Food Chemistry 341 (2021) 128232

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effect directed synthesis of a new tyrosinase inhibitor with anti-browning activity

Ignacio Cabezudo[a], I. Ayelen Ramallo[a], Victoria L. Alonso[b], Ricardo L.E. Furlan[a,*]

[a] Farmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Suipacha 531, 2000 Rosario, Argentina

[b] Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina

ARTICLE INFO

Keywords:
Tyrosinase
Browning
Inhibition
Bioautography
Thiosemicarbazone

ABSTRACT

The inhibition of enzymatic browning is an attractive target to elevate the quality of foods. The objective of this work is to describe a novel platform for the discovery of tyrosinase inhibitors, based on (a) one-pot preparation of a library of thiosemicarbazide compounds, (b) biological evaluation using tyrosinase TLC bioautography, (c) inhibitor identification via mass spectrometry coupled to bioautography. During these proof-of-concept experiments, the approach led to the straightforward identification of a new thiosemicarbazone with improved tyrosinase inhibition properties and fresh-cut apple slices antibrowning effect when compared to kojic acid. In conclusion, the platform represents an interesting strategy for the discovery of this type of inhibitors.

1. Introduction

Enzymatic browning is a major issue in most fresh fruits and vegetables, as it causes unfavourable effects on their safety, organoleptic properties, and nutritional values (Moon et al., 2018). Therefore, the inhibition of enzymatic browning is an attractive target to elevate the quality and safety of foods. Browning is caused mainly by polyphenol oxidases, copper-containing enzymes related to the hydroxylation of monophenols to o-diphenols and the oxidation of diphenols to quinones, which undergo subsequent reactions that form brown pigments (Mi Moon, Young Kim, Yeul Ma, & Lee, 2019; Moon et al., 2018). Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase widely distributed in microorganisms, animals, and plants (Shao et al., 2018). Tyrosinase inhibitors are usually considered of interest as anti-browning agents for the industry that processes vegetables (Carcelli et al., 2020; Xu, Liu, Zhu, Yu, & Cao, 2017).

A large number of tyrosinase inhibitors have been reported in the literature, from both synthetic and natural sources; nevertheless, most of them could be unsuitable for practical applications. The inhibition of metalloenzymes such as tyrosinase, depends in part on the ability to coordinate the metal ions in the active site (Chen et al., 2019). Thiosemicarbazones and thiocarbazones are well-known metal-chelating compounds, such as copper, present in the tyrosinase active site (Rogolino et al., 2017), and they have been explored for tyrosinase inhibition properties (Carcelli et al., 2020; Hałdys & Latajka, 2019; Xie, Dong, Yu, & Cao, 2016).

To the best of our knowledge, all the synthetic tyrosinase inhibitors reported to date have been prepared and biologically evaluated individually. Alternatively, the one-pot preparation of compound mixtures as sources of potential tyrosinase inhibitors has not been explored. Although efficient from a synthetic perspective, an important drawback of this strategy is the innate necessity for inhibitor identification from the mixtures that display interesting properties.

TLC bioautography is an assay format particularly well suited for the evaluation of enzyme inhibition properties of complex mixtures (Bräm & Wolfram, 2017). TLC bioautographic methods combine chromatographic separation and *in situ* enzyme activity determination, facilitating the localisation of inhibitors within a mixture. This assay format has been used to detect tyrosinase inhibitors from plant extracts (Hsu, Chan, Chen, Lin, & Cheng, 2018; Zhou, Tang, Wu, & Cheng, 2017) and chemically engineered extracts as well (Paula García, Salazar, Ramallo, & Furlan, 2016; Solís, Salazar, Ramallo, García, & Furlan, 2019). Nevertheless, its utility for the evaluation of synthetic libraries has not been explored. In addition, TLC has demonstrated to be a straightforward approach to link chromatographic and biological data with respective mass spectra of bioactive compounds (Azadniya & Morlock, 2018; Ciura, Dziomba, Nowakowska, & Markuszewski, 2017; Jamshidi-Aidji & Morlock, 2016; Krüger, Hüsken, Fornasari, Scainelli, & Morlock, 2017; Ramallo, Salazar, & Furlan, 2015; Zhang et al., 2017).

The aim of this study was to evaluate the combination of mixture

* Corresponding author.
E-mail addresses: icabezudo@fbioyf.unr.edu.ar (I. Cabezudo), aramallo@fbioyf.unr.edu.ar (I. Ayelen Ramallo), alonso@ibr-conicet.gov.ar (V.L. Alonso), rfurlan@fbioyf.unr.edu.ar (R.L.E. Furlan).

https://doi.org/10.1016/j.foodchem.2020.128232
Received 8 July 2020; Received in revised form 24 September 2020; Accepted 24 September 2020
Available online 28 September 2020
0308-8146/

library preparation, tyrosinase TLC bioautography, and algorithm aided mass spectrometry analysis, as a platform for the discovery of tyrosinase inhibitors. Using this platform, a new mixed type tyrosinase inhibitor with a K_i = 0.29 ± 0.11 μM and interesting anti-browning effect on apple slices was discovered.

2. Materials and methods

Mushroom tyrosinase, dimethyl sulfoxide (DMSO), L-tyrosine (Tyr), 2-thiophenecarboxaldehyde, 4-hydroxy-3-methoxybenzaldehyde (**A1**), 3,4,5-trimethoxybenzaldehyde (**A2**), anthracene-9-carbaldehyde (**A3**), 5-ethoxyfuran-2-carbaldehyde (**A4**), 5-hydroxyfuran-2-carbaldehyde (**A5**), 2,3-dihydroxypropanal (**A6**), 4-(diethylamino)-2-hydroxybenzaldehyde (**A7**), hexanal (**A8**), hydrazinecarbothioamide (**T1**) and hydrazinecarbothiohydrazide (**T2**) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium phosphate dibasic and sodium phosphate monobasic was purchased in Cicarelli (San Lorenzo, Argentina). Agar was purchased from Britania (Buenos Aires, Argentina). HPLC-grade acetonitrile was purchased from Carlo Erba Regents (Milan, Italy). All other reagents were analytical grade, and the water used was re-distilled and ion-free.

Thin-layer chromatography was carried out on aluminum-backed silica gel 60 F_{254} (Merck, Darmstadt, Germany). Chromatograms were run and processed using equipment from CAMAG (Muttenz, Switzerland). Separation of synthetic mixtures, controls, and pure compounds was performed on 7 cm height TLC layers. Samples were applied in 4 mm bands onto the TLC plate using a CAMAG Automatic TLC Sampler 4 (ATS 4) under air flux. After solvent evaporation, TLC images were captured under white light and UV light with a CAMAG TLC Visualizer. For bioautographic purposes, agar containing enzyme and medium was distributed evenly over the TLC layer. Colour development and control experiments were carried out as described in Section 2.3, and plate images were captured under white light again. The chromatograms were scanned using a TLC scanner 4, and optical density graphs were plotted.

^{1}H NMR (300 MHz), ^{13}C NMR (75 MHz) were measured on a Bruker Avance II 300 MHz spectrometer. LC-MS and direct infusion MS experiments were carried out using an Ultimate 3000 RSLC (Dionex, Thermo Scientific) coupled with an ESI triple quadrupole mass spectrometer (TSQ Quantum Access Max (QQQ), Thermo-Scientific). The following ionization conditions were used: ESI, positive-ion mode; drying gas (N_2) temp., 300 °C; drying gas flow rate, 10 L/min; nebulizer pressure, 10 UA, and cap. voltage, 4.5 kV.

2.1. Synthesis of individual thiosemicarbazones

Thiosemicarbazones **1** and **A7T1** were prepared by refluxing in ethanol followed by product crystallization. 2-(thiophen-2-ylmethylene)hydrazine-1-carbothioamide (**1**), was obtained as yellow crystals, yield rate was 40%. ^{1}H NMR and ^{13}C NMR spectra were coincident with literature (Xie et al., 2016).

2-(4-(diethylamino)-2-hydroxybenzylidene) hydrazine-1-carbothioamide (**A7T1**) was obtained as bright yellow crystals, and the yield rate was 82%. ^{1}H NMR (DMSO-d_6, TMS, 300 MHz): δ (ppm) 11.07 (1H, s, NH), 9.49 (1H, s, Ar-OH), 8.19 (1H, s, CH), 7.87 (1H, s, CH = N), 7.66 (1H, s, NH_2), 7.52 (1H, d, j = 9.0 Hz, Ar-H), 6.22 (1H, dd, j = 9.0, 2.5 Hz, Ar-H), 6.09 (1H, d, 2.5 Hz, Ar-H), 3.34 (4H, q, j = 6.5 Hz,CH_2), 1.10 (6H, t, j = 6.5 Hz, CH_3). ^{13}C NMR (DMSO-d_6, TMS, 75 MHz): δ (ppm) 178.38 (C═S), 158.09 (Ar-OH), 150.03 (Ar-N), 142.27 (CH = N), 128.94 (CH, Ar), 107.35 (Ar-CHN), 103.91 (CH, Ar), 97.23 (CH, Ar), 43.77 (CH_2), 12.51 (CH_3). MS (ESI): m/z 267.12 $[M + H]^+$.

2.2. Thiosemicarbazone library preparation and analysis

Aldehydes **A1**-**A8** and thiosemicarbazide (**T1**) and thiocarbazide (**T2**) stock solutions were prepared in DMSO (30 mg/mL for **A1**, **A2**, **A4**-**A8**, and **T1**; 15 mg/mL for **A3** and **T2**). Adequate volumes of each building block solution were mixed and diluted in EtOH (2 mL) to obtain final concentrations of 0.80 mM for each aldehyde, 3.2 mM for **T1**, and 1.6 mM for **T2**, maintaining a 1:1 proportion between the equivalents of thiosemicarbazone and aldehyde reactive groups. The reaction was stirred under reflux for three hours. The library was stored at 8 °C.

LC-MS analysis of the library was carried out using a reversed-phase HPLC Agilent Zorbax Eclipse column (50 mm × 3.0 mm XDB C18, particle size 1.8um) following step-wise gradient: 0 min (water 9:1 ACN); 3 min (water 9:1 ACN); 23–25 min (water 1:9 ACN). Water contained 0.5% formic acid, column temperature was maintained at 30 °C, and autosampler tray temperature was set at 25 °C.

2.3. Tyrosinase bioautography assays

For a 70 cm^2 TLC sheet, the staining solution for tyrosinase was prepared by dissolving agar (135 mg) at 90 °C in sodium phosphate buffer (20 mM, pH 6.8, 11.2 mL). Whenever the TLC sheets size was different, the quantities were modified proportionally according to the technique reported (Paula García & Furlan, 2015). The solution was allowed to cool to 55 °C, the L-tyrosine solution (2.5 mM, 2.8 mL) was added, and the whole mixed by hand. At 35 °C, tyrosinase solution (3800 U/mL, 130 μL) was added, and the obtained solution was mixed by rotation by hand and then carefully poured on the already developed TLC sheet (Paula García & Furlan, 2015).

2.3.1. Spiking experiments

The library was diluted 2.4 times using EtOH. Aliquots were spiked with increasing amounts (1.6 and 3.2 μL) of an **A7T1** solution in EtOH (0.4 mg/mL), for a final addition of 0.015 and 0.036 μg of **A7T1** on each TLC spot. These two samples and a library control were spotted and eluted with DCM:MeOH (97:3) and evaluated by TLC-tyrosinase bioautography, according to section 2.3.

2.4. BioMSId evaluation of thiosemicarbazone compounds

2.4.1. Sample preparation

For inhibitor detection in the mixture library, three TLC plates were spotted with 6 μL samples of the library. They were eluted with three different mobile phases: DCM:MeOH (97:3), hexane:EtOAc (4:6), and toluene:$CHCl_3$:EtOH (5:5:2). Each TLC was tested for tyrosinase inhibition using the described bioautography conditions, and circular portions of the agar layer from inhibition and background zones were taken using a glass tube (internal diameter 0.5 cm). Each gel portion was extracted with EtOH (2 × 1 mL) using an ultrasonic bath for 5 min. The ethanol was removed under air current; the solid was dissolved in ACN (0.5 mL) and filtered with 0.45 μm RC syringe filters. The resulting solutions were infused directly into the ESI chamber at a rate of 25 μL/min during 5 min for LRMS analysis. The spectra were read with Thermo Xcalibur 2.2 SP1.48; each mass spectra comprising 0.23 min data collection were averaged and the mass lists, conformed by m/z and respective intensity values, were exported and saved in ASCII format.

2.4.2. Parameter setting for BioMSId algorithm

BioMSId was implemented according to Ramallo et al. (Ramallo et al., 2015). BioMSId algorithm was run under MATLAB environment (R2020a trial use). For spectra comparison, the tolerance parameter value was set at 0.04 m/z and the analysed mass range was 164–490 m/z. First, the routine control spectrum of the equipment was subtracted from background/halo spectra. Second, the spectra were filtered at an intensity threshold equal to 3.3 × 10^5 to eliminate low-intensity signals. Third, the background spectra were subtracted from halo spectra, to filter signals that were unrelated with the library chemical components under the inhibition halo (phosphate buffer, L-tyrosine,

tyrosinase). Finally, all subtracted spectra were compared, searching common signals related to the inhibitors.

2.5. Quantification of tyrosinase inhibitory activity

Enzyme activity was continuously determined at 37 °C during 30 min following the increase in absorbance at 475 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) determined by the formation of dopachrome (Choudhary & Thomsen, 2001) in 96-well microplate using a VersaMax™ microplate reader (Molecular Devices LLC, California, USA). All measurements were performed in triplicate using L-tyrosine as substrate, kojic acid as reference inhibitor, and Na_2HPO_4-NaH_2PO_4 as buffer medium (100 mM, pH 6.8). Wells containing DMSO without inhibitor were used as control of maximum enzymatic rates. All data sets were processed using Prism V5.01 software (GraphPadSoftware Inc., La Jolla, CA, USA).

IC_{50} determinations. Serial DMSO dilutions of kojic acid, **1**, and **A7T1** were prepared following equally spaced points. Wells were filled: 10 μL DMSO solution (end plate concentration ranged between 0.048 and 500 μM), and mushroom tyrosinase in buffer phosphate (15.6 U/mL, end concentration in well). The reaction was started by addition of L-tyrosine (0.63 mM, end concentration in well) completing 270 μL end volume in well. The plate was shaken for 10 s, and the increase in absorbance was immediately monitored, as explained in the previous section. Percentage of inhibition of tyrosinase activity was calculated as follows:

$$\%\text{inhibition} = \{[(C_t - C_0) - (t_t - t_0)]/(C_t - C_0)\} \times 100$$

where C_t is the absorbance of a DMSO solution after incubation, C_0 is the absorbance of DMSO before incubation, t_t is the absorbance of sample solution after incubation, and t_0 the absorbance of sample solution before incubation.

Inhibition type determination. Five serial DMSO dilutions of kojic acid, **1**, and **A7T1** were prepared in equally spaced points. Wells were filled: 10 μL DMSO solution (end plate concentration ranged between 0.18 and 16.78 μM), and mushroom tyrosinase in buffer phosphate (15.6 U/mL, end concentration in well). The reaction was started by addition of L-tyrosine (0.31–1.26 mM end concentration in well) completing 270 μL end volume in well. The plate was shaken for 10 s and was immediately monitored by measuring the increase in absorbance.

2.6. Browning colorimetric assays

Granny Smith and *Pink Lady* apples and eggplants were washed and cut into small identical slices of 5 mm width using a mandolin slicer, which were dipped in 300 mL of 15 μM of inhibitor solution, for 3 min and then drained. Inhibitor solutions were prepared in 20 mM phosphate buffer (pH 6.8) (Wu, Cheng, Li, Wang, & Ye, 2008). This procedure was repeated for **A7T1** and kojic acid, while control samples were dipped only in buffer phosphate. Samples were then placed on absorbent paper and stored at room temperature, and changes of colour were measured during 24 h.

For colorimetric determinations, standardized images of apples were obtained using CAMAG TLC visualizer. Then images were imported into the Fiji software and converted to CIE L*a*b* space, where L* indicates lightness, a* indicates chromaticity on a green (-) to red (+) axis, and b* chromaticity on a blue (–) to yellow (+) axis. In order to compare the apple slice samples, L*, a*, and b* coordinates were obtained, and from them, the browning index (BI) was calculated using the formula (Perez-Gago, Serra, Alonso, Mateos, & del Río, 2005):

$$BI = [100(X-0.31)]/0.17$$

$$\text{Where } X = (a* + 1.75L*)/(5.645\ L* + a* - 3.012b*)$$

For differences among control and the two inhibitor treatments applied to apple slices, one-way analysis of variance (ANOVA) with the *post hoc* Bonferroni's Multiple Comparison Test were performed on the obtained a* and BI data using the Graph Pad Prism software. A *p*-value lower than 0.05 was selected as the decision level for statistically significant differences.

2.7. Cell viability assay

Cell viability after treatment was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretazolium bromide (MTT) reduction assay. Briefly, Vero cells (5.000 cells per well) were incubated in 96-well plate in the presence of increasing concentrations of **A7T1** for 48 h (9.375 to 300 μM). Then 20 μL of MTT solution (5 mg/mL in PBS) were added to each well and incubated for 1 h at 37 °C. After this incubation period, MTT solution was removed and precipitated formazan was solubilized in 100 μL of DMSO. Optical density (OD) was spectrophotometrically quantified ($\lambda = 540$ nm). DMSO was used as blank and each treatment was performed in triplicates. Values were normalized using the blank as 100% growth. IC_{50} was calculated from the log (concentration) *vs.* Viability (%) curves using the non-linear fit log(inhibitor) *vs.* response, Variable slope (four parameters) in GraphPad Prism.

3. Results

3.1. In situ inhibition evaluation and MS analysis of a reference tyrosinase inhibitor

In order to evaluate if the TLC bioautography-MS analysis could be applied for the identification of tyrosinase inhibitors, we used thiosemicarbazone **1** as a positive control of the method. This potent tyrosinase inhibitor was reported recently by the group of Cao (Xie et al., 2016). Compound **1** was prepared from 2-thiophenecarboxaldehyde and thiosemicarbazide in refluxing EtOH.

The tyrosinase inhibition by **1** was evaluated using a TLC-bioautography. In this assay format, a TLC sheet is covered by a thin layer of agar-gel containing the enzyme tyrosinase and the substrate tyrosine (Paula García & Furlan, 2015). Due to enzyme-catalyzed dye formation, the gel is colored in reddish-brown, and the presence of enzyme inhibitors spotted on the TLC surface can be detected as clear spots. In order to evaluate the assay sensitivity towards the model compound, increasing amounts of thiosemicarbazone **1** were spotted on the TLC, ranging between 0.04 μg and 0.75 μg. The observed inhibition spots and the optical densities (475 nm, absorption wavelength for tyrosinase products) were proportional to the amount of inhibitor up to 0.63 μg, when apparent saturation was observed (Fig. 1).

To evaluate if compound **1** could be linked to the observed tyrosinase inhibition through MS analysis, two gel samples were taken from the TLC bioautography, one from the inhibition zone and one from the background, i.e., a gel area without any spots. Samples were extracted with EtOH, the solvent was evaporated, and the sample was dissolved in ACN for MS analysis. The analysis was performed using MatLab, to subtract the background as described in previous work from our group and lead to a clean signal $m/z = 208.00$ corresponding to the $[M + Na]^+$ ion from **1** (Fig. S1). Altogether, the results indicated that this type of tyrosinase inhibitors can be detected, when spotted on a TLC plate and that the observed bioactivity could be linked to an MS signal.

3.2. Library preparation and analysis

In order to establish conditions for library preparation and analysis, the model thiosemicarbazone **1** was now prepared in ethanol from DMSO solutions (30 mg/mL) of 2-thiophenecarboxaldehyde and thiosemicarbazide (**T1**). Product formation was followed by TLC-bioautography analysis through both, naked eye TLC observation and TLC scanning at 475 nm (Fig. 2). At 3 h of reaction, the product reached a

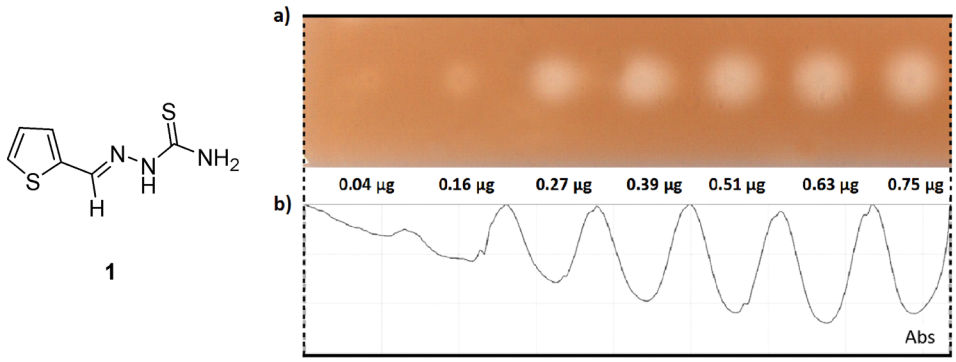

Fig. 1. Analysis of tyrosinase inhibition produced by increasing amounts of thiosemicarbazone **1**: a) tyrosinase bioautography; b) tyrosinase bioautography scan at 475 nm.

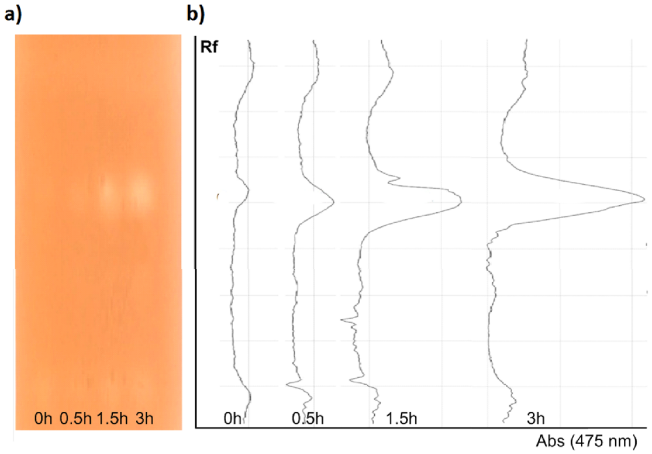

Fig. 2. Tyrosinase activity-based detection of product **A9T1** in the reaction mixture at different times: a) tyrosinase bioautography; b) tyrosinase bioautography scan at 475 nm.

constant concentration. Product formation could not be detected by TLC analysis under UV light at 254 and 365 nm, indicating lower sensitivity than the bio-based detection (Fig. S2).

The reaction conditions and building-block concentrations used for the preparation of compound **1** were then used to prepare a library of thiosemicarbazones and thiocarbazones, Fig. 3a. Adequate volumes of DMSO solutions of aldehydes **A*i*** (15 mg/mL for **A3** and 30 mg/mL for the rest), thiosemicarbazide **T1** (30 mg/mL), and thiocarbazide **T2** (15 mg/mL) were mixed and diluted in EtOH to a final volume of 2 mL (Fig. 3b). The reaction mixture was stirred for 3 h under reflux and analyzed by LC-MS, detecting the presence of 43 thiocarbazone and thiosemicarbazone products out of the 52 possible products (Table S1).

3.3. Bioautography detection of tyrosinase inhibitors

Tyrosinase inhibition by the library was evaluated by TLC-bioautography using DCM:MeOH (97:3) as mobile phase. Mixtures of building blocks **A1**-**A8**, and **T1** and **T2** were also spotted, separately, as controls. The assay showed three potentially interesting inhibition zones: two weak inhibition zones with Rf values between 0.02 and 0.18 and 0.72–0.88, and one more intense inhibition zone of intermediate polarity in the Rf range 0.35–0.55 (Fig. 3c). Inhibition observed in the more polar zone could be produced by **T1** and/or **T2** since a similar inhibition was observed in the TLC lane for these building blocks (Fig. S3). Similarly, comparison of the tyrosinase inhibition based chromatograms also suggested that the least polar inhibition zone, observed at Rf 0.72–0.88, was due to some of the aldehyde building blocks since a similar inhibition was detected in the **A1**-**A8** control mixture (Fig. S3).

The most intense inhibition zone was observed in the intermediate polarity region (Rf between 0.35 and 0.55), which could be indicative of one (or more) thiocarbazone or thiosemicarbazone inhibitor present in the library (Fig. 3c). This inhibition spot showed similar intensity to that of the spot produced by 0.51 µg of the known inhibitor **1**. Taking into consideration the library sample applied to the TLC for analysis, the compound responsible for tyrosinase inhibition in that zone could have similar potency to **1**.

3.4. Tyrosinase inhibitor identification using BioMSId

Identification of the library member responsible for the inhibition spot by MS analysis of a sample from the bioautography can be more complicated than in the previous analysis with model compound **1** because of the number of compounds present. In addition to MS signals from bioassay related reagents, now the co-extraction of inactive library members and starting materials with similar TLC migration properties could increase further the complexity of the MS spectrum obtained.

Therefore, to gain insight into the identity of the compound responsible for the observed activity in the library, the BioMSId (BIOautography coupled to Mass Spectrometry for the IDentification of compounds) strategy was applied (Ramallo et al., 2015). To implement BioMSId, library samples were chromatographed on TLC using three different solvent systems, and the three TLC plates were then tested for tyrosinase activity (Fig. S4). Samples from the inhibition zones and the background were taken from each TLC plate and subjected to MS analysis. The obtained spectra were processed with a MATLAB algorithm that compares the spectra searching for common signals that could be linked to the structure of the active compound (García, Ramallo, Salazar, & Furlan, 2016; Ramallo et al., 2015).

One MS signal was linked by BioMSId to the inhibition spot, with an m/z value of 267.12 that could correspond to the $[M + H]^+$ signal of compound **A7T1** (Fig. 4c). This is the only library member with MW of 266, the two compounds with closest MW values are **A6T2A8** ($[M + H]^+$ m/z = 261.13) and **A2T1** (m/z = 270.08), undoubtedly different from the one observed. The signals corresponding to the active compound were picked out from a crowded zone of the spectra (Fig. 4a and 4b). Nevertheless, they could be correctly assigned to the compound responsible for the observed activity (Fig. 4c).

3.5. Synthesis and inhibitory properties of compound A7T1

In order to confirm the identity and further study tyrosinase inhibition and anti-browning properties, compound **A7T1** was prepared in 82% yield from the aldehyde **A7** and thiosemicarbazide (**T1**) in refluxing ethanol. As expected, **A7T1** showed the same MS signal observed previously after BioMSId application to the library (m/z = 267.12, Fig. S5). To corroborate that **A7T1** played an important role in the tyrosinase inhibition produced by the library, a library

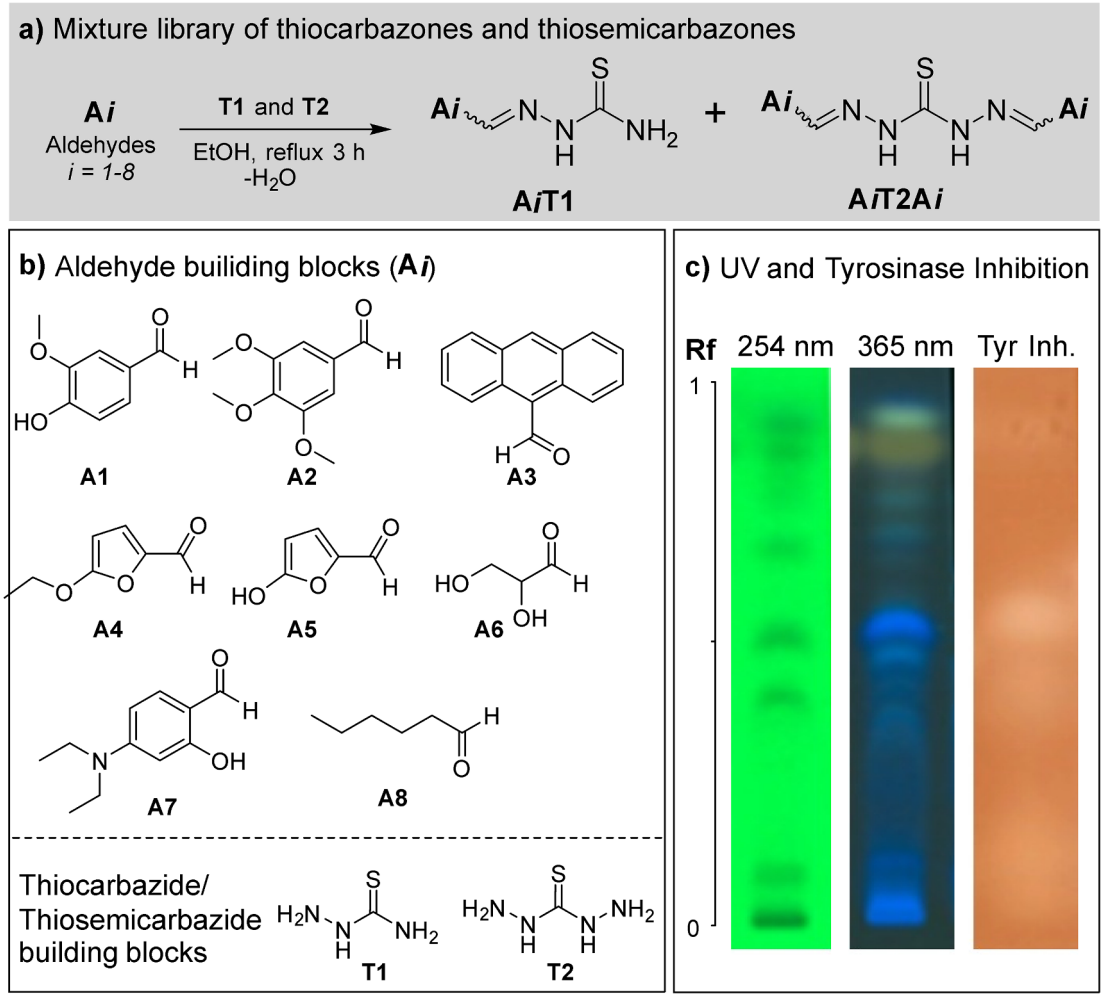

Fig. 3. Preparation and biological analysis of a mixture library of thiosemicarbazones and thiocarbazones: a) library; b) building blocks; c) TLC-UV-tyrosinase bioautography analysis of the library.

sample was spiked with **A7T1** and evaluated by TLC-bioautography observing a clear increase in the inhibition spot intensity in tyrosinase bioautography (Fig. S6).

The IC_{50} value for **A7T1** is 0.46 ± 0.05 μM, significantly lower to the IC_{50} of kojic acid (22.84 ± 0.26 μM) and similar to that of compound **1** (IC_{50} = 0.36 ± 0.05 μM) determined using the same conditions. The IC_{50} plots of **A7T1**, **1**, and kojic acid are included in SI (Fig. S7).

The Michaelis-Menten plot for the enzymatic reaction in the presence of different concentrations of compound **A7T1** is shown in Fig. 5a. This inhibitor presented the best fit using a mixed-type inhibition model, according to the Graph Pad Prism software. Compound **A7T1** binds not only to the enzyme (competing with the substrate) but also to the substrate-enzyme complex. The affinity of the inhibitor for the free enzyme (K_i = 0.29 μM ± 0.11 μM) is higher than the affinity of the inhibitor for the substrate-enzyme complex ($K_{is} = \alpha K_i$, with α = 5.40, therefore K_{is} = 1.56 μM). The Lineweaver-Burk plot for **A7T1** is shown in Fig. 5b and slope and intercept of the Lineweaver-Burk plot can be found in Supplementary material, Fig. S8.

Two aminobenzaldehyde-thiosemicarbazones have been reported as tyrosinase inhibitors: 4-dimethylaminobenzaldehyde-thiosemicarbazone (IC_{50} = 1.54 μM, mix-type inhibitor) and 4-dimethylaminobenzaldehyde-*N*-phenyl-thiosemicarbazone (IC_{50} = 1.78 μM, non-competitive inhibitor) (Yang et al., 2013). Compared to **A7T1**, both compounds exhibited higher IC_{50} values.

3.6. Anti-browning effect

To confirm the potential of tyrosinase inhibitory activity of **A7T1** in anti-browning applications, *Granny Smith* apple slices were chosen as food models. Slices were dipped in solutions of **A7T1** and kojic acid (a reported anti-browning agent), and the enzymatic browning reaction was measured. Readings of images were transformed to a CIELAB scale (L*, a*, b*), and browning index (BI) was calculated. Earlier research studies have found that a* value was more sensitive to browning in fresh-cut apple (Rojas-Graü, Sobrino-López, Tapia, & Martín-Belloso, 2006). The browning index (BI), calculated from L*, a* and b*, is defined as brown-color purity, usually used as an indicator of the extent of browning in apple-cuts (Perez-Gago et al., 2005).

Therefore, the browning of the three groups at 6 h and 24 h was compared through a* and BI (Fig. 6a and b). Apple slices treated with 15 μM **A7T1** possessed the slower browning degree than control and 15 μM kojic acid during the entire storage period. At 24 h, the mean BI value in untreated slices almost doubles that in the slices treated with **A7T1**. This effect is most pronounced in a* whose mean value in the control quadruples that observed for **A7T1** treated slices. This is consistent with the results of surface color observations (Fig. 6c), and supported statistically though one-way analysis of variance (ANOVA) with the *post hoc* Bonferroni's Multiple Comparison Test, that found significant differences between **A7T1** treated slices *vs* control slices for a* and BI ($p < 0.05$) (Tables S2 and S3 respectively). Similar browning reducing effects were observed in slices of eggplants and *Pink Lady*

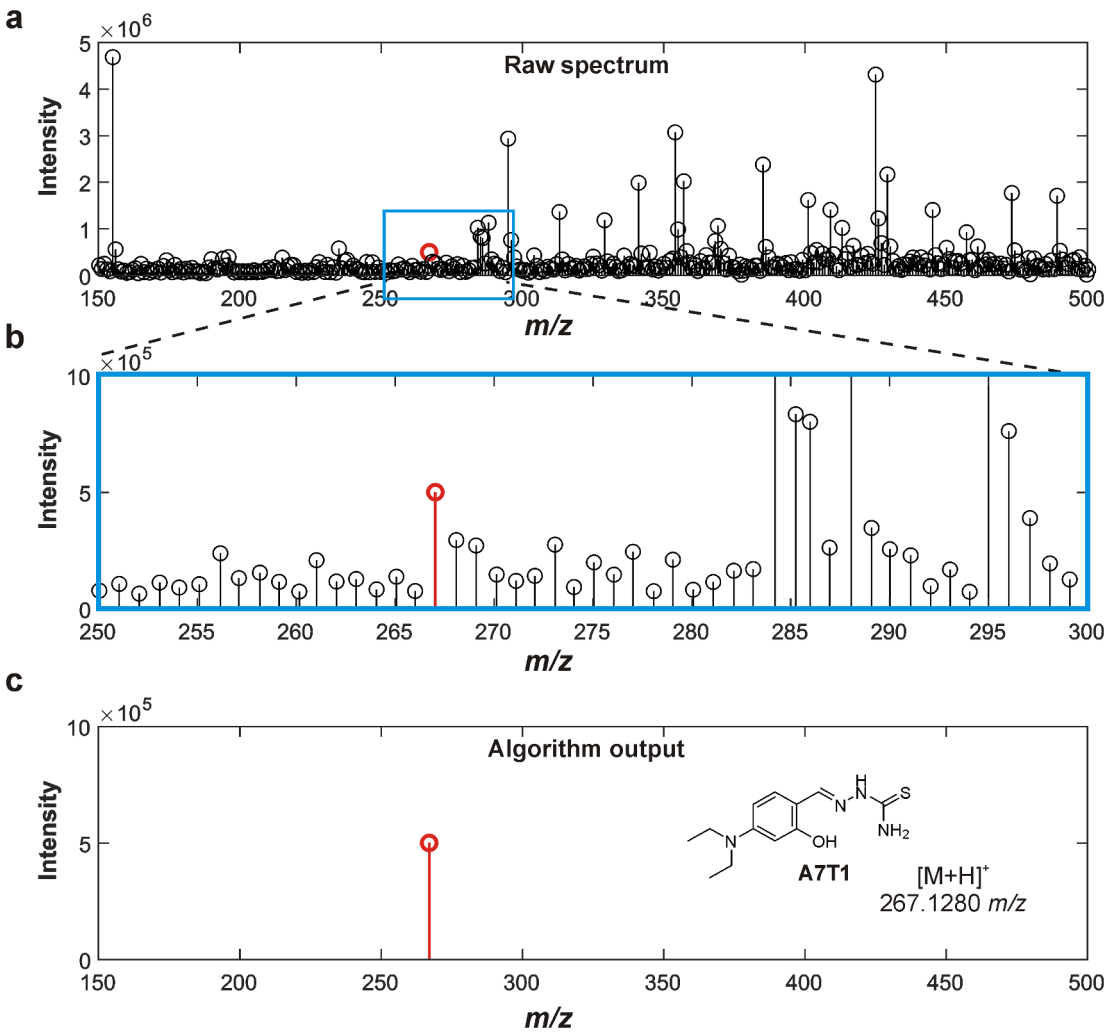

Fig. 4. BioMSId identification of **A7T1** as the tyrosinase inhibitor in the library: a) raw LRMS spectrum; b) amplification of the area from which the compound is finally identified; c) algorithm output.

apples (Fig. S9). The ANOVA and the *post hoc* Bonferroni's Multiple Comparison Test found again significant differences between **A7T1** treated slices *vs* control slices for a* and BI ($p < 0.05$) (Tables S4–S7).

3.7. Cellular assay

The cytotoxicity of compound A7T1 against Vero Cells was evaluated using an MTT assay. The IC_{50} value was 145.00 ± 1.28 μM (Table S4 and Fig. S10). This concentration is more than 300 times higher than the concentration required for inhibition of 50% of the enzyme activity (0.46 ± 0.05 μM).

4. Conclusions

For the first time, library-preparation, TLC bioautography, and mass spectrometry were applied in tandem for the discovery of a bioactive compound. The strategy led to the straightforward identification of a new tyrosinase inhibitor with interesting inhibitory properties and anti-browning effects that deserves further studies in order to evaluate its applicability

Since the approach involves bioguided synthesis, milligram scale preparation and characterization is carried out only after a bioactive molecule has been identified. Going beyond this proof-of-principle

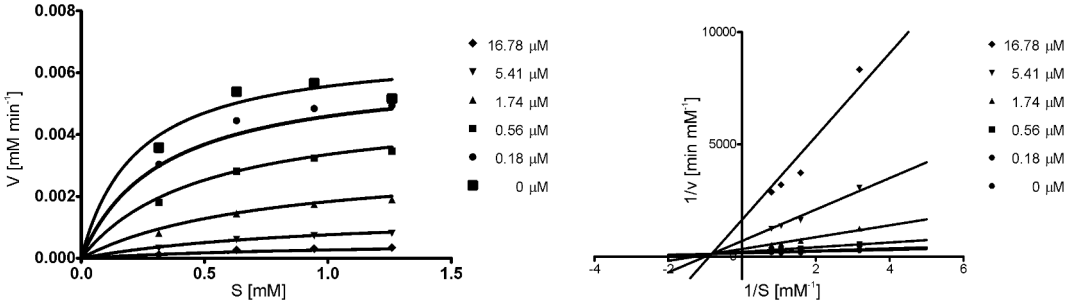

Fig. 5. Tyrosinase activity assay: a) Michaelis-Menten plot: effects of different concentrations of **A7T1** on the reaction rate as a function of substrate concentration; b) Lineweaver-Burk plot for **A7T1**.

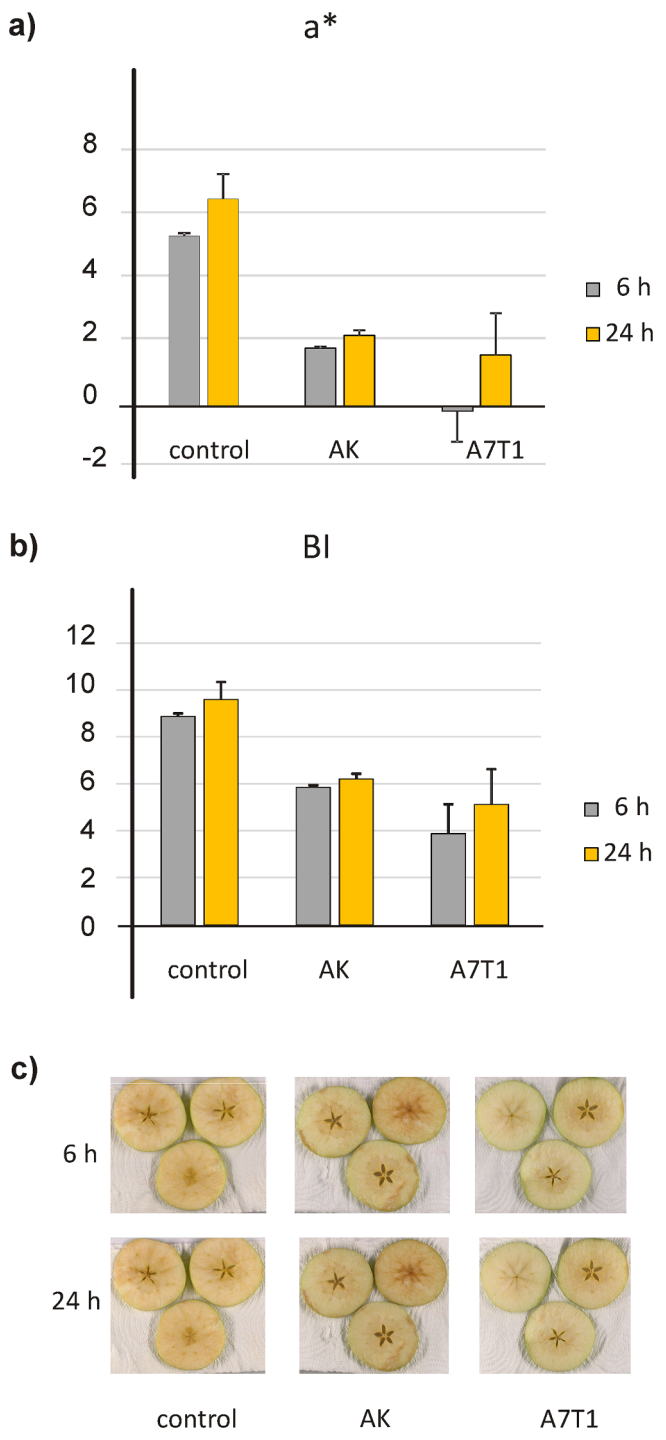

Fig. 6. Browning of apple slices in control samples and samples treated using 15 μM of kojic acid and **A7T1**: a) comparison using a*; b) comparison using BI; c) some images used in the analysis of apple slice browning.

study with anti-browning thiosemicarbazones, the effect-directed based platform can be applied for the discovery of other bioactive compounds relevant for food. Using appropriate high-yield simple chemical reactions and existing TLC-bioautography targets, novel antioxidants, glycosidase and lipase inhibitors, or antimicrobials could be identified.

CRediT authorship contribution statement

Ignacio Cabezudo: Methodology, Validation, Investigation, Data curation, Writing - original draft. **I. Ayelen Ramallo:** Methodology, Software, Validation, Formal analysis, Data curation, Writing - review & editing, Visualization. **Victoria L. Alonso:** Investigation, Formal analysis. **Ricardo L.E. Furlan:** Conceptualization, Writing - review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Financial support for this work was provided by Fondo para la Investigación Científica y Tecnológica (Argentina, grant number PICT2015-3574), and Universidad Nacional de Rosario (Argentina, grant number 80020180300114UR). The authors thank the Instituto de Química de Rosario (IQUIR, CONICET-UNR) for providing access to NMR equipment. Ignacio Cabezudo thanks CONICET for his fellowship. I. Ayelen Ramallo, Victoria L. Alonso, and Ricardo L. E. Furlan are CONICET researchers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128232.

References

Azadniya, E., & Morlock, G. E. (2018). Bioprofiling of Salvia miltiorrhiza via planar chromatography linked to (bio)assays, high resolution mass spectrometry and nuclear magnetic resonance spectroscopy. *Journal of Chromatography A, 1533*, 180–192. https://doi.org/10.1016/j.chroma.2017.12.014.

Bräm, S., & Wolfram, E. (2017). Recent Advances in Effect-directed Enzyme Assays based on Thin-layer Chromatography. *Phytochemical Analysis, 28*(2), 74–86. https://doi.org/10.1002/pca.2669.

Carcelli, M., Rogolino, D., Bartoli, J., Pala, N., Compari, C., Ronda, N., ... Fisicaro, E. (2020). Hydroxyphenyl thiosemicarbazones as inhibitors of mushroom tyrosinase and antibrowning agents. *Food Chemistry, 303*, Article 125310. https://doi.org/10.1016/j.foodchem.2019.125310.

Chen, A. Y., Adamek, R. N., Dick, B. L., Credille, C. V., Morrison, C. N., & Cohen, S. M. (2019). Targeting Metalloenzymes for Therapeutic Intervention. *Chemical Reviews, 119*(2), 1323–1455. https://doi.org/10.1021/acs.chemrev.8b00201.

Ciura, K., Dziomba, S., Nowakowska, J., & Markuszewski, M. J. (2017). Thin layer chromatography in drug discovery process. *Journal of Chromatography A, 1520*, 9–22. https://doi.org/10.1016/j.chroma.2017.09.015.

García, P., & Furlan, R. L. E. (2015). Multiresponse Optimisation Applied to the Development of a TLC Autography for the Detection of Tyrosinase Inhibitors. *Phytochemical Analysis, 26*(4), 287–292. https://doi.org/10.1002/pca.2562.

García, P., Ramallo, I. A., Salazar, M. O., & Furlan, R. L. E. (2016). Chemical diversification of essential oils, evaluation of complex mixtures and identification of a xanthine oxidase inhibitor. *RSC Advances, 6*(62), 57245–57252. https://doi.org/10.1039/C6RA05373D.

García, P., Salazar, M. O., Ramallo, I. A., & Furlan, R. L. E. (2016). A New Fluorinated Tyrosinase Inhibitor from a Chemically Engineered Essential Oil. *ACS Combinatorial Science, 18*(6), 283–286. https://doi.org/10.1021/acscombsci.6b00004.

Hałdys, K., & Latajka, R. (2019). Thiosemicarbazones with tyrosinase inhibitory activity. *MedChemComm, 10*(3), 378–389. https://doi.org/10.1039/C9MD00005D.

Hsu, K.-D., Chan, Y.-H., Chen, H.-J., Lin, S.-P., & Cheng, K.-C. (2018). Tyrosinase-based TLC Autography for anti-melanogenic drug screening. *Scientific Reports, 8*(1), 401. https://doi.org/10.1038/s41598-017-18720-0.

Jamshidi-Aidji, M., & Morlock, G. E. (2016). From Bioprofiling and Characterization to Bioquantification of Natural Antibiotics by Direct Bioautography Linked to High-Resolution Mass Spectrometry: Exemplarily Shown for Salvia miltiorrhiza Root. *Analytical Chemistry, 88*(22), 10979–10986. https://doi.org/10.1021/acs.analchem.6b02648.

Krüger, S., Hüsken, L., Fornasari, R., Scainelli, I., & Morlock, G. E. (2017). Effect-directed fingerprints of 77 botanical extracts via a generic high-performance thin-layer chromatography method combined with assays and mass spectrometry. *Journal of Chromatography A, 1529*, 93–106. https://doi.org/10.1016/j.chroma.2017.10.068.

Mi Moon, K., Young Kim, C., Yeul Ma, J., & Lee, B. (2019). Xanthone-related compounds as an anti-browning and antioxidant food additive. *Food Chemistry, 274*, 345–350. https://doi.org/10.1016/j.foodchem.2018.08.144.

Moon, K. M., Lee, B., Cho, W.-K., Lee, B.-S., Kim, C. Y., & Ma, J. Y. (2018). Swertiajaponin as an anti-browning and antioxidant flavonoid. *Food Chemistry, 252*, 207–214.

https://doi.org/10.1016/j.foodchem.2018.01.053.
Perez-Gago, M. B., Serra, M., Alonso, M., Mateos, M., & del Río, M. A. (2005). Effect of whey protein- and hydroxypropyl methylcellulose-based edible composite coatings on color change of fresh-cut apples. *Postharvest Biology and Technology, 36*(1), 77–85. https://doi.org/10.1016/j.postharvbio.2004.10.009.
Ramallo, I. A., Salazar, M. O., & Furlan, R. L. E. (2015). Thin layer chromatography-autography-high resolution mass spectrometry analysis: Accelerating the identification of acetylcholinesterase inhibitors. *Phytochemical Analysis, 26*(6), 404–412. https://doi.org/10.1002/pca.2574.
Rogolino, D., Cavazzoni, A., Gatti, A., Tegoni, M., Pelosi, G., Verdolino, V., ... Carcelli, M. (2017). Anti-proliferative effects of copper(II) complexes with hydroxyquinoline-thiosemicarbazone ligands. *European Journal of Medicinal Chemistry, 128*, 140–153. https://doi.org/10.1016/j.ejmech.2017.01.031.
Rojas-Graü, M. A., Sobrino-López, A., Tapia, M. S., & Martín-Belloso, O. (2006). Browning Inhibition in Fresh-cut'Fuji'Apple Slices by Natural Antibrowning Agents. *Journal of Food Science, 71*(1), S59–S65. https://doi.org/10.1111/j.1365-2621.2006.tb12407.x.
Shao, L.-L., Wang, X.-L., Chen, K., Dong, X.-W., Kong, L.-M., Zhao, D.-Y., ... Zhou, T. (2018). Novel hydroxypyridinone derivatives containing an oxime ether moiety: Synthesis, inhibition on mushroom tyrosinase and application in anti-browning of fresh-cut apples. *Food Chemistry, 242*, 174–181. https://doi.org/10.1016/j.foodchem.2017.09.054.
Solís, C. M., Salazar, M. O., Ramallo, I. A., García, P., & Furlan, R. L. (2019). A Tyrosinase Inhibitor from a Nitrogen-Enriched Chemically Engineered Extract. *ACS Combinatorial Science, 21*(9), 622–627. https://doi.org/10.1021/acscombsci.9b00064.
Wu, J.-J., Cheng, K.-W., Li, E. T. S., Wang, M., & Ye, W.-C. (2008). Antibrowning activity of MRPs in enzyme and fresh-cut apple slice models. *Food Chemistry, 109*(2), 379–385. https://doi.org/10.1016/j.foodchem.2007.12.051.
Xie, J., Dong, H., Yu, Y., & Cao, S. (2016). Inhibitory effect of synthetic aromatic heterocycle thiosemicarbazone derivatives on mushroom tyrosinase: Insights from fluorescence, 1H NMR titration and molecular docking studies. *Food Chemistry, 190*, 709–716. https://doi.org/10.1016/j.foodchem.2015.05.124.
Xu, J., Liu, J., Zhu, X., Yu, Y., & Cao, S. (2017). Novel inhibitors of tyrosinase produced by the 4-substitution of TCT. *Food Chemistry, 221*, 1530–1538. https://doi.org/10.1016/j.foodchem.2016.10.140.
Yang, M.-H., Chen, C.-M., Hu, Y.-H., Zheng, C.-Y., Li, Z.-C., Ni, L.-L., ... Chen, Q.-X. (2013). Inhibitory kinetics of DABT and DABPT as novel tyrosinase inhibitors. *Journal of Bioscience and Bioengineering, 115*(5), 514–517. https://doi.org/10.1016/j.jbiosc.2012.11.019.
Zhang, L., Shi, J., Tang, J., Cheng, Z., Lu, X., Kong, Y., & Wu, T. (2017). Direct coupling of thin-layer chromatography-bioautography with electrostatic field induced spray ionization-mass spectrometry for separation and identification of lipase inhibitors in lotus leaves. *Analytica Chimica Acta, 967*, 52–58. https://doi.org/10.1016/j.aca.2017.03.008.
Zhou, J., Tang, Q., Wu, T., & Cheng, Z. (2017). Improved TLC Bioautographic Assay for Qualitative and Quantitative Estimation of Tyrosinase Inhibitors in Natural Products. *Phytochemical Analysis, 28*(2), 115–124. https://doi.org/10.1002/pca.2666.