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Qualitative Analysis by High-Performance Thin-Layer Chromatography–Bioautography of Ecuadorian *Chenopodium quinoa* Willd. Leaves: Influence of Variety, Phenological Stage, and Place of Cultivation on Free Radical Scavenging and α-Amylase Activity

Verónica Taco [1,2], Claudio Palmieri [2], Dayana Borja [1], Elena Villacrés [3], Pierre Duez [2] and Amandine Nachtergael [2,*]

[1] Faculty of Chemical Sciences, Central University of Ecuador (UCE), Av. Universitaria, Quito 170129, Ecuador; vjtaco@uce.edu.ec (V.T.)
[2] Unit of Therapeutic Chemistry and Pharmacognosy, Faculty of Medicine, Pharmacy and Biomedical Sciences, University of Mons (UMONS), Avenue du Champ de Mars, 25, 7000 Mons, Belgium; pierre.duez@umons.ac.be (P.D.)
[3] Nutrition and Food Quality Department, National Agricultural Research Institute, Santa Catalina, Quito 170518, Ecuador; elena.villacres@iniap.gob.ec
* Correspondence: amandine.nachtergael@umons.ac.be; Tel.: +32-65372212

Abstract: The present study aimed to qualitatively assess the influence of *Chenopodium quinoa* Willd. varieties (INIAP-Tunkahuan, INIAP-Pata de Venado varieties and Chimborazo genotype), phenological stages (40, 60, and 80 days), and places of cultivation (Pichincha and Chimborazo Ecuadorian provinces) on the leaf and seed phenolic composition and biological properties. Their nutraceutical potential was assessed through qualitative analyses of (i) their polyphenols by high-performance thin-layer chromatography (HPTLC); and (ii) their free radical scavenging (quenching of 2,2-diphenyl-1-picrylhydrazyl free radical, DPPH•) and α-amylase inhibitory properties (iodine visualization of starch hydrolysis) by HPTLC–bioautography. Compared to seeds, the quinoa leaf methanolic extracts present a high content of polyphenols with free radical scavenging activity, and compounds with an α-amylase inhibitory property; both biological activities indicate a remarkable potential of quinoa leaves, which may be relevant for the treatment of diabetes but also for the chemoprevention and/or treatment of pathologies related to oxidative stress. In quinoa leaves harvested after 80 days of cultivation, regardless of the place of production and the variety, a high content of bioactive compounds was observed. Future research is undoubtedly needed to further promote quinoa leaves as a dietary vegetable or to develop them into a nutritional supplement. This would empower quinoa smallholders in Andean regions to promote the sustainable development of this culture in its places of origin.

Keywords: amaranthaceae; DPPH•; polyphenols; antioxidant detection; α-amylase enzyme inhibitor screening

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1. Introduction

Quinoa (*Chenopodium quinoa* Willd, Amaranthaceae) is an important crop recognized as an ally for global food security in the 21st century and stands out among the crops for its resistance to abiotic stresses such as drought, hot temperature, high altitude, and saline soils. It has a broad genetic diversity, which allows it to adapt to various tough environments,

such as highlands and frost [1–3]. Quinoa seeds are rapidly gaining global popularity as a functional food and nutraceutical because they contain a variety of bioactive compounds (flavonoids, saponins, peptides, phytoecdysteroids, phytosterols, lectins, among others) with antioxidant, cytotoxic, antidiabetic, and anti-inflammatory broad spectra properties, demonstrated in vitro [4–7].

Today, biologically active compounds isolated from plants are still important sources for modern drug and nutraceutical formulations. Koseoglu Yilmaz et al. previously reported the phenolics, flavonoids, and biological activities of acetone and methanol extracts of a scarcely studied species, *C. album subsp. album var. micropyhllum* (note: the botanical status of this plant is not recognized in www.worldfloraonline.org (accessed on 12 December 2024). Acetone and methanol extracts showed similar DPPH free radical scavenging activities, whereas the cupric ion-reducing capacity of the acetone extract was the highest. The methanol extract was found to contain significant amounts of hesperidin and rutin [8].

Peru, Bolivia, and Ecuador are the leading quinoa-producing countries in the Andean region, as they have the largest quinoa cultivation areas and are key centers of domestication and biodiversity [9,10]. While quinoa is now grown in many countries worldwide, preserving agrobiodiversity remains a crucial task. This preservation not only safeguards the plant's genetic diversity but also protects the cultural heritage of the indigenous farmers in the Andean region [11]. Today, quinoa faces growing global demands. Sudden spikes in consumer interest led to rising prices, prompting land-use changes at both farm and national levels, resulting in rapid production increases. This phenomenon, known as a "boom", is often followed by a "bust", where prices and production sharply decline [12]. These effects are beginning to surface in Ecuador. Quinoa boom and bust cycles could have unforeseen consequences for consumers, including food insecurity in the regions of production and origin, simplified local food systems, and a potential reduction in the genetic diversity that has been preserved for centuries. Addressing this challenge requires a more inclusive approach that fosters active decision-making and evaluates future scenarios. One way to enhance the sustainable development of quinoa is by diversifying its use through research that explores the nutraceutical potential of other plant parts, such as the leaves, and by empowering smallholders to engage in sustainable production practices [11,12]. In this context, only limited information is available on the nutraceutical potential of quinoa leaves, even though their polyphenolic content is reported to be even higher compared to seeds [5,13]. The young quinoa leaves (harvested in the phenological vegetative stage, around 90 days of cultivation) are used as vegetables in some places in Asia and North and South America [13,14]. The advantages of promoting the consumption of quinoa leaves would be to (i) reduce the growth time before harvesting, i.e., a maximum of 3 months for leaves compared to 6 to 9 months for seeds, which, for producers, would lessen the risks of climatic threats; (ii) diversify the economy of quinoa producers; and (iii) promote health, as quinoa leaves may help in chemoprevention of cancer and other diseases related to oxidative stress; indeed, a previous in vitro study reported a probable high bioavailability of quinoa leaf polyphenols with cytostatic and anti-invasive effects on prostate cancer cells [13].

Polyphenolic compounds are a diverse and widely occurring group of plant phytochemicals; they are potent antioxidants and reactive oxygen species (ROS) quenchers [15,16] through both direct and indirect mechanisms, notably hydrogen donation, metal chelation, antioxidant enzyme upregulation, nuclear factor erythroid 2-related factor 2 (Nrf2) activation, sirtuin activation and/or pro-oxidant enzyme inhibition [17]. Such properties presumably imply preventive and/or therapeutic effects on a wide range of diseases, from cancer to neurodegenerative disorders [18]. Among polyphenols, flavonoids have

been extensively investigated and are already successfully applied to the treatment of osteoarthritis and certain vascular diseases; their interest has led to the development of new product delivery systems likely to increase their efficacy [17,19]. Also, a high number of in vitro and in vivo studies indicate that a diet rich in flavonoids may contribute to reducing the incidence of diabetes; flavonoids notably reduce glycemia, inhibit α-amylase and α-glucosidase, and protect the pancreas [20]. The inhibition of starch-hydrolyzing enzymes delays the elevation of postprandial blood sugar levels. Most of the commercially available synthetic inhibitors, such as acarbose, miglitol, and voglibose, strongly inhibit α-amylase and α-glucosidase, leading to colonic fermentation of undigested saccharides, abdominal distention, meteorism bloating, flatulence, and possibly diarrhea [21,22]. Therefore, there is interest in screening natural sources for novel enzyme inhibitors that may present fewer side effects [5]. Natural sources investigated for flavonoids' antidiabetic potential, specifically quercetin and kaempferol, include apples, berries, red onions, cherries, grapefruits, teas, cruciferous vegetables, *Hypericum ascyron* L., and *Myrica rubra* (Lour.) Siebold & Zucc [20,23].

When studying natural sources of active compounds, such as quinoa leaves, it is necessary to know the influence of production factors on their quality and efficacy, i.e., on their polyphenolic profiles and their biological activities. Indeed, a versatile analytical system is required to study such influences [24]. In this sense, high-performance thin-layer chromatography (HPTLC) and bioautographic assays on HPTLC plates play a relevant role in rapidly fingerprinting and screening plant extracts for various bioactivities, e.g., antibacterial, antifungal, antioxidant, or enzyme inhibition [25,26]. HPTLC–bioautography, applied to these complex matrices, enables the fast detection and localization of the compounds responsible for tested activities. Furthermore, this technique is effective, relatively inexpensive, and can be performed in small research laboratories without access to sophisticated equipment [27]. A distinct advantage of HPTLC assays resides in the chromatographic separation that precedes the application of detection reagents, which reduces or avoids interferences, notably from colored compounds such as leaf chlorophylls.

Ecuador exhibits significant morphological variability in quinoa crops, making it important to determine the chemical profile of compounds with biological activities.

Our previous study [28] allowed us to optimize the parameters necessary for obtaining reproducible results in the assay of α-amylase inhibitory activity, focusing on extracts derived from a single quinoa variety. Based on these data, the present paper aims to study the variabilities inherent to *Chenopodium quinoa* cultivars, culture stations, and phenological stages. Additionally, this study proposes a qualitative method for the rapid detection of phenolic compounds and associated bioactivities in quinoa leaves, while minimizing interference from colored molecules such as chlorophyll, which can affect results obtained using spectrophotometric methods.

2. Material and Methods

2.1. Plant Material

In this study, INIAP-Tunkahuan (IT) and INIAP-Pata de Venado (PV) quinoa varieties and the Chimborazo (C) quinoa genotype from Ecuador were investigated. The INIAP varieties, selected for their low saponin content, were grown at the Instituto Nacional de Investigaciones Agropecuarias (INIAP), Santa Catalina Experimental Station, Pichincha Province (altitude 3050 m.a.s.l., 0°22′01″ S 78°33′17″ W; during quinoa leaves growth and harvest, average temperature 12 °C, relative humidity 78%, rainfall 20 mm) and in Calpi, Chimborazo Province (altitude 3060 m.a.s.l., 1°38′48″ S 78°43′47″ W; during quinoa leaves growth and harvest, average temperature 14 °C, relative humidity 74%, rainfall 37 mm). The Chimborazo genotype has a high saponin content and is cultivated in Calpi; it is

endemic to Ecuador and exclusively cultivated in Chimborazo Province, whose name is due to its proximity to the Chimborazo volcano. The cultivation cycles of INIAP-Tunkahuan and INIAP-Pata de Venado varieties and Chimborazo genotype are 150–170, 130–150, and 240–270 days, respectively. The phenological growth of the different quinoa varieties and genotype is presented in Figure 1, according to previous descriptions of stages [27,28]; Table 1 describes the investigated samples with their phenological stages at harvest times. After harvest, quinoa leaves were washed with distilled water, and the excess water was removed with a paper towel. They were then lyophilized, ground, and stored at −20 °C. Seed quinoa samples were stored at −20 °C and ground before analysis. All material was powdered in a laboratory mill (PX-MFC 90 D, Kinematica AG, Malters, Switzerland) and sieved to obtain particle sizes ≤ 0.5 mm.

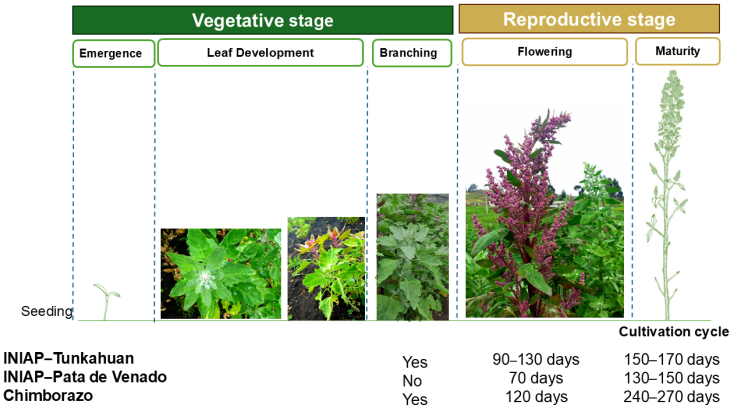

Figure 1. Phenology growth of quinoa plant (adapted from Sosa-Zuniga et al. [29] and Yzarra et al. [30]).

Table 1. Description of quinoa methanolic extracts, sweet and bitter varieties, investigated in this work.

Quinoa	Description	Place of Cultivation	Variety or Genotype	Characters	Phenological Stage	Track N° in Chromatogram
Sweet	Quinoa leaves (QL)	Pichincha (P)	INIAP-Tunkahuan (IT)	Green leaves	40 days [a]	1
					60 days [b]	2
					80 days [c]	3
			INIAP-Pata de Venado (PV)	Green leaves	40 days [a]	4
					60 days [b]	5
					80 days [c]	6
		Chimborazo (C)	INIAP-Tunkahuan (IT)	Green leaves	60 days [b]	7
					80 days [c]	8
			INIAP-Pata de Venado (PV)	Green leaves	60 days [b]	9
					80 days [c]	10
	Quinoa seed (QS)	Pichincha (P)	INIAP-Tunkahuan (IT)	White seeds	6 months	a, b, c
			INIAP-Pata de Venado (PV)	White seeds	5 months	d, e, f
Bitter	Quinoa leaves (QL)	Chimborazo (C)	Chimborazo (C)	Mix of yellow, green, and red leaves	60 days [b]	11
				Mix of yellow, green, and red leaves	80 days [c]	12
				Yellow leaves (y)	80 days [c]	13
				Green leaves (g)	80 days [c]	14
				Red leaves (r)	80 days [c]	15

[a] Vegetative stage: six true leaves are visible on the plant. [b] Vegetative stage: amounts of leaves significantly increase. [c] Vegetative stage: large amounts of leaves.

2.2. Chemicals and Reagents

2-Aminoethyl diphenylborinate (97%) (NP reagent), α-amylase from *Bacillus licheniformis* (Cat. No. A4582-5mL), soluble starch (ACS reagent), iodine (≥99.8%), 2,2-di (4-tert-octylphenyl)-1-picrylhydrazyl (DPPH•), rutin hydrate (≥ 94%), quercetin hydrate (≥95%), kaempferol (≥90%), and chlorogenic acid (≥95%) were purchased from Sigma-Aldrich (Darmstadt, Germany); hyperoside (≥98%) and isoquercitroside (≥99%) were obtained from Extrasynthese (France); polyethyleneglycol 400 (PEG 400), methanol (99%), absolute ethanol (≥99.8%), methyl ethyl ketone (GPR reactapur), formic acid (98%), and ethyl acetate (ACS reagent) were purchased from VWR Chemicals. Water was obtained using milli-Q grade water Plus Millipore (18.0 MΩ·cm).

2.3. Extraction

To prepare the extracts, 0.150 g of quinoa leaf powder or 0.300 g of quinoa seed powder were mixed with 3 mL of methanol–water (80:20, *w*/*w*), vortexed for 2 min, heated at 40 °C for 1 h, sonicated at room temperature (RT) for 30 min, and centrifuged (4000× *g*, 20 min, 25 °C; UniCen MR, Herolab, Germany) [31]. The supernatants were stored in an amber glass 5 mL vial with a screw cap and analyzed the following day.

2.4. Preparation of Solutions

Standard solutions were separately prepared in methanol at the following concentrations: 0.8 mg/mL of quercetin, 1.6 mg/mL of rutin, 0.5 mg/mL of hyperoside, 1.0 mg/mL of isoquercitroside, 1.0 mg/mL of kaempferol, and 1.2 mg/mL of chlorogenic acid. For polyphenols detection, a 10 g/L solution of diphenylboric acid 2-amino ethyl ester, and a 50 g/L solution of PEG 400 were prepared in methanol. For radical scavengers' detection, a 0.05% (*w*/*v*) DPPH• solution was prepared in methanol and protected from light. The α-amylase solution (~5 U/mL) was prepared by mixing 50 µL of α-amylase stock solution with 20 mL of water and diluting it to the required concentrations with ethanol–water (10:90, *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 to room temperature slowly by stirring. That solution was viscous and difficult to apply using the automatic Camag® Derivatizer; therefore, 10 mL of ethanol was added, and the solution was stirred for 2 h at RT to reduce its viscosity [32].

2.5. High-Performance Thin-Layer Chromatography

HPTLC was performed according to the general chapter 2.8.25 of the *European Pharmacopoeia* 10 [32]. Fingerprinting of polyphenolic compounds was performed according to the procedure reported by Liu et al. [31]; antioxidant and α-amylase inhibitory zones were visualized using the protocols reported by Agatonovic-Kustrin et al. [33] with some optimizations [32]. Chromatographic layers were HPTLC silica gel 60 F_{254} plates, size 20 × 10 cm (Merck, Darmstadt, Germany). Samples were applied by spray, using an Automatic TLC Sampler (ATS 4, Camag, Basel, Switzerland); 4, 6, and 8 µL of the sample were sprayed onto the plate for the polyphenols detection protocol, the free radical scavenging assay (DPPH•), and the α-amylase inhibition assay, respectively; 15 bands of 8 mm were applied per plate, 8 mm from the plate's lower edge; the plates were equilibrated under a 33% relative humidity and developed over a pathway of 70 mm from the lower edge in an Automated Multiple Development chamber (AMD2, Camag) with formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, *v*/*v*/*v*/*v*). The plates were dried automatically for 5 min after development and heated at 105 °C for 60 min using a TLC Plate Heater (Camag). All reagents were sprayed with the Derivatizer Camag®, selecting the appropriate nozzle. Upon derivatization, the plates were documented as digital images under short-wave UV

light (254 nm), long-wave UV light (365 nm), and white light using the TLC Visualizer 2. The Camag® systems were driven by visionCATS software, version 2.5.

Post-Chromatographic Derivatization

Polyphenolic compounds: 2 mL of aminoethyl diphenylborinate solution was applied on the warm plate (green nozzle, level 3) followed by 2 mL of polyethyleneglycol 400 solution (blue nozzle, level 2).

Free radical scavenging assay (DPPH•): 2 mL of DPPH solution was applied on a warm plate (green nozzle, level 3). Images were recorded 90 s, 30 min, and 100 min after derivatization.

α-Amylase inhibition assay: 3 mL of α-amylase solution was applied (yellow nozzle, level 4) on a cooled plate that was then incubated at 37 °C for 30 min in a humid chamber. Then, 2 mL of starch solution was applied (yellow nozzle, level 6), and the plate was incubated at 37 °C in a humid chamber for 10 min and treated with iodine vapors for 2 min (1 g of solid iodine in a lidded 27.0 × 26.5 × 7.0 cm TLC development chamber).

3. Results and Discussion

3.1. Polyphenols HPTLC Fingerprinting

Phenological stages, cultivation sites, and varieties were evaluated by HPTLC methods for their influence on polyphenolic profiles, ROS scavenging, and α-amylase inhibition of quinoa leaves' polar extracts. The HPTLC fingerprints of quinoa leaves and quinoa seeds were compared, considering that quinoa seeds have been extensively studied in recent years for their nutritional and health relevance [7].

Quinoa seeds and leaves contain many secondary metabolites, such as polyphenols, terpenoids, steroids, and nitrogen-containing compounds. Polyphenols, including phenolic acids, flavonoids, and tannins, constitute bioactive secondary metabolites that are reported to play significant roles, notably for antimicrobial, antioxidant, anti-inflammatory, antitumor, and anticarcinogenic activities [6,34]. Most recent studies on quinoa leaves primarily report the total content of polyphenols and flavonoids. Notably, the contents of these secondary metabolites are higher in young leaves compared to quinoa seeds [35,36]. Gawlik-Dziki et al. reported that, in the leaves of *Chenopodium quinoa* variety Faro, the predominant phenolic acids were ferulic, sinapic, chlorogenic, and gallic acids, while rutin, kaempferol, and isorhamnetin were the most abundant flavonoids [13].

Considering that phenolic acids and flavonoids, based on kaempferol and quercetin aglycones, are the most abundant compounds in quinoa, with previously reported biological activities, the present HPTLC study investigated major representative compounds from these groups. This approach aimed to determine not only their Rf values but also the colors they developed with NP reagent–PEG, allowing for the interpretation of the HPTLC fingerprints of sweet and bitter quinoa leaf extracts. Characteristic colors of different polyphenols (standard solutions) upon treatment with NP reagent–PEG are shown in Figure 2. The yellow/orange spots correspond to quercetin and its derivatives hyperoside, isoquercitroside, and rutin; the green and blue spots correspond to kaempferol and chlorogenic acid, respectively [31].

According to previous studies on the extraction of polyphenols, hydromethanolic solvents are particularly suited to extract these compounds. And so, in this study, the mixture methanol–water 80:20 (*v*/*v*) was retained. The HPTLC fingerprints of polyphenols from sweet and bitter quinoa leaf extracts (Table 1) indicate (Figure 3) an abundance of glycosides based on the flavonols quercetin and kaempferol (yellow and green spots, respectively) and of phenolic acids (blue spots). These compounds have been extensively studied, mostly in vitro, for their chemopreventive and anticarcinogenic effects [6,13]. The red spots

probably correspond to chlorophylls [37]. Interestingly, Gawlik-Dziki et al. associated the presence of high levels of the flavonols kaempferol, isorhamnetin, and rutin in a quinoa leaf extract (young leaves at 90 days; ethanol 50%, *v*/*v*) with an inhibition of prostate cancer cell proliferation, motility, and competence for gap junctional communication [13,31].

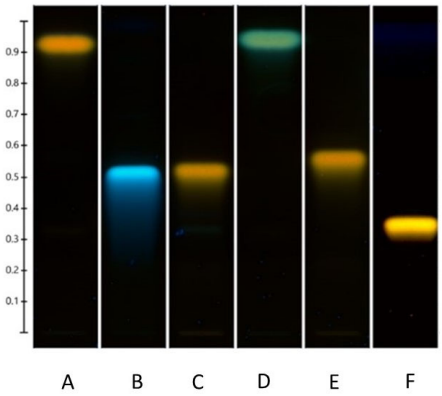

Figure 2. HPTLC chromatogram of standard phenolic compounds: quercetin, 3.2 µg (**A**); chlorogenic acid, 4.8 µg (**B**); hyperoside, 2.0 µg (**C**); kaempferol, 4.0 µg (**D**); isoquercitroside, 4.0 µg (**E**); rutin, 6.4 µg (**F**). Mobile phase: formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, *v*/*v*/*v*/*v*); treatment with NP reagent–PEG and visualization under UV_{365nm}.

The HPTLC profiles of INIAP-Tunkahuan (Figure 3; tracks 1, 2, 3) and INIAP-Pata de Venado (Figure 3; tracks 4, 5, 6) leaves from Pichincha vary according to phenological stages. As exemplified by peak profiles of INIAP-Tunkahuan (Figure 3), the later vegetative stages (60 and 80 days vs. 40 days, respectively) lead (i) to increased peak areas for kaempferol derivatives, by 8 and 23% (green peak 1) and by 26 and 29% (green peak 2), and quercetin derivatives, by 16 and 17% (yellow peak 1) and by 31 and 35% (yellow peak 2); and (ii) to decreased peak areas for phenolic acids, by 41 and 55% (blue peak 1) and by 18 and 70% (blue peak 2). These results are consistent with those of Buitrago et al. [14], who recorded 1.3 times higher contents of flavonoids (90 days versus 30 and 180 days) in quinoa leaf ethanolic extracts (ethanol 96% *v*/*v*). Similarly, Baldeon et al. confirmed that the total polyphenol content in quinoa leaves varies with the phenological stage. Interestingly, they reported that the highest levels of total phenolic compounds in four Peruvian quinoa cultivars (Blanca Junin, Pasankalla, Salcedo INIA, and Blanca Criolla) were observed during the second phenological stage, 42 days after sowing. Another study on young Peruvian quinoa leaves from three cultivars (Titicaca, Puno, and Vikinga) demonstrated that the contents of ferulic acid, isoquercetin, and rutin significantly varied depending on plant density, harvesting moment, and cultivar [38,39].

The influence of phenological stages on leaf flavonoid content and/or profile was also shown for plant species from many different families, e.g., Moraceae (*Morus alba* L. [40]), Passifloraceae (*Passiflora alata*, Dryander [41]), and Portulacaceae (*Portulaca oleracea* L. [42]).

The HPTLC fingerprints of sweet quinoa leaves appear quite similar at days 60 and 80, indicating that there are no significant effects of variety (INIAP-Tunkahuan or INIAP-Pata de Venado) or cultivation place (Pichincha or Chimborazo); these harvesting times correspond to the end of the vegetative stage (cf. Figure 1), when leaves are most abundant and locally consumed as vegetables [13]. In both sites, over all the cultivations of samples, the environmental conditions were quite similar (cf. Section 2.1), precluding any of the well-known meteorological impacts on polyphenol profiles [43]. The results of this study differ from those reported in the seeds of two Peruvian species (*Chenopodium quinoa*, six ecotypes, and *Chenopodium pallidicaule* Aellen, three varieties), in which the contents of the

most relevant flavonoids, quercetin and kaempferol glycosides, as well as phenolic acids, differed according to both species, ecotypes, and varieties [44].

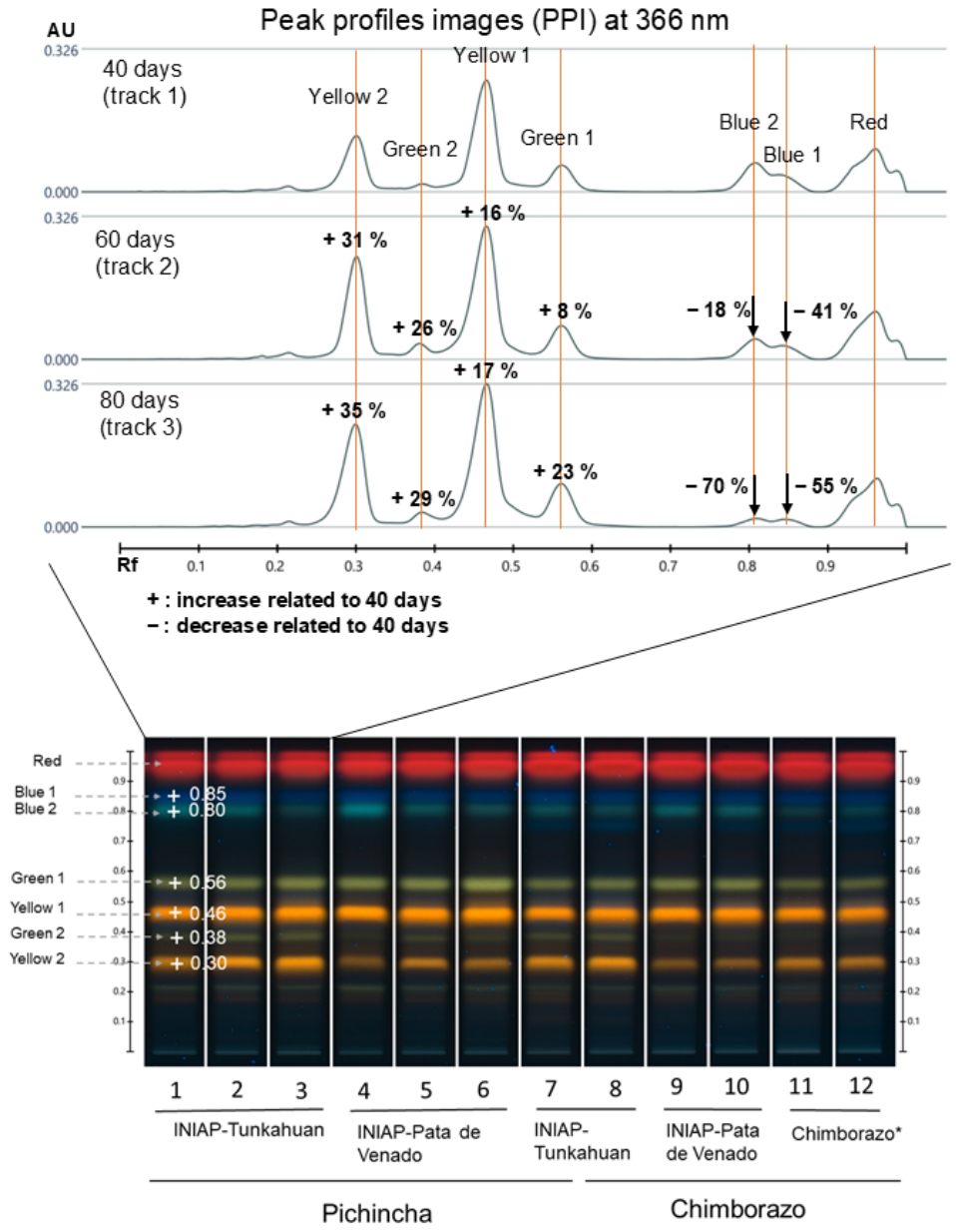

Figure 3. HPTLC fingerprints of polyphenols in Ecuadorian quinoa leaf extracts (methanol–water, 80:20, *w*/*w*; sample–solvent ratio, 1:20 *w*/*v*; application volumes, 4 µL). Mobile phase: formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, *v*/*v*/*v*/*v*). Derivatization with NP and PEG; examination under UV_{365nm}. Quinoa leaves from Pichincha: INIAP-Tunkahuan (tracks 1, 2, 3), INIAP-Pata de Venado (tracks 4, 5, 6). Quinoa leaves from Chimborazo: INIAP-Tunkahuan (tracks 7, 8), INIAP-Pata de Venado (tracks 9, 10), Chimborazo* genotype (11, 12).

The chromatographic profiles of Chimborazo bitter quinoa leaves (Figure 3; tracks 11, 12) were comparable to those of the sweet varieties. The endemic Chimborazo bitter genotype has the particularity of developing morphotypes bearing leaves with three clearly differentiated colors, yellow, green, and red [45], and is then considered a *"mestiza"* genotype. Figure 4 shows the polyphenol profiles of extracts obtained from leaves separately collected according to color (these samples were obtained in a specific experiment where quinoa plants of these three colors were separately cultivated); these can be compared with samples composed by mixing the three types of leaves (Figure 3; tracks 11, 12). Interestingly,

regardless of the morphotype color, their polyphenolic fingerprints appear quite similar; the color difference could be attributed to carotenoids, which would be interesting to further investigate.

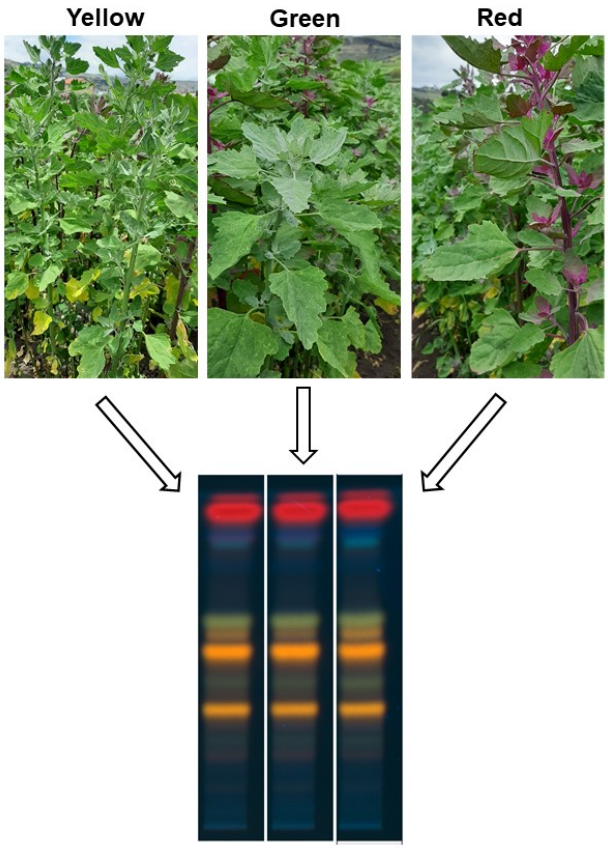

Figure 4. HPTLC fingerprints of polyphenols in yellow, green, and red leaves of the Chimborazo bitter quinoa variety extracts (methanol–water; 80:20, *w*/*w*; sample–solvent ratio, 1:20 *w*/*v*; application volumes, 4 µL). Mobile phase: formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, *v*/*v*/*v*/*v*). Derivatization with NP and PEG; examination under UV_{365nm}. Samples as per Table 1.

3.2. DPPH• Assay

Phenolic compounds are a diverse and widely occurring group of phytochemicals in plant foods, reputed for their beneficial effects on health. They are of considerable interest due to their antioxidant and enzyme-inhibiting properties, as well as their ability to scavenge free radicals and reactive oxygen species. Flavonol glycosides isolated from *Chenopodium quinoa* seeds have demonstrated antioxidant activity in the DPPH• assay. Zhu et al. reported the isolation of six flavonol glycosides from quinoa seeds, all of which exhibited antioxidant activity in this test. Notably, the two identified quercetin 3-glycosides showed significantly stronger activity than the four kaempferol 3-glycosides present [13,34,46]. Also, Gawlik-Dziki et al. reported that hydroethanolic extracts of quinoa leaves (50% *v*/*v* ethanol), which contained considerable amounts of ferulic, sinapic, chlorogenic, and gallic acids, as well as rutin, kaempferol, and isorhamnetin, were able to prevent lipid oxidation. However, their reducing power and antioxidant activity, measured using an $ABTS^{+\bullet}$ decolorization assay, were significantly lower [13].

The direct HPTLC-DPPH• assay was applied here to assess the free radical scavenging activity of quinoa leaf extracts, with the distinct advantages of easiness and rapidity [33,40]. The intensely violet DPPH• is reduced into the yellow 2,2-diphenyl-1-picrylhydrazine (DPPH-H), upon reaction with a hydrogen atom donor. The quinoa compounds with free radical scavenging properties were separated on the HPTLC plate, appearing as yellow

zones against the violet DPPH• background; the intensity of reagent decolorization is proportional to a compound's free radical scavenging potency and amount [40]. Figure 5a profiles the compounds that contribute to the free radical scavenging activity of sweet and bitter quinoa leaves, compared with rutin. Ninety seconds after derivatization, the same three yellow zones (Rf 0.32, 0.48, and 0.85) appear in all quinoa leaf samples, regardless of harvest time (40, 60, and 80 days), variety, and place of cultivation (Pichincha and Chimborazo). Baldion et al. assessed the influence of the phenological profile on the antioxidant capacity of leaves from four Peruvian quinoa varieties (Blanca Junín, Blanca Criolla, Pasankalla, and Salcedo). They reported the highest $ABTS^{+\bullet}$ radical scavenging capacity in Pasankalla (1492 µmol TEAC/g) at the second stage, 44 days after sowing. During the same stage, Blanca Junín (1127 µmol TEAC/g), Salcedo (1042 µmol TEAC/g), and Blanca Criolla (946 µmol TEAC/g) also reached their maximum values. This study observed that the highest $ABTS^{+\bullet}$ radical scavenging capacities coincided with the highest total phenolic content at the same stage, a trend also reported previously in Peruvian Andean medicinal leaves, where correlations between $ABTS^{+\bullet}$ and total phenolic content were established. Additionally, studies by Gawlik-Dziki et al. (2013) on quinoa leaves indicated strong relationships between the concentration of phenolic compounds and antioxidant activity, including $ABTS^{+\bullet}$ antiradical capacity [13,38].

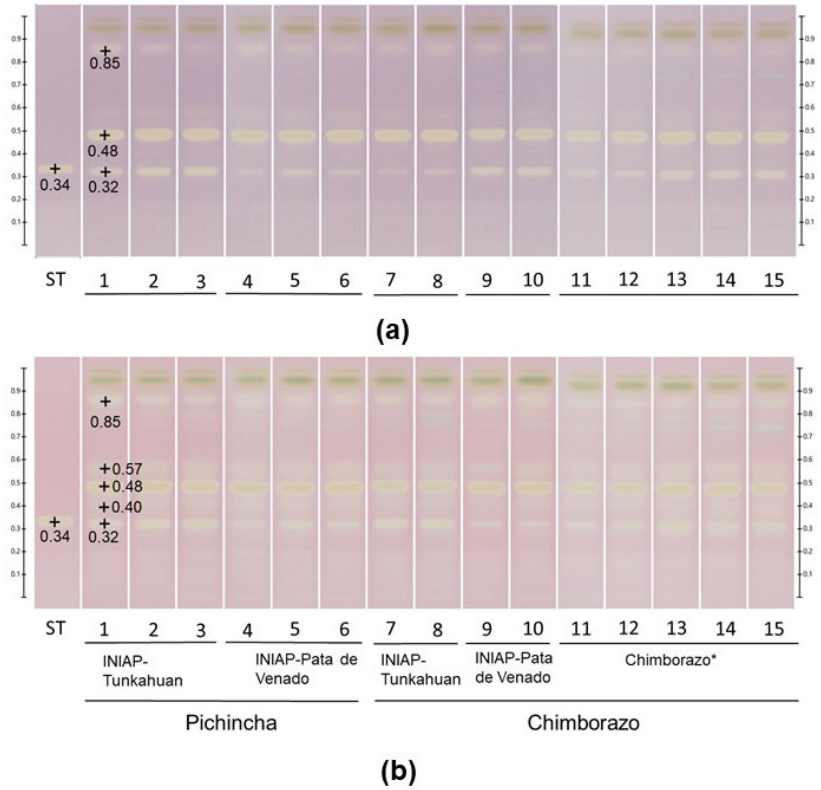

Figure 5. Free radical scavenging activity of Ecuadorian quinoa leaf extracts (methanol–water; 80:20, *w*/*w*; sample–solvent ratio, 1:20 *w*/*v*; application volumes, 6 µL). Mobile phase: formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, *v*/*v*/*v*/*v*). Derivatization with DPPH• for 90 s (**a**) and 100 min (**b**) and examination under visible light. Rutin 0.50 µg (track, ST). Quinoa leaves from Pichincha: INIAP-Tunkahuan (tracks 1, 2, 3), INIAP-Pata de Venado (tracks 4, 5, 6); quinoa leaves from Chimborazo: INIAP-Tunkahuan (tracks 7, 8), INIAP-Pata de Venado (tracks 9, 10), Chimborazo variety (11, 12) and Chimborazo* genotype yellow, green, and red colors (tracks 13, 14, 15). Samples as per Table 1.

Upon derivatization, the plate was maintained at RT in the dark for 30 min (Figure S1, Supplementary Materials) and 100 min (Figure 5b); with time, the intensity of

the discolored bands increased, with a higher number of yellowish zones appearing. Recent studies on HPTLC bioautography by Agatonovic-Kustrin et al. [33] and Islam et al. [47] indicate that the rates of reaction between DPPH$^{\bullet}$ and substrates vary widely, according to their antioxidant potency and amounts, and that times between 30 and 60 min are needed to complete the reaction.

Additionally, regardless of the color of Chimborazo bitter quinoa leaves, as shown in Figure 5a,b, they show similar fingerprints of free radical scavenging activity (composite samples in tracks 11, 12; yellow, green, and red leaves in tracks 13, 14, 15, respectively).

3.3. α-Amylase Inhibitory Activity

The HPTLC bioautography with "micro droplet" spraying was optimized in our laboratory for the screening of compounds with α-amylase inhibitory activity [31] to considerably reduce the reagent needs (from 40–200 mL to 2–3 mL) and plate smearing usually associated with dipping methods [33,48,49]. A clear and defined blue zone (Rf 0.38; marked with a solid red line; Figure 6a) highlighting the position of α-amylase inhibitor(s) was observed in all samples, regardless of the harvest time, variety, and place of cultivation. Slight blue areas (Rf 0.53 or 0.55; marked with a red dotted line), possibly due to a low effectiveness or concentration of α-amylase inhibitor(s), were observed in some samples, especially in samples with a harvest time of 80 days, regardless of the variety or cultivation site. To clearly see these blue zones, the applied sample volumes were increased (Figure 6b). A previous study suggested that the prominent blue area at the lower edge of the plates may indicate incomplete removal of migrated formic acid, leading to enzyme denaturation and preventing starch degradation [28]. Hence, only the zone above formic acid can be inspected for active compounds. In an in vitro assay, Hemalatha et al. reported a strong inhibition of α-amylase from hydromethanolic quinoa bran extracts (80% *v*/*v* methanol), an activity attributed to phenolic acids (vanillic, ferulic, and chlorogenic acids) and flavonoids (the flavonols quercetin, kaempferol, myricetin, rutin; the flavones luteolin and apigenin; the flavanone naringenin; the flavan-3-ol catechin; and the isoflavone daidzein); moreover, the inhibitory activity of quinoa milled fractions ranged in the order of bran > hulls > whole seed > dehulled seed > milled seed. A significant correlation was also noted between α-amylase inhibitory activity and total phenolic compounds [5].

On the other hand, Coronado-Olano et al. reported both α-amylase and α-glucosidase inhibitory activities in three commercial varieties of *Chenopodium quinoa* (Salcedo INIA and Negra Collana) and two commercial varieties of *Chenopodium pallidicaule* Aellen locally known as "*cañihua*" (INIA 406-ILLPA and Cupi). Salcedo INIA, Negra Collana, and Cupi showed a significantly higher inhibition of α-amylase compared to INI 406-ILLPA. Furthermore, in the varieties of quinoa and cañihua, a significant correlation was observed between α-amylase inhibition and total phenolics, total flavonoids, DPPH$^{\bullet}$ radical scavenging activity, gallic acid, and chlorogenic acid [50].

On our HPTLC chromatograms, at least two blue zones were observed in most of the samples marked with a solid red line (Figure 6b). Interestingly, a third but faint blue zone in the samples of the INIAP-Tunkahuan variety, 80 days from both Pichincha and Chimborazo, was observed (marked with a red dotted line). This may be attributed to the higher phenolic compound content in quinoa leaves harvested at 80 days compared to those collected at 40 and 60 days after sowing. These qualitative results that indicate an inhibition of α-amylase by quinoa leaf extracts should be further confirmed through in vivo studies to validate a potentially interesting antidiabetic activity.

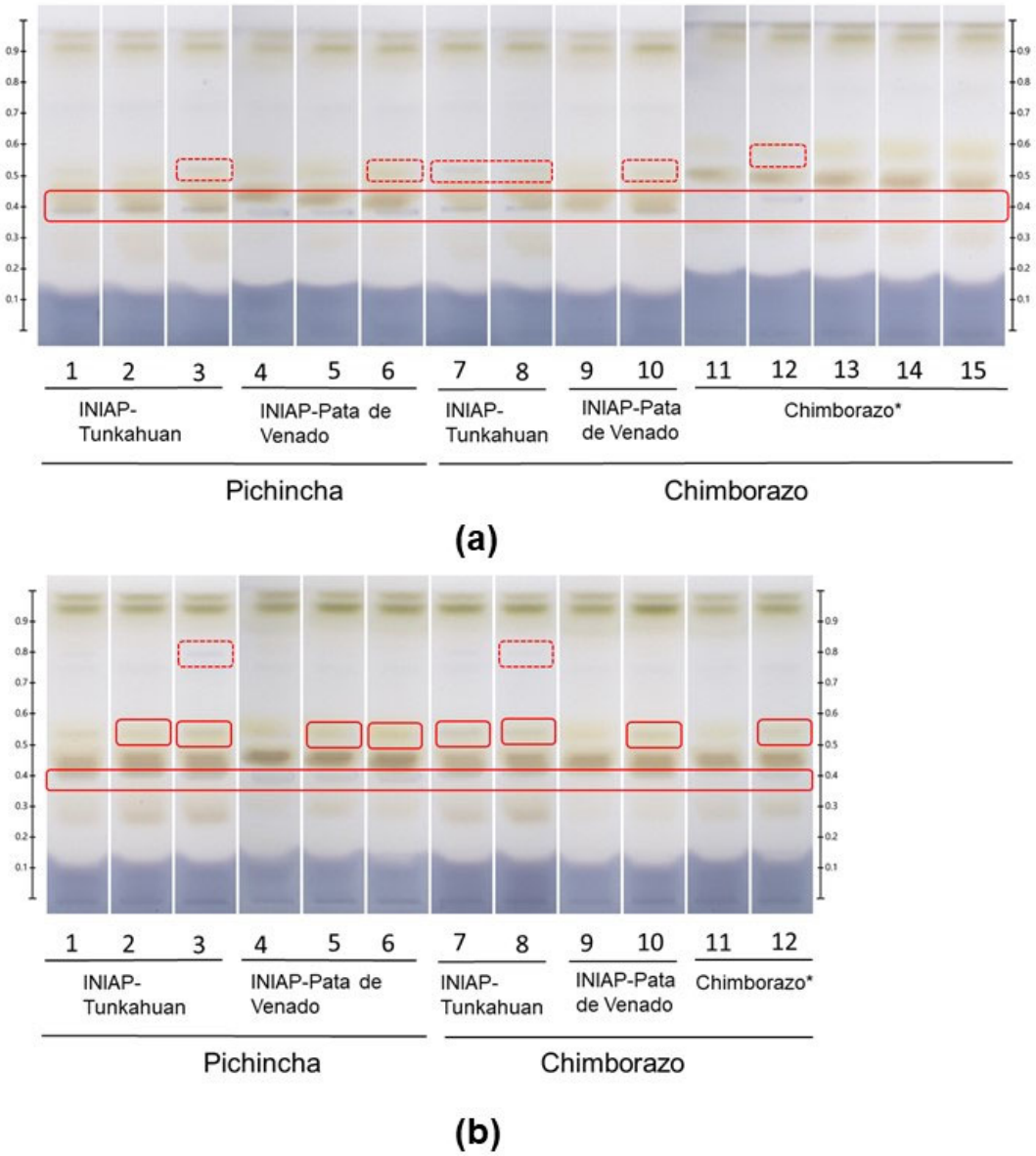

Figure 6. α-Amylase inhibitory activity of Ecuadorian quinoa leaf extracts [methanol–water; 80:20, *w*/*w*; sample–solvent ratio, 1:20 *w*/*v*; application volumes, 8 µL (**a**) and 14 µL (**b**)]. Mobile phase: formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, *v*/*v*/*v*/*v*). Derivatization with α-amylase, starch, and iodine; examination under visible light. Quinoa leaves from Pichincha: INIAP-Tunkahuan (tracks 1, 2, 3), INIAP-Pata de Venado (tracks 4, 5, 6); quinoa leaves from Chimborazo: INIAP-Tunkahuan (tracks 7, 8), INIAP-Pata de Venado (tracks 9, 10), Chimborazo variety (11, 12), and Chimborazo* genotype, yellow, green and red colors (tracks 13, 14, 15). Samples as per Table 1. The red marks indicate α-amylase inhibition zones. The large blue smear at the bottom of the plate (Rf 0.0 to 0.2) is due to inhibition of α-amylase by the formic acid of the mobile phase. Samples as per Table 1.

As shown in Figure S2, the development with a more polar mobile phase (formic acid–water–methyl ethyl ketone–ethyl acetate; 10:20:40:30, *v*/*v*/*v*/*v*) allows a better resolution of α-amylase inhibitory bands and a reduction in the formic acid band height at the bottom of the plate.

The major advantage of bioautography is its ability to quickly screen many samples for various bioactivities, such as antioxidant and enzyme inhibition, while using minimal reagent quantities (3 and 2 mL). This method ensures a uniform background and high detection sensitivity. The optimized fast screening technique enables the rapid localization of

bioactive compounds within complex plant matrices and colored extracts, as demonstrated with quinoa leaves. This is particularly beneficial because it eliminates the need for sample pretreatment before analysis, streamlining the process significantly.

3.4. Comparison of HPTLC Fingerprints of Quinoa Leaves Versus Seeds

Due to their radical scavenging properties, polyphenols such as flavonoids and phenolic acids are considered major contributors to the antioxidant activity of quinoa leaves [13], but previous reports [51,52] indicate that there is not necessarily a correlation between polyphenol content and DPPH• scavenging. For quinoa leaves, however, there is a clear correlation, as the radical scavenging bands correspond to glycosides of quercetin, kaempferol, and phenolic acids (Figure 3, in all tracks, yellow, green, and blue spots, respectively).

Several in vitro studies indicate a strong inhibition of α-amylase and α-glucosidase by quinoa seed extracts [5,6], and Graf et al. reported that quinoa seeds possess in vivo antidiabetic properties, notably attributed to leached phytoecdysteroids and flavonoids [4]. By contrast, little information is available so far on the eventual antidiabetic properties of quinoa leaves. Interestingly, our HPTLC–bioautographies show that the α-amylase inhibitors we evidence in leaves (blue zones in all tracks of Figure 6, Rf: 0.40) do not correspond to the major polyphenol (yellow bands in Figure 3, Rf: 0.30 y 0.46).

Given the demonstrated chemopreventive interest of seeds [53], their profiles were compared with those obtained from leaves; as the bitter quinoa seeds, Chimborazo variety, were not available, only the sweet varieties could be compared (Figure 7; IT, tracks a, b, c and PV, tracks d, e, f). These HPTLC profiles indicate much less intense bands in seed extracts, for the detection of polyphenols, free radical quenchers, and α-amylase inhibitors.

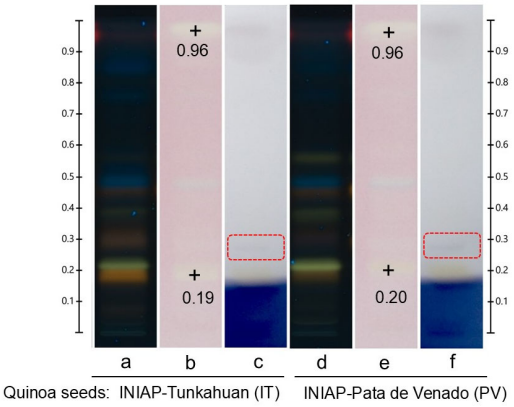

Figure 7. HPTLC fingerprints of polyphenols (derivatization with NP and PEG; examination under UV_{365nm}): tracks a (IT) and d (PV); free radical scavenging activity (derivatization with DPPH• for 100 min and examination under visible light): tracks b (IT) and e (PV); α-amylase inhibition (derivatization with α-amylase, starch, and iodine; examination under visible light): tracks c (IT) and f (PV) of Ecuadorian quinoa seed extracts (methanol–water; 80:20, *w*/*w*; sample–solvent ratio, 1:40 *w*/*v*, and application volumes, 8, 12, and 16 µL, respectively). Mobile phase: formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, *v*/*v*/*v*/*v*). Samples as per Table 1. The red marks indicate α-amylase inhibition zones. The large blue smear at the bottom of the plate (Rf 0.0 to 0.2) is due to inhibition of α-amylase by the formic acid of the mobile phase. Samples as per Table 1.

These results indicate a major interest in further investigating quinoa leaves, which could be an asset for producers to develop new products with high added value.

4. Conclusions

Quinoa leaves harvested after 80 days of cultivation consistently exhibited higher levels of polyphenols and α-amylase inhibition, regardless of production location or variety.

Moreover, the content of free radical scavenging compounds appeared independently of variety, cultivation time, and production site.

The HPTLC–bioautography methods described in the present study will be a useful tool for the quality control of raw materials in the future production of health-promoting, high-value products based on quinoa leaves. Reliable, fast, and easy to perform, they provide in situ information about the biological activity of separated compounds. Compared to the often-praised quinoa seeds, the much less consumed quinoa leaves present a high content of polyphenols with free radical scavenging activity, and an interesting α-amylase inhibitory property; these biological activities indicate a remarkable potential of quinoa leaves, which may be relevant for the management of diabetes but also for the chemoprevention and/or treatment of pathologies related to oxidative stress. Therefore, further research is essential to explore the potential of quinoa leaves as a dietary vegetable, in the development of functional foods, or for the extraction of bioactive compounds destined for the food and/or pharmaceutical industries. Such initiatives could significantly benefit quinoa smallholders in the Andean regions, promoting economic empowerment and sustainability.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/nutraceuticals5010001/s1, Figure S1: Free radical scavenging activity of Ecuadorian quinoa leaf extracts; Figure S2: α-Amylase inhibitory activity of Ecuadorian quinoa leaf extracts.

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