JPC – Journal of Planar Chromatography – Modern TLC
https://doi.org/10.1007/s00764-021-00140-6

SHORT COMMUNICATION

Optimized high-performance thin-layer chromatography–bioautography screening of Ecuadorian *Chenopodium quinoa* Willd. leaf extracts for inhibition of α-amylase

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Received: 17 August 2021 / Accepted: 14 December 2021

Keywords High-performance thin-layer chromatography (HPTLC) · Low reagent consumption · α-Amylase bioassay · Bioautography · Quinoa leaves (*Chenopodium quinoa* Willd. leaves)

1 Introduction

Bioautographic assays on high-performance thin-layer chromatography (HPTLC) plates play a relevant role to rapidly identify bioactive compounds from plants, enabling the fast detection and localization of the compounds responsible for the tested activity in complex plant matrices [1]. Furthermore, this technique is effective and relatively inexpensive and can be performed in small research laboratories without access to sophisticated equipment [2], but, as in any analytical process, attention must be paid to the possible sources of variability that may affect the results. Derivatization implies another step in the process, consequently causing an increase in variability. Using an automated derivatization device such as the CAMAG® Derivatizer (Muttenz, Switzerland) provides reproducible results (relative standard deviation, RSD < 5% between assays) equal to those obtained with an immersion device, yet consuming considerably less reagent [3]. The major advantage of bioautography is that it allows the fast screening of a large number of plants for various bioactivities, for example, antibacterial, antifungal, antioxidant, enzyme inhibition, etc. [4].

The steady increase in diabetes is becoming a major burden to health care systems worldwide [5, 6]. The inhibition of starch-hydrolyzing enzymes like α-amylase delays the elevation of postprandial blood sugar levels; this is a validated therapeutic target for the prevention and treatment of type 2 diabetes mellitus, as indicated by the widespread use of acarbose [7]. Imbalanced glycemic index and glucose intolerance are indeed improved by inhibiting enzymes involved in carbohydrate digestion, such as pancreatic α-amylase, which degrades amylaceous polysaccharides (starch, glycogen, amylose, amylopectins) and various maltooligosaccharides [8] into a mixture of smaller oligosaccharides such as maltose, maltotriose, and maltotetraose [9]. Most of the commercially available synthetic inhibitors, such as acarbose, miglitol, and voglibose, have strong amylase and glucosidase inhibitory properties, causing an excessive inhibition of enzymes and subsequent abnormal fermentation of undigested saccharides in the colon, resulting in abdominal distention, meteorism bloating, flatulence, and possibly diarrhea [10, 11]. Those side effects, which depend on the ingested polysaccharides, with major differences according to populations [12], somewhat limit the use of alpha-amylase inhibitors. Therefore, there is an interest in screening natural sources for novel enzyme inhibitors [8], possibly with less side effects. Plants investigated for α-amylase inhibitory activities include *Tamarindus indica* (leaves), *Vaccinium myrtillus* (leaves), *Balanites aegyptiaca* (bark), *Camellia sinensis* (leaves), *Khaya senegalensis* (bark), *Mitragyna inermis* (leaves), *Rosmarinus officinalis* (leaves), *Securidaca longepedunculata* (root), *Salvia officinalis* (leaves), *Trigonella foenum-graecum* (seeds), *Anacardium occidentale*, *Lagerstroemia speciosa*, *Averrhoa bilimbi*, *Pithecellobium jiringa*, *Parkia speciosa*, and *Phyllanthus amarus* [8]. Plant constituents theaflavins, catechins, and chalcones notably inhibit α-amylase and/or α-glucosidase [8].

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Published online: 10 January 2022

Quinoa (*Chenopodium quinoa* Willd.), a pseudocereal traditionally consumed by Andean cultures in South America, is rapidly gaining popularity as a functional food and nutraceutical [13, 14]. Quinoa is a rich source of bioactive compounds, notably flavonoids, saponins, and phytoecdysteroids, with a wide range of potential beneficial health effects, e.g., a reduction in risks for cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, etc. [15–18]. On the other hand, little information is available concerning the nutraceutical potential of the green parts of quinoa, such as the leaves that have been treated until now as worthless waste but that are, in fact, edible [15].

The present study aimed at improving an HPTLC–bioautographic method to detect the α-amylase inhibitory activity [5] of quinoa leaf extracts by determining optimal and reproducible chromatographic conditions (mobile phase, plate drying, incubation times) and especially derivatizing conditions, reagent preparation, and application using the CAMAG® Derivatizer, an environmentally friendly and safe handling through a closed system with low reagent consumption and homogenous reagent distribution [3].

2 Experimental

2.1 Principle of HPTLC α-amylase inhibitory activity assay

The inhibitory activity of plant extracts against α-amylase was determined on HPTLC plates, according to the method of *Agatonovic-Kustrin et al.* [5] (Fig. 1), using starch as substrate and iodine vapors for the visualization of bands. The reagent preparation was modified to allow automatic spraying on the plates.

Upon sample application, development, and drying, three steps are needed for the evaluation of enzyme inhibition: (1) homogeneous impregnation of the plates with α-amylase and incubation at 37 °C for eventual binding between enzyme and inhibitors; (2) homogeneous impregnation of the plates with starch and incubation at 37 °C to allow starch digestion by the enzyme; and (3) visualization of the plate with iodine vapors, based on the blue coloration of complex formed between iodine and undigested starch (blue bands corresponding to compounds inhibiting α-amylase). The effect of several key factors on the enzyme assay was investigated to select the optimal parameters and evaluate the potential of Ecuadorian quinoa leaves as a source of antidiabetic compounds.

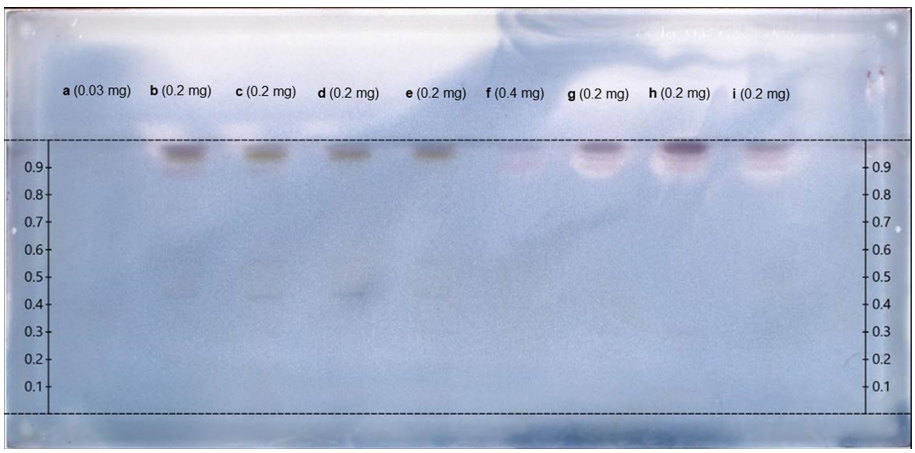

Fig. 1 Initial HPTLC plate before optimization with different varieties of quinoa leaves; extracts were performed either in methanol or in deep eutectic solvents (DES); in the latter case, the DES was eliminated by solid-phase extraction on C18 [19]. *Mobile phase* formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, *V/V*). *Derivatization* spray with α-amylase (3 mL, ~ 20 U/mL, nozzle yellow, level 4) and immersion in starch (1% *w/V*) in aqueous solutions, followed by exposure to iodine vapors (iodine saturated tank; 2 min). *Tracks* (*in brackets, mg of sample applied on plate*) **a**, Acarbose (5 mg/mL); Quinoa leaf MeOH extracts (50 mg/mL), **b**, Iniap Tunkahuan sweet variety; **c**, Chimborazo bitter variety color yellow; **d**, Chimborazo bitter variety color green; **e**, Chimborazo bitter variety color red; **f**, Quinoa seed MeOH extract Iniap Tunkahuan variety. The extracts of quinoa described in tracks **g**, **h**, and **i** were obtained using NADES solvents (green solvents); DES application to quinoa compound extraction is part of another study in progress

2.2 Chemicals and preparation of solutions

All reagents were of analytical grade. α-Amylase from *Bacillus licheniformis* (Cat. N°. A4582-5 mL), starch, iodine (≥ 99.8%), and absolute ethanol (≥ 99.8%) were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). Total starch assay kit K-TSTA-100A was bought from Megazyme® (including thermostable α-amylase, amyloglucosidase, D-glucose standard, and maize starch control; Wicklow, Ireland). Acarbose was extracted from the drug Glucobay® 50 mg (Bayer, Brussels, Belgium) by dissolving one tablet of Glucobay 50 mg in methanol−water (1:1, *V/V*) to a final concentration of 5 mg/mL (standard solution). The α-amylase solution (~ 5 U/mL) was prepared by mixing 50 µL of α-amylase (Cat. N°. A4582-5 mL) with 20 mL of water (stock solution, stored at 4 °C for a week) and diluted to the required concentrations about 60 min before use with 10% ethanol (*V/V*). A homogeneous solution of starch was prepared by dissolving 1 g of starch in 40 mL of water (70 °C, stirring at 350 rpm for 30 min), adding water up to 100 mL and cooling slowly by stirring to room temperature (RT). That solution was viscous and difficult to apply using CAMAG® Derivatizer; therefore, in order to reduce its viscosity, 10 mL of ethanol (≥ 99.8%) was added and the solution was stirred for 2 h at RT.

2.3 Preparation of quinoa samples

Quinoa leaves, Pata de Venado sweet variety from Ecuador, were collected at three different cultivation times (40, 60, and 80 days) in the Santa Catalina Experimental Station of the Instituto Nacional de Investigaciones Agropecuarias (INIAP) located in Pichincha, Ecuador. The extracts were prepared as follows: 0.2 g of lyophilized and milled quinoa leaves was mixed with 4 mL of methanol−water (80:20, *w/w*), vortexed for 5 min, heated at 40 °C for 1 h, ultrasonicated at RT for 30 min, and centrifuged at 4000 g for 40 min [19]. The supernatant was directly applied on HPTLC plates.

2.4 High-performance thin-layer chromatography−bioautography

HPTLC was performed according to the procedure of the European Pharmacopeia 10 [20]. The conditions described in this section and in 2.4.1 were established after the method optimization process. Chromatographic layers were HPTLC silica gel 60 F_{254} plates (Merck, Germany). Sample was applied using automatic TLC sampler (ATS 4), tracks with a band length of 8.0 mm, track distance of 11.4 mm, and application volume of 14 µL. Chromatography was performed in the Automatic Developing Chamber 2 (ADC 2) with chamber saturation for 20 min under relative humidity maintained at 33%, using a saturated solution of magnesium chloride, development with the mobile phase formic acid−water−methyl ethyl ketone−ethyl acetate (10:20:40:30, *V/V*) to 70 mm from the lower edge, drying for 5 min.

2.4.1 Post-chromatographic derivatization

The developed plate was heated at 105 °C for 60 min using the TLC plate heater. Derivatization was achieved in three steps: (1) a 3 mL solution of α-amylase (5 U/mL of α-amylase in ethanol 10%) was applied on the plate (CAMAG® Derivatizer with yellow nozzle, level 4), incubation at 37 °C for 30 min, (2) a 2 mL solution of starch (1% *w/V* of starch in ethanol 10%) was applied on the plate (CAMAG® Derivatizer with yellow nozzle, level 6), incubation at 37 °C for 10 min, and (3) detection using iodine vapors for 2 min (1 g of solid iodine in a 20 × 20 cm development chamber). Documentation was performed using TLC Visualizer 2 under white light after the derivatization. The CAMAG® systems were driven by the visionCATS version 2.5 software.

3 Results and discussion

In most of the reviewed articles about bioautography detection on thin-layer chromatography [5, 21, 22], authors either dip the plate inside a chamber filled with a "reagent" (reagent solution or microorganism suspension) or manually spray this "reagent" over the plate. The main disadvantages are, for dipping, the high amounts of reagent needed (from 40 to 200 mL, depending on the tank) and the smears induced by immersion waves (Fig. 1); and, for spraying, the difficulties in ensuring homogeneity and plate-to-plate reproducibility. The situation is particularly difficult for aqueous "reagents," needed for enzyme- and microorganism-based methods.

The CAMAG® Derivatizer was used for automated reagent application in the derivatization of thin-layer chromatographic supports and set a new standard of reproducibility by employing a unique "micro droplet" spraying technology (patented by CAMAG®), consisting of a nozzle that generates an extremely fine reagent aerosol, which evenly distributes in the closed chamber and gradually settles on the HPTLC plate [3]; this technology was recently developed to ensure homogeneity and convenience in applying derivatization reagents with different viscosities and low reagent volume consumption (2–3 mL) and presents an attractive solution for bioautography with aqueous reagents; in this study, we aim at defining the experimental conditions for an enzyme-based assay.

3.1 Development of the bioautography reagents system

A series of α-amylase and starch solutions (different concentrations and ethanolic dilutions) were tested with the automated "micro droplet" spraying unit to obtain a viscosity suitable for homogeneous spraying, while keeping concentrations amenable for detection. The final starch solution described in Sect. 2.2 was stable and could be stored at RT for several weeks. The activity of the enzyme is not hampered by the presence of 10% ethanol; this was verified by pre-incubating α-amylase solution (Megazyme® kit) with 10% ethanol for 0 and 60 min, at RT, before the addition of α-glucosidase. The % of total starch obtained for the kit reference starch assay, without (88.3%) and with ethanol (87.8% and 86.4% at times 0 and 60 min after ethanol addition, respectively), indicates an efficient digestion of control starch in all cases [23]. Besides, in our HPTLC assay, we presume that the ethanol partly evaporates during incubation, leading to a reduction in its concentration.

Fig. 2 Incubation system consisted in: a container filled with water, such that the plate did not come into contact with the water, but enough to maintain humidity inside the container when it was covered

3.2 Investigation of plate drying spray conditions and incubation time

To optimize the experimental parameters, such as incubation time and concentration of reagent, acarbose (10 µL, 5 mg/mL) was applied with a micropipette in the center of TLC silica gel plates (3 × 10 cm). The plates were developed in the automatic developing chamber, using the HPTLC conditions described in Sect. 2.4 and using mobile phase 1 (MF1) formic acid−water−methyl ethyl ketone−ethyl acetate (10:10:30:50, *V/V*). The plates were then dried at 105 °C on the TLC plate heater for 60 or 90 min with the goal of eliminating the mobile phase, especially formic acid (a denaturing agent that inhibits α-amylase). The plates were cooled to RT, sprayed with 3 mL of α-amylase solution (5 U/mL, yellow nozzle, level 4) and placed over four supports inside a closed 500-mL polypropylene container, previously filled with 100 mL of water and equilibrated at 37 °C for 2 h (the support prevented contact between plate and water) (Fig. 2). Incubation was performed for 30 min at 37 °C for the primary reaction between the enzyme and any inhibitors present in a sample (acarbose in this case). After incubation, the plates were sprayed with 2 mL of starch solution (yellow nozzle, level 6), incubated as above for different times (5, 10 or 15 min) for enzyme–substrate reaction and then placed for 2 min in a chamber saturated with iodine vapors (Fig. 3).

Figure 3 shows blue zones on all plates at the application spots, indicating the expected acarbose inhibition of α-amylase [5]. Note that, in our solvent system developed for quinoa samples, there is practically no migration of acarbose. The bioautography with α-amylase and starch, using the automated "micro droplet" spraying unit and the solutions we developed, was fully efficient with a very uniform

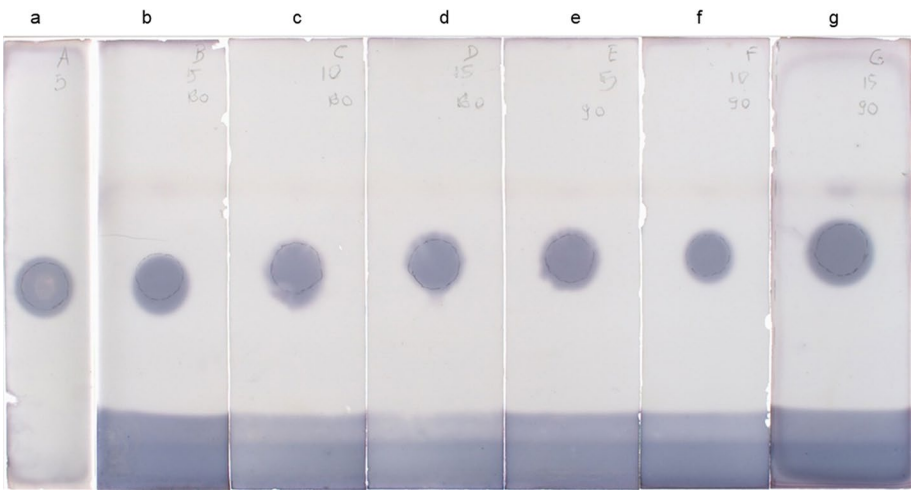

Fig. 3 Acarbose (10 µL, 5 mg/mL) applied at the center of the plate. **a** Control plate, without mobile phase development. Plates **b**, **c**, and **d** were developed, heated for 60 min at 105 °C and incubated at 37 °C during 5, 10, and 15 min, respectively, for enzyme–substrate reaction. Plates **e**, **f**, and **g** were heated for 90 min at 105 °C and incubated at 37 °C during 5, 10, and 15 min, respectively. All plates were incubated for 2 min in a chamber saturated with iodine vapors

background, consuming only small volumes of reagents (for a 10×20 cm HPTLC plate, 3 mL and 2 mL of α-amylase and starch solutions, respectively). At the lower edge of plates (b) to (g), the large blue area may indicate that migrated formic acid could not be removed, denaturing the enzyme and preventing starch degradation. Also, we observed that the plates heated to 60 and 90 min had a large blue area. Therefore, there was no evidence that a longer time favors the total removal of solvents that denature the enzyme in that area; for that reason, a heating time of 60 min was kept for following experiments. Figure 3b–d indicates that, regardless of the time of the incubation at 37 °C, the acarbose blue color clearly develops. Taking these results into account and considering that, in the original method [5], a time of 10 min was selected, our subsequent experiments were performed with this incubation time.

3.3 Investigation of α-amylase concentrations

The next experiment was designed to test the effect of different concentrations of α-amylase (2.0, 2.5 and 3.0 U/mL) on the bioautography of quinoa samples. Indeed, the amount of α-amylase on the plate conditions the detectability of the method; a low-concentration inhibitor, or a weaker inhibitor, most probably would not inhibit a too high of an enzyme activity. Using previously selected conditions, samples of quinoa leaf methanolic extracts, Pata de Venado variety, collected after 80 days of cultivation, were applied (6 µL of methanolic extract whose concentration was 50 mg/mL considering the mass of the sample and the volume of solvent used for extraction) with a micropipette at the bottom of the plate. Acarbose was then applied in the middle of the plates that were developed as in the previous experiment.

Figure 4a shows that quinoa leaf methanolic extract was separated in many spots of different colors after the development with MF1; the developed and dried plate was dipped into the chamber saturated with iodine vapors, proving that spots in the sample do not generate blue color with iodine. Figure 4b–d shows the effect of α-amylase

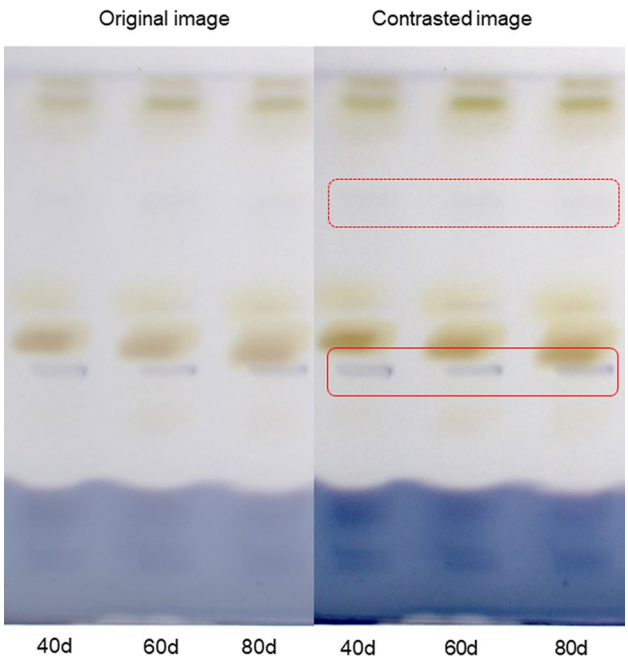

Fig. 5 HPTLC chromatograms of quinoa leaf methanolic extracts (8 µL, 50 mg/mL), Pata de Venado Ecuadorian variety harvested at different cultivation times (40, 60, and 80 days, i.e., tracks 40d, 60d, and 80d). Development using formic acid−water−methyl ethyl ketone−ethyl acetate (10:10:30:50, *V/V*) with spraying of α-amylase solution (2.5 U/mL), followed by 30-min incubation at 37 °C, spraying of starch solution and 10-min incubation at 37 °C. Plates were then placed for 2 min in a chamber saturated with iodine vapors at room temperature. Images were treated using the automatic contrasting tool of the visionCATS software. The red marks indicate α-amylase inhibition zones

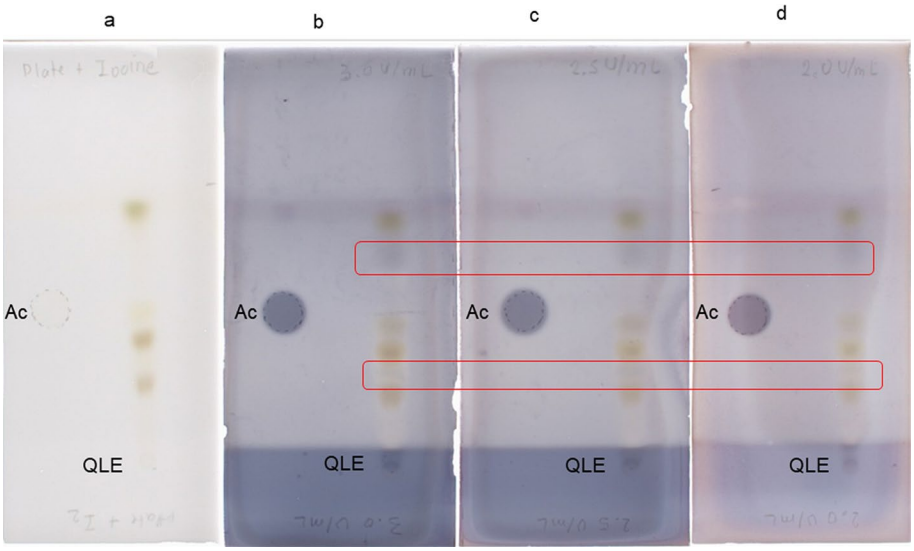

Fig. 4 Different concentrations of α-amylase solution sprayed on the plate. Acarbose (Ac) (6 µL, 5 mg/mL) and quinoa leaf methanolic extracts (QLE) (6 µL, 50 mg/mL) were applied on the plate. Development using MF1 without spraying (**a**); and with spraying of α-amylase solutions (**b**, 3.0 U/mL), (**c**, 2.5 U/mL) and (**d**, 2.0 U/mL), followed by 30-min incubation at 37 °C, spraying of starch solution and 10-min incubation at 37 °C. All plates were then incubated for 2 min in a chamber saturated with iodine vapors. The red marks indicate α-amylase inhibition zones

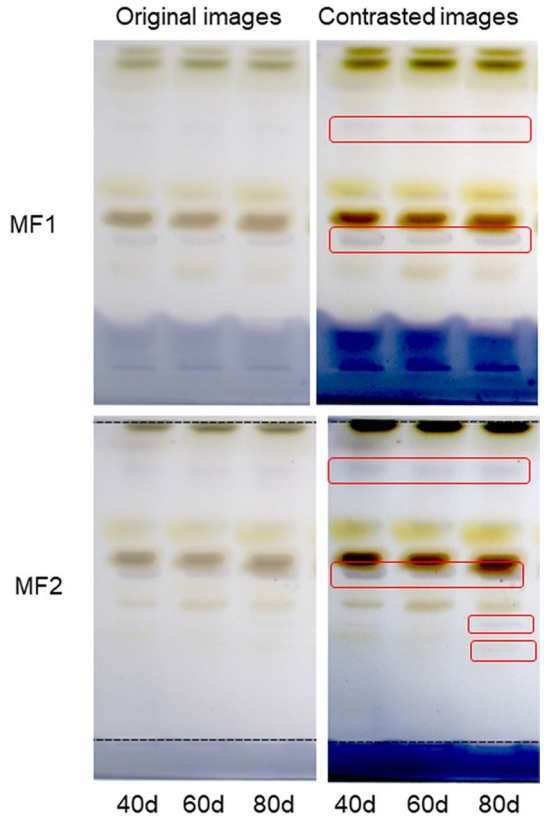

Fig. 6 HPTLC chromatograms of quinoa leaf methanolic extracts (14 µL, 50 mg/mL), Pata de Venado Ecuadorian variety harvested at different cultivation times (40, 60, and 80 days, i.e., tracks 40d, 60d, and 80d). Development using MF1 and MF2 with spraying of α-amylase solution (2.5 U/mL), followed by 30-min incubation at 37 °C, spraying of starch solution and 10-min incubation at 37 °C. Plates were then placed for 2 min in a chamber saturated with iodine vapors at room temperature. Images were treated using the automatic contrasting tool of the visionCATS software. The red marks indicate α-amylase inhibition zones

concentration. All three plates indicate at least two blue zones in the quinoa leaf methanolic extract. Plate (c) showed the best results as the blue zones in the sample appear more clearly than on plate (d). Plate (b) presented a light blue background that was more intense than plate (c), making it difficult to clearly see the blue zones in the samples. A concentration of α-amylase solution of 2.5 U/mL, combined with 10-min incubation, was selected as a compromise between a bluish background and sensitivity.

According to Fig. 5, quinoa leaf methanolic extracts were positive for α-amylase inhibitors; a clear and defined blue zone (marked with a solid red line) was observed in all samples regardless of the harvest time. However, a slight blue area (marked with a red dotted line) was observed in all three times, possibly due to a low concentration of α-amylase inhibitors in that zone. In order to clearly see the blue zone marked with a dotted line, the injected sample volume was increased from 8 to 14 µL in subsequent experiments.

3.4 Investigation of mobile phase for the resolution of chromatographic separation of quinoa samples

Two mobile phases were tested on HPTLC plates, MF1: formic acid−water−methyl ethyl ketone−ethyl acetate (10:10:30:50, *V/V*) and MF2: formic acid−water−methyl ethyl ketone−ethyl acetate (10:20:40:30, *V/V*) (Fig. 6). For this experiment, 14 µL of three different samples of quinoa leaves methanolic extracts (40, 60, and 80 days of cultivation) was applied. The sample volume was increased from 8 to 14 µL to see the blue zones on the plate more clearly.

Figure 6 shows at least two blue zones in all samples eluted with the MF1 mobile phase. Interestingly, when the plate was developed with the more polar MF2, the large blue area at the bottom of the plate was reduced, and in the 80-day sample, four blue zones were perfectly separated having evidence of consistent results.

4 Conclusion

In this study, we have presented the optimization of HPTLC bioautography for testing α-amylase inhibition. This method, based on an automated and close "micro droplet" spraying unit, allows the detection of hypoglycemic activities with low reagent (α-amylase and starch solutions) consumption (3 and 2 mL), ensuring homogeneity of background and sensitivity of detection. This optimized fast screening method allows rapid localizing of α-amylase inhibitory compounds in complex plant matrices and colored extracts, as exemplified by quinoa leaves, which represents a great advantage because it avoids the need for pretreatment of the sample before it is analyzed. It will be interesting to define a reference inhibitor that migrates on silica with various mobile phases, so as to have an internal control of the on-plate reaction.

The modifications of reagents applied here could easily be adapted to other enzyme-based bioautographies that most often rely on aqueous reagents and enzyme solutions. The reduction in reagent volumes is particularly interesting for costly enzymes and/or substrates, and friendly with the environment by reducing the generated waste.

Acknowledgements This work was supported by the Académie De Recherche Et d'Enseignement Supérieur (ARES) in Belgium. We would like to thank the CAMAG® company for the lending of a Visualizer system and their technical support; Instituto Nacional de Investigaciones Agropecuarias (INIAP) and Maquita Cushunchic (MCCH) in Ecuador for providing the quinoa leaf samples for this study; Cedrick Mutombo and Benjamín Martínez for their technical support.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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