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Rapid autographic method for detection of enzymatic browning inhibitors based on enzyme immobilization

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ABSTRACT

This work describes a TLC-coupled bioautographic assay suitable for the separation and detection of apple polyphenol oxidase (PPO) inhibitors from natural extracts. PPO was immobilised in agar containing L-DOPA as substrate and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) to enhance colour development. The inhibition was detected as white spots on reddish background. Minimum amount of PPO inhibitors detected was 0.0125 μg of 4-hexylresorcinol, 0.025 μg of ascorbic acid, 0.5 μg of cysteine and 1 μg of kojic acid. The assay was compatible with normal and reverse phase TLC systems and allows detecting compounds that directly had action on the enzyme as well as agents that could convert quinones back to their reduced form. The chromatographic run evidenced the different nature of enzymatic browning inhibitory compounds from garlic and onion extracts. Using natural enzymes will provide a fast and cheap alternative for target specific exploration of natural enzymatic inhibitors.

1. Introduction

Browning is a major concern with regard to sensorial quality in food (Son, Moon & Lee, 2001). Although different sulphites have been employed for years to control non-enzymatic browning, the World Health Organization has recommended a limited use or nonuse of sulphites in treatment of fresh fruit and vegetables (Jukanti, 2017). In addition, there is an increasing demand by consumers for the substitution of synthetic food ingredients by natural substances. Therefore, there is an increasing interest in the search of enzymatic browning inhibitors from natural origin (Roldán, Sánchez-Moreno, de Ancos, & Cano, 2008; Gendel, 2012). The most important enzyme involved in browning is polyphenol oxidase (PPO). Natural products such as honey, pineapple juice, paper mulberry, rhubarb juice, onion and garlic extracts can inhibit PPO activity. PPO inhibition was produced also by residual water from processing vegetables from BRASSICACEAE family (Arzani, Khoshghalb, Malakouti, & Barzegar, 2010; de la Rosa et al., 2011; Kim, Kim, & Park, 2005; Lee, Lee, & Park, 2007; Lozano-de-Gonzalez, Barrett, Wrolstad, & Durst, 1993; Zheng, Cheng, Chao, Wu, & Wang, 2008; Zocca, Lomolino, & Lante, 2010). However, none of these agents is commercially used to prevent browning in fresh-cut fruits and vegetables highlighting the importance for the discovery of natural and efficient polyphenol oxidase inhibitors (Jukanti, 2017; Lee et al., 2007).

Polyphenol oxidase catalyses two types of oxidative reactions: hydroxylation of monophenols to o-diphenols (cresolase or monophenolase activity) and oxidation of o-diphenols to quinones (catecholase or diphenolase activity) (Kavrayan & Aydemir, 2001). Further condensation of quinones leads to brown melanin pigments (Martinez & Whitaker, 1995). PPOs from vegetal sources exist in multiple molecular forms (isoforms). Such isoforms differ in physical, chemical and/or enzymatic properties such as electrophoretic mobility, optimum temperature and pH, substrate specificity and isoelectric point (Yoruk & Marshall, 2003). These enzymes have been termed polyphenol oxidases in spite of its plant or fungal origin; although this designation does not distinguish between those that have cresolase activity and those that do not. For example: tyrosinase from mushroom has both cresolase and catecholase activities (Marusek, Trobaugh, Flurkey, & Inlow, 2006), apple PPO is more active toward o-diphenols rather than monophenols (Zhou, Smith & Lee, 1993) and sweet potato PPO has not been shown to possess cresolase activity (Klabunde, Eicken, Sacchettini, & Krebs, 1998; Marusek, Trobaugh, Flurkey, & Inlow, 2006). Variation in substrate specificity of PPO among different vegetal species is mainly due

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to the different endogen polyphenol composition (Yoruk & Marshall, 2003).

Most inhibitor searching studies have used the commercially available mushroom PPO and ignored the differences in substrate affinity and in inhibitor response of PPOs from different sources. For example: some natural inhibitors of banana PPO such as caffeic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, S-methyl-L-cysteine, did not inhibit mushroom PPO. On the contrary some mushroom PPO inhibitors such as 3,4 dihydroxybenzaldehyde, 2,6-dihydroxybenzoic acid, phenylacetic acid and 2,6 dihydroxy-toluol do not affect banana PPO (Ferrar & Walker, 1996). On the other hand, onion extracts showed different affinity for pear and banana PPO when the enzyme was extracted by the same technique (Kim et al., 2005; Lee, 2007). Since these differences in inhibition are relevant when addressing enzymatic browning control, the use of a target food system as PPO source is an interesting approach in order to avoid enzyme specificity problems.

The search for natural inhibitors has been based mostly on spectrophotometric assays (Kim et al., 2005; Lee et al., 2007). This methodology has the advantage of quantitative measurement, but natural extracts are complex mixtures with dark colour which difficult accurate absorbance measurements. Thin layer chromatography (TLC) coupled with biological detection assay is designed to separate possible active molecules and to measure its activity on chromatographic plates, facilitating the localisation and target-directed isolation of the active constituents on a complex mixture (Ramallo, García, & Furlan, 2015; Salazar & Furlan, 2007). Fig. 1 shows a schematic description of a bioautographic system used to detect enzymatic inhibition activity. The different components of a mixture are separated running a chromatography on a TLC plate. A staining solution containing enzyme-substrate system that develops a visible colour is then applied on the plate. After incubation, active compounds are detected as white spots on coloured background. TLC-bioautography has reached a broad scope of enzymatic inhibitory activities such as, acetylcholinesterase (Ramallo, Salazar, & Furlan, 2015; Ramallo, Salazar, & Furlan, 2015; Rhee, Van De Meent, Ingkaninan, & Verpoorte, 2001), xanthine oxidase (Ramallo, Zacchino & Furlan, 2006), α- and β-glucosidases (Ramallo, Gonzalez Sierra, & Furlan, 2012; Salazar & Furlan, 2007; Salazar, Micheloni, Escalante, & Furlan, 2011), lipase (Hassan, 2012) and mushroom tyrosinase (García & Furlan, 2015; Wangthong, Tonsiripakdee, Monhaphol, Nonthabenjawan, & Wanichwecharungruang, 2007).

In this work, we describe the development of a bioautographic assay suitable for the separation and detection of enzymatic browning inhibitors from complex natural sources based on PPO extracted from apple.

2. Materials and methods

2.1. Fruits and materials

Onion (*Allium cepa*), garlic (*Allium sativum*) and Blue Bow Delicious apples were purchased on the local market. 3-Metil-2-benzothiazolinona hidrazona (MBTH), poly(vinylpolypyrrolidone) (PVPP), 4-hexylresorcinol (4HR) and catechol were purchased from Sigma (St Louis, MO, USA). L-DOPA and kojic acid were purchased from Saporiti (Buenos Aires, Argentina). Cysteine was purchased from Mallinckrodt (USA). Ascorbic acid, methanol, acetone, ethyl acetate, di-sodium hydrogen phosphate and sodium di-hydrogen phosphate were purchased from Cicarelli (Rosario, Argentina). Aluminium-backed silica gel 60 F_{254} and reverse phase RP_{18} TLC layers were purchased from Merck (Darmstadt, Germany). Agarose was purchased from Biodynamics (Buenos Aires, Argentina). All reagents used were analytical grade.

2.2. Onion and garlic extract preparation

Onion or garlic (50 g) were homogenized using a home mixer (Moulinex model D63, Moulinex, France) with 50 mL deionised water for 5 min, and the mixture was heated at 100 °C for 10 min and filtered through cheesecloth. The filtrate was centrifuged at 12,000 × *g* for 20 min at 4 °C and the supernatant was freeze dried and stored for further experiments (Lee et al., 2007).

2.3. Preparation and partial purification of PPO from apple

Apples were peeled and sliced. 50 g of apple were mixed with 50 mL of 50 mmol/L sodium phosphate buffer (pH 6.8) and PVPP was added to a final concentration of 4%. The mixture was homogenized with a home mixer. Then it was centrifuged at 12,000 × *g* for 20 min at 4 °C and the supernatant was stored at −20 °C for further experiments.

2.4. Polyphenol oxidase activity quantification

Due to the use of a natural source of enzyme, the activity was measured after the extraction in order to ensure consistency. The polyphenol oxidase activity was measured spectrophotometrically by using a λ25 spectrophotometer (Perkin Elmer, Norkwalk, CT). The reaction mixture consisted of 0.1 mL of apple extract containing polyphenol oxidase and 1.9 mL of 50 mmol/L phosphate buffer at pH 6.8. The reaction was started by adding 1.0 mL of 0.2 mol/L catechol solution to the cuvette and the absorbance at 420 nm was recorded continuously for 2 min (Zauberman et al., 1991). The total volume of the assay was 3.0 mL. The temperature was maintained at 25 °C. One enzymatic unit (U) of PPO activity was defined as an increase in the absorbance at 420 nm by 0.001/min when using catechol as substrate. Substrate blanks were prepared by excluding the enzyme, from the reaction mixture and buffer solution was used to complete the final volume.

2.5. PPO staining solution

This section describes the selected protocol used to prepare PPO

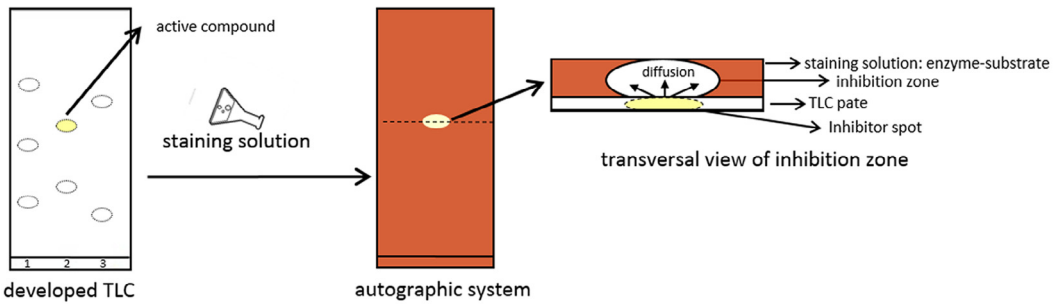

Fig. 1. Schematic description of the bioautographic assay principle designed to detect enzyme inhibition. Enzyme substrate system is applied in agar gel. On autographic system reddish colour represents enzyme active zone and white enzyme inhibition zone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

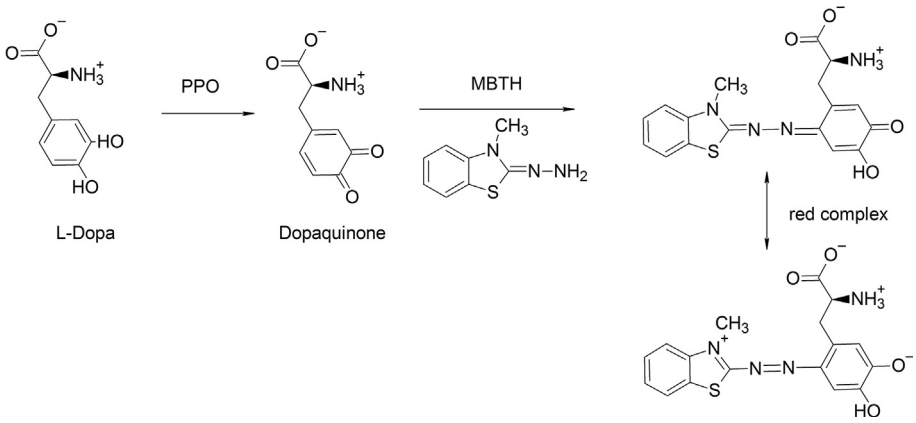

Fig. 2. Oxidation of L-DOPA to dopaquinone catalysed by PPO and reaction with MBTH to produce red complex.

staining solution that produced the best results. The following quantities are expressed as mass of reagent or volume per cm^2 of TLC plate: 1.05 mg agar and 10 μg of MBTH were dissolved in 0.13 mL of 50 mmol/L phosphate buffer pH: 6.8 at about 80 °C. Then 20 μL of 15 mmol/L of L-DOPA aqueous solution were added. The agar solution was allowed to cool to 45 °C, 2.71 μL of PPO were added (4.0 U) and the resulting solution was thoroughly mixed.

2.6. Analysis of known inhibitors and limit of detection

When the sample is a pure compound, there is no need for previous separation. During the analysis of pure PPO inhibitors, TLC plates were used as support for sample applications but previous thin layer chromatography was not carried out. 5 μL of inhibitor solutions of different concentrations were spotted on a 4x7 cm TLC plate (normal phase $60F_{254}$ or reverse phase RP18) to obtain spots with decreasing amounts of inhibitor. After solvent evaporation, 4.2 mL of staining solution were poured on the plate and gel formation was observed. After solidification, the PPO activity produced a red colour and enzyme inhibition was detected as clear spots against a dark red background. Since false negative results could be produced by direct reaction of certain compounds whit MBTH and/or L-DOPA, a control experiment was carried out without enzyme.

2.7. Chromatography and image analysis

The chromatograms were run and processed using equipment from CAMAG (Muttenz, Switzerland). Separation of natural extracts were performed on 6 × 4 cm TLC layers. 10 μL of each sample containing 1 mg of extract or 0.5 μg of ascorbic acid as positive control were applied in 5 mm bands onto the TLC plate using a CAMAG Automatic TLC Sampler 4 (ATS 4) under nitrogen flux. Elution was carried out with *n*-ethyl acetate:methanol (50:50) using an Automatic Developing Chamber 2 (ADC 2).

After solvent evaporation, 3.6 mL of the PPO staining solution were distributed evenly over the TLC layer. Colour development and control experiment were carried out as described in Section 2.6. TLC images were captured under white light with a CAMAG TLC Visualizer. The chromatograms were scanned using a TLC scanner 4 and optical density graphs were plotted.

3. Results and discussion

Initial attempts were made spraying the enzyme and substrate solution directly on a TLC layer. In those experiments, homogeneous enzyme distribution over the plate surface was difficult to obtain, and colour development could not be observed. Hence we decided to apply the enzyme-substrate system in an agar gel layer (Fig. 1). Different substrates were tested and different reagent addition sequences were evaluated to enhance the contrast between the colour developed by enzyme activity and the colour of the zones wherein the enzyme is inhibited. Once de detection system was set up, the limit of detection was analysed on normal phase and reverse phase TLC plates and finally the assay was used to detect PPO inhibitory compounds in natural extracts (onion and garlic).

3.1. Substrate selection and assay optimization

Initially catechol was tested as PPO substrate and ascorbic acid, spotted on the TLC plate (1 μg/spot), was used as enzyme inhibitor. Agar was dissolved at 80 °C in phosphate buffer containing catechol, the solution was allowed to cool to 45 °C and PPO was added. This staining solution was poured evenly on the TLC plate. A control experiment was carried out without enzyme. The plates were allowed to rest in a wet chamber for colour development. The colour produced was too dim to be useful for assay development. Therefore 3-methyl-2-benzothiazolinone hydrazone (MBTH) was evaluated to enhance the detection of the quinones produced by the enzyme activity. MBTH reacts with quinones to produce a red complex that could result in better gel staining and improved contrast (Böyükbayram, Kiralp, Toppare, & Yağci, 2006). TLC plates, treated in the same conditions as described above, were immersed in MBTH solution. Different concentrations of substrate, MBTH and PPO were tested along with different immersion times. In this series of experiments a reddish colour staining was observed on the gel, even in the control plates without PPO. This colour probably resulted from the direct reaction of catechol, or a catechol derivative produced under non-enzymatic conditions, with MBTH. In order to avoid unspecific gel staining, L-DOPA was tested as an alternative substrate with good affinity for apple PPO (Rocha & Morais, 2001). The colour reaction is described in Fig. 2. Three different reagent addition sequences were tested. All solutions were made in phosphate buffer and PPO was added to the agar at 45 °C.

1) The molten agar solution containing L-DOPA and PPO was poured on the TLC plate and once it was solid, the plate was immersed in a solution of MBTH.
2) The same procedure was carried out but adding MBTH and PPO to the molten agar and immersing the plate on L-DOPA solution.
3) Finally agar was added to MBTH solution in buffer, the mixture was heated to 80 °C and allowed to cool to 45 °C, when L-DOPA solution and PPO were added. The resulting staining solution was layered onto a TLC plate.

The three systems were incubated at room temperature on a wet chamber for 60 min before reading. A spot of 1 μg of ascorbic acid was used as inhibitor and controls without PPO were run in the three

sequences. The best results were observed when the last sequence was used.

Using the last sequence, different reagent concentrations were tested to enhance colour development and contrast. MBTH 1.6×10^{-4}, 8×10^{-5} and 4×10^{-5} mmoles/cm^2 were tested. The amounts of L-DOPA tested were: 3×10^{-4}, 1.4×10^{-4}, 0.74×10^{-4} and 0.35×10^{-4} mmoles/cm^2. Enzyme activities used were 4, 2 and 1 U/cm^2. The highest colour difference between inhibition zone and enzyme active zone were obtained with 1.05 mg/cm^2 of agar, 8×10^{-5} mmol/cm^2 of MBTH, 1.4×10^{-4} mmol/cm^2 of L-DOPA and 4.0 U/cm^2 of PPO.

3.2. Detection limit

The assay was run in presence of four known inhibitors of enzymatic browning: 4-hexylresorcinol, ascorbic acid, kojic acid and cysteine (Son et al., 2001). Both, normal phase 60F and reverse phase RP_{18} TLC plates were tested in order to check the assay compatibility with different separation systems. The mechanism of browning inhibition is different for the compounds tested: ascorbic acid and cysteine reduce dopaquinone to dopahydroquinone thus reverting the reaction pathway (Friedman, 1996); kojic acid acts as inhibitor on the enzyme (Jukanti, 2017) and is able to reduce o-quinone to o-diphenol thus preventing the formation of the final pigment (Kim et al., 2005). Finally, 4-hexylresorcinol has been described as a competitive inhibitor (Álvarez-Parrilla et al., 2007).

In order to evaluate the sensitivity of the assay, the inhibitors were spotted in decreasing amounts on the TLC layer. The minimum amount detected was 0.0125 μg for 4HR, 0.025 μg for ascorbic acid, 0.5 μg for cysteine and 1 μg for kojic acid on silica gel 60F TLC plates. Fig. 3 shows the inhibition spots for the different amounts used for 4-HR and ascorbic acid. The optical density graph makes clearly noticeable the peak corresponding to 0.0125 μg of 4HR. García and Furlan (2015) found a minimal inhibitory amount of 0.0125 μg of kojic acid when they developed a commercial tyrosinase immobilization assay on agar. When comparing the inhibitory effect of kojic acid on PPO from different sources it has been found that is far more effective on mushroom enzymes compared to fruit PPO (Ferrar & Walker, 1996; Palma-Orozco, Marrufo-Hernández, Sampedro, & Nájera, 2014). Other enzymatic autographies have shown a wide range of sensitivities for example, Ramallo, Zacchino, and Furlan (2006) could detect 0.005 μg of allopurinol in a xanthine oxidase inhibition assay, 0.1 μg of conduritol β-epoxide was the limit when studying β-glucosidase (Salazar & Furlan, 2007), and Hassan (2012) detected 0.01 μg of orlistat in a lipase inhibition autography.

When running the assay on RP_{18}-TLC plates inhibition spots were also observed indicating that the method is compatible with both stationary phases. For the tested inhibitors, the minimum amount detected was always higher compared to normal phase plates: 0.25 μg for 4HR, 0.5 μg for ascorbic acid and 1 μg for cysteine, kojic acid was not detected even at 1 μg. The decrease in sensibility, observed when using reverse phase, could be due to a homogeneous gel layer formation is more difficult on the RP-TLC surface. In addition the repulsion between the hydrophilic and the hydrophobic layers at the interface between the gel and the plate surface hampers the migration of compounds from the TLC plate to the gel (García, Ramallo, & Furlan, 2017; Ramallo et al., 2015). The application of aqueous matrices in the form of gels on hydrophobic surfaces of RP_{18}-TLC plates broadens the separations possibilities in the study of natural mixtures. Therefore, the compatibility of the technique with both stationary phases allows the search for new inhibitors present in aqueous extracts that are better resolved in reverse phase.

3.3. Analysis of onion and garlic extracts

Onion and garlic extract were analysed by PPO autography after development of the chromatography on 60F silica plates and results are shown in Fig. 4. Both extracts showed clear browning inhibition zones at $R_f = 0.55$ for garlic and at R_f near to cero for onion, whereas no reaction was detected in control plates. It has been suggested that low molecular weight compounds are responsible for the inhibitory effect on different fruit PPO observed in species from *Allium* genus (Arzani et al., 2010; Lee et al., 2007). However, these compounds have not yet been isolated and identified (Kim et al., 2005). The difference in migration distance for the inhibition zones observed in our TLC-coupled assay suggests that the molecules that inhibited PPO in garlic and onion are different.

When the plates where analysed under UV light at 254 and 365 nm it could be seen that, for garlic, a mayor spot remained in the starting point and other compounds migrated and were responsible for the

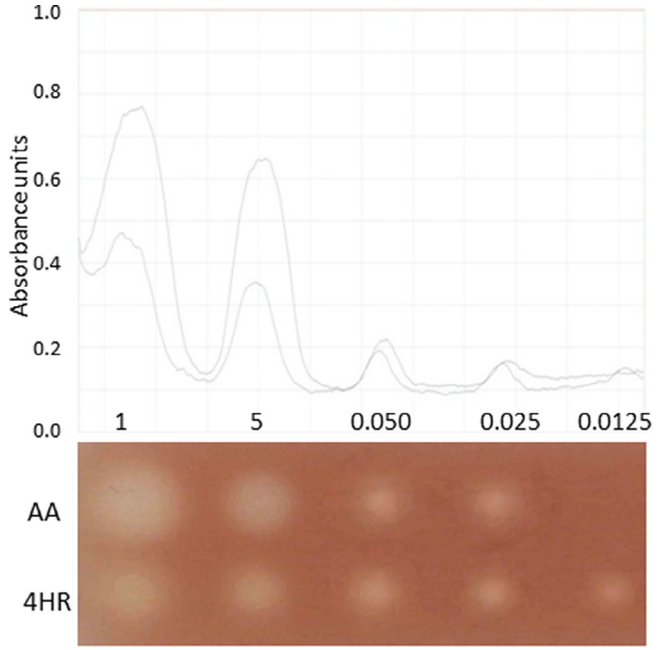

Fig. 3. TLC layer spotted with decreasing amounts of ascorbic acid (AA) and 4-hexylresorcinol (4HR). Spot mass (μg) is indicated at the bottom of the optical density graph (upper line AA, lower line 4HR).

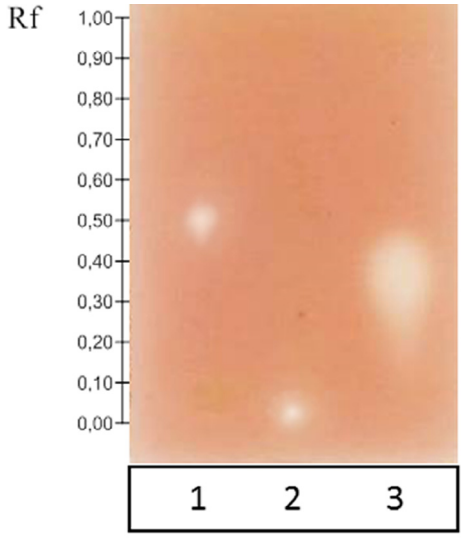

Fig. 4. TLC chromatograms of extracts from *Allium sativum* (1000 μg, lane 1) and *Allium cepa* (2000 μg, lane 2) and ascorbic acid (0.5 μg, lane 3) revealed using PPO autographic assay.

inhibition observed (see Complementary figure). On the other hand, onion extract migrated in the form of a wide band and the compounds that inhibited apple PPO remained in the starting point. Ramallo et al. (2015) have recently proposed an innovative strategy to facilitate the identification of active compounds of natural extracts coupling high-resolution mass spectrometry to enzymatic autography, which could facilitate the rapid identification of compounds present in complex mixes as these extracts.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.07.025.

When studying the inhibitory effect of onion extract on enzymes from different fruits such as taro, pear and banana it was found that, as occurred with synthetic compounds, the effectiveness depended on the PPO source. For example 3.1 mg/mL of onion extract inhibited 77% of banana PPO activity (Lee, 2007) and 54% when PPO was extracted from taro (Lee et al., 2007). In the case of PPO extracted from pear it was needed a concentration of 60 mg/mL of onion extract to reach 54% inhibition (Kim et al., 2005). These results empathize the importance of targeting the correct enzyme source when searching for antibrowning applications.

4. Conclusions

Polyphenol oxidases are enzymes that have differences depending on whether they are obtained from vegetal or fungal sources. Therefore in the autographic study of new inhibitors from natural extracts it is important the use of the PPO of the food under study.

The gel entrapment method was more appropriate for the development of enzymatic based autographic assays than direct spray application because allowed to accurately measure the amount of enzyme and substrate used and an evenly surface distribution of reagents. The inclusion of colour enhance reagents, as MBTH, was a useful tool to improve contrast between background and inhibition zones. In spite of a reduction in sensitivity, the assay was compatible with normal and reverse phase TLC systems. The proposed method allows detecting compounds that directly had action on the enzyme as well as reducing agents that could convert recently formed quinones back to their reduced form.

This simple and easy autographic technique evidenced the different nature of enzymatic browning inhibitory compounds from garlic and onion. Given that, many enzymes can be immobilised by gel entrapment whilst conserving their activities autographic methods based on natural sources would then broaden the range of enzymes that could be used and therefore represent a fast and cheap alternative for exploration of natural enzymatic inhibitors.

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The authors declare no conflict of interest on the research or on the writing of this paper.

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