max} reaction rate of the PPO was drastically reduced in the presence of high concentrations of **I1** (0.0157 mM min^{-1}) achieving almost a total inhibition. In addition, the Michaelis-Menten constant values progressively decreased as the inhibitor concentration increased with statistically significant differences with respect to the control. Therefore, as the inhibitor favors binding to the enzyme-substrate complex, the observed inhibition can be considered a mixed type non-competitive-uncompetitive inhibition (Palmer & Bonner, 2011). The catalytic constant (K_{cat}) and the catalytic efficiency (K_{cat}/Km) of the enzyme also plummeted from values close to 1000 to values below 250 for both constants. In fact, the catalytic constant drops 94% when the concentration of **I1** in the medium is 1.5 mM, which implies that the efficiency of the enzyme is much lower in its presence.

Same v_{max} and K_m parameters were found (without statistical differences) for **I2** at double concentration (1.50 mM) compared to those

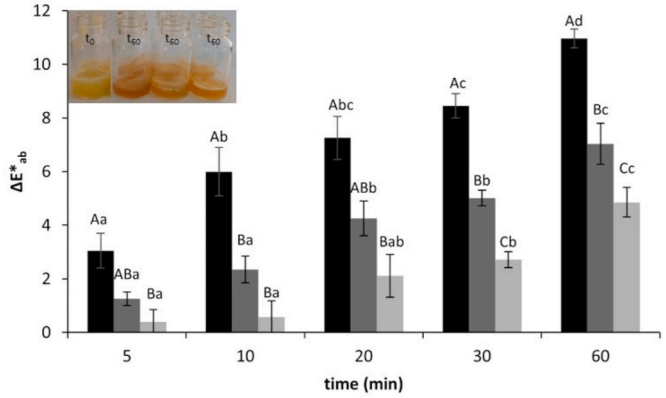


Fig. 3. Colour changes in apple juice of control (black), in presence of 1.12 mM of **I1** (grey) and 2.25 mM of **I1** (light grey) over time. A–C: different letters indicate significant differences in ΔE_{ab}* between **I1** concentrations. a–c: different letters indicate significant differences between times ($p < 0.05$). Inset: Colour evolution in apple smoothie followed by images after 60 min. First the control, and then the sample treated with 1.12 mM and 2.25 mM of **I1** respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

found for **I1** at 0.65 mM. Although the type of inhibition is assumed to be the same (mixed type), it appears that the pyrone group offers additional inhibition power that the amine group cannot perform (see Table 1). However, **I2** also achieves a high inhibition on the PPO in the concentrations studied since it manages to reduce the catalytic constant of the enzyme (1350 min^{-1}) to almost half when the concentration is 0.67 mM.

However, the last compound, **I3**, behaves differently, exhibiting a non-competitive inhibition on the PPO enzyme. The Michaelis−Menten constant maintains the control values when altering the inhibition concentration, but the v_{max} drops off. Contrasted with the other two inhibitors, **I3** is not capable of a strong inhibition on the PPO, being necessary to double the concentration to produce an equal K_{cat} as **I2**.

Besides, the IC_{50} parameter for **I2** and **I3** is much higher than for **I1** which was calculated at 0.30 mM. In previous studies it was observed how the molecules that presented the same central ring inhibited the PPO enzyme to a lesser extent, making it necessary to increase the central cavity to improve the IC_{50} to around 0.30 mM. However when the central cavity is functionalized with the group similar to kojic acid, the IC_{50} rose to 0.30 mM (Muñoz-Pina, Ros-Lis, Delgado-Pinar, et al., 2020). However, this value cannot reach the IC_{50} values obtained for either kojic acid (0.011 mM) or other azamacrocyclic ligands under the same conditions (Muñoz-Pina, Ros-Lis, Delgado-Pinar, et al., 2020). Nevertheless, this IC_{50} value of the ligand is substantially better than the value found for other commonly used organic acids found in literature, such as citric acid (20 mM), cinnamic (2.10 mM), or benzoic acid (5.2 mM) (Liu et al., 2013; Öz, Colak, Özel, Saglam Ertunga, & Sesli, 2013; Shi, Chen, Wang, Song, & Qiu, 2005).

3.3. Browning inhibition of I1 on apple juice

Considering the positive results obtained for II **I1** in model system studies with PPO, we further studied its anti-browning ability in fresh apple juice as a real food system. It is also important to note that preliminary studies of **I1** with two cell lines, HELA and ARPE-19, do not reveal any toxicity (López-Martínez et al., 2016). The liquified juice was mixed with two different concentrations of **I1** (1.12 mM and 2.25 mM) and the colour change was followed over time. Fig. 3 shows the colour differences (ΔE_{ab}*) using the initial colour as reference.

Once the apples are liquified, the enzymatic browning process begins rapidly, and the colour change of the control (in absence of inhibitor) is well visible to the human eye in 5 min (ΔE_{ab}* = 3). However, in the presence of the inhibitor at 2.25 mM the colour changes are not noticeable. At a lower concentration (1.12 mM), the inhibitor delayed the enzymatic browning, reaching a notable change of colour with a ΔE_{ab}* close to 2.5 at 10 min, similar to the control at 5 min.

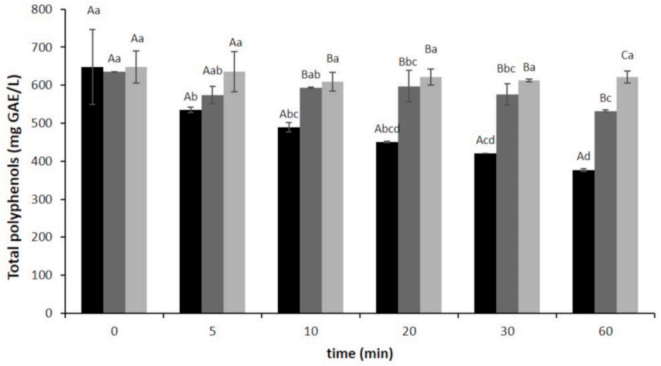


Fig. 4. Total phenol content of apple juice of control (black), in presence of 1.12 mM of **I1** (grey) and 2.25 mM of **I1** (light grey) during the time. A–C: different letters indicate significant differences in total polyphenols between concentrations. a–c: different letters indicate significant differences between times ($p < 0.05$).

Even though the speed of PPO decreased in presence of the inhibitor, the enzymatic browning reaction did not stop completely. The colour change in the juice treated with the lowest **I1** concentration (1.12 mM) varies among the 40 and 65% when compared to the untreated sample at the same time. By raising the inhibitor concentration in the juice to 2.25 mM, the enzymatic browning is further delayed with a ΔE_{ab}* of 0.6 after 10 min of stirring compared to 6.0 found for the control. The threshold of colour change to be qualified as "great" is passed within 10 min in absence of inhibitor and 60 min at 1.12 mM. However, it is not reached if a [**I1**] = 2.25 mM is used even after 1 h of exposition to the air under stirring. When measuring the initial browning rate, values for the slope of 0.601, 0.219 and 0.084 ΔE_{ab}* min^{-1} were calculated for the control and the juices containing 1.12 mM and 2.25 mM respectively ($R^2 > 0.95$). Therefore, reductions in the initial enzymatic browning rate of 86% can be observed in presence of [**I1**] equal to 2.25 mM.

3.4. Effect of I1 on total phenolic content (TPC) and antioxidant capacity (DPPH) on apple juice

Apple and their juices contain high concentrations of diverse phenolic compounds such as chlorogenic acid, gallic acid, catechin and quercetin, which are well known for their beneficial effects on human health (Barreira, Arraibi, & Ferreira, 2019). Thus, the effect of the

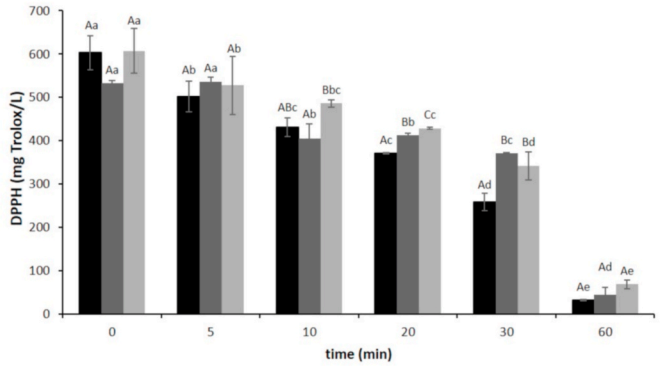


Fig. 5. Antioxidant activity (DPPH) of apple juice of control (black), in presence of 1.12 mM of **I1** (grey) and 2.25 mM of **I1** (light grey) during the time. A–C: different letters indicate significant differences in DPPH between **I1** concentrations. a–c: different letters indicate significant differences between times ($p < 0.05$).

inhibitor over total phenolic content and antioxidant capacity (DPPH) was measured.

As shown in Fig. 4, the initial content of total phenols was about 600 mg of GAE L^{-1} just after liquefying the apple which is in agreement with the range presented in the literature for the same apple variety (Sauceda-Gálvez et al., 2020; Suárez-Jacobo et al., 2011). However, this value decreased significantly ($p < 0.05$) as time progressively evolved, reducing the content of phenols by almost 40% after 60 min of stirring. This trend was repeated when the samples were treated with 1.12 mM inhibitor, however, the degradation rate of phenol was slowed down and only 15% was lost after 60 min. Nonetheless, the decrease in the total phenolic content in the apple juice stopped and remained stable after 60 min in the presence of 2.25 mM of **I1** ($p < 0.05$). The results also showed a good negative correlation (−0.756) between the TPC and enzymatic browning (ΔE_{ab}^*) for $p < 0.05$, as expected from the oxidation of phenolic compounds used as PPO substrates (Persic, Mikulic-Petkovsek, Slatnar, & Veberic, 2017). This correlation is stronger for the control (−0.926) than for the juices containing inhibitor (−0.636 for 1.12 mM and −0.266 for 2.25 mM), suggesting that the PPO inhibition induced by **I1** could be more intense than we can deduce from the enzymatic browning.

The antioxidant capacity measured with DPPH can be found in Fig. 5. As time increases and oxidation occurs, the antioxidant activity of fresh, cloudy apple juice significantly decreases. When **I1** is added, even though after 20 and 30 min this effect is significantly diminished, at longer times the loss of radical scavenging species is comparable to the control.

According to Pearson's correlations, the reduction of antioxidant properties expressed by the DPPH method is significantly correlated with the enzymatic browning for the control (−0.931) and both concentrations of inhibitor (−0.871 for 1.12 mM and −0.906 for 2.25 mM). However, if we analyse the correlation with the total phenolic content in the presence of 2.25 mM of **I1,** no significant correlation is found (0.139 $p > 0.05$). This behaviour can be explained considering that the DPPH test measures not only phenols but also other compounds present in the apple juice such as vitamins (vitamin C and E), heteropolysaccharides and polypeptides (Liu, Shi, Colina Ibarra, Kakuda, & Jun Xue, 2008; Liu et al., 2020). Similar results were found on fresh lettuce when testing citric acid and oxalic acid as inhibitors, where no correlation was found between antioxidant activity and total phenolic content in the presence of the inhibitors (Altunkaya & Gökmen, 2008). Also, in a recently published article (Wu et al., 2020) the authors correlated the content of nine different phenolic compounds found in apple juice and the antioxidant activity determined by the DPPH method. The study showed how significant positive correlations were only obtained for the content of caffeic acid and phlorizin for $p < 0.05$ (0.677 and 0.532 respectively). Although no correlation was found for chlorogenic acid or epicatechin, which were the most abundant phenolic compound besides the phlorizin in apple juice. Thus, the oxidation process in the presence of **I1** could damage more polyphenols such as caffeic acid, which have a greater impact on the antioxidant activity than TPC.

4. Conclusion

The effect on the PPO enzyme of three different azamacrocyclic inhibitors has been tested. The inhibition studies at different pHs suggest that the interaction with the PPO enzyme is not due to electrostatic forces but through other complex interactions. Furthermore, the presence of hydroxypyridinone attached to the macrocycle was relevant to enhance the inhibitor power especially at pH 5.5 and 6.5. Kinetic studies denote a mixed type of inhibition of **I1** with an IC_{50} (0.30 mM) much higher than the one calculated for **I2** and **I3**. The inhibitory activity of **I1** was validated on freshly obtained apple juice as a real food system. The inhibitor can reduce the enzymatic browning by more than 50% and the decrease in total phenolic content in apple juice was stopped when used 2.25 mM. Furthermore, **I1** delays, at least during the first 30 min, the antioxidant capacity measured with DPPH.

Declaration of competing interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112778.

Author contribution

S.M, A.D, J.V.R, B.V, E.G, A.A and A.A have participated in the conceptualization, investigation and writing of the manuscript.

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