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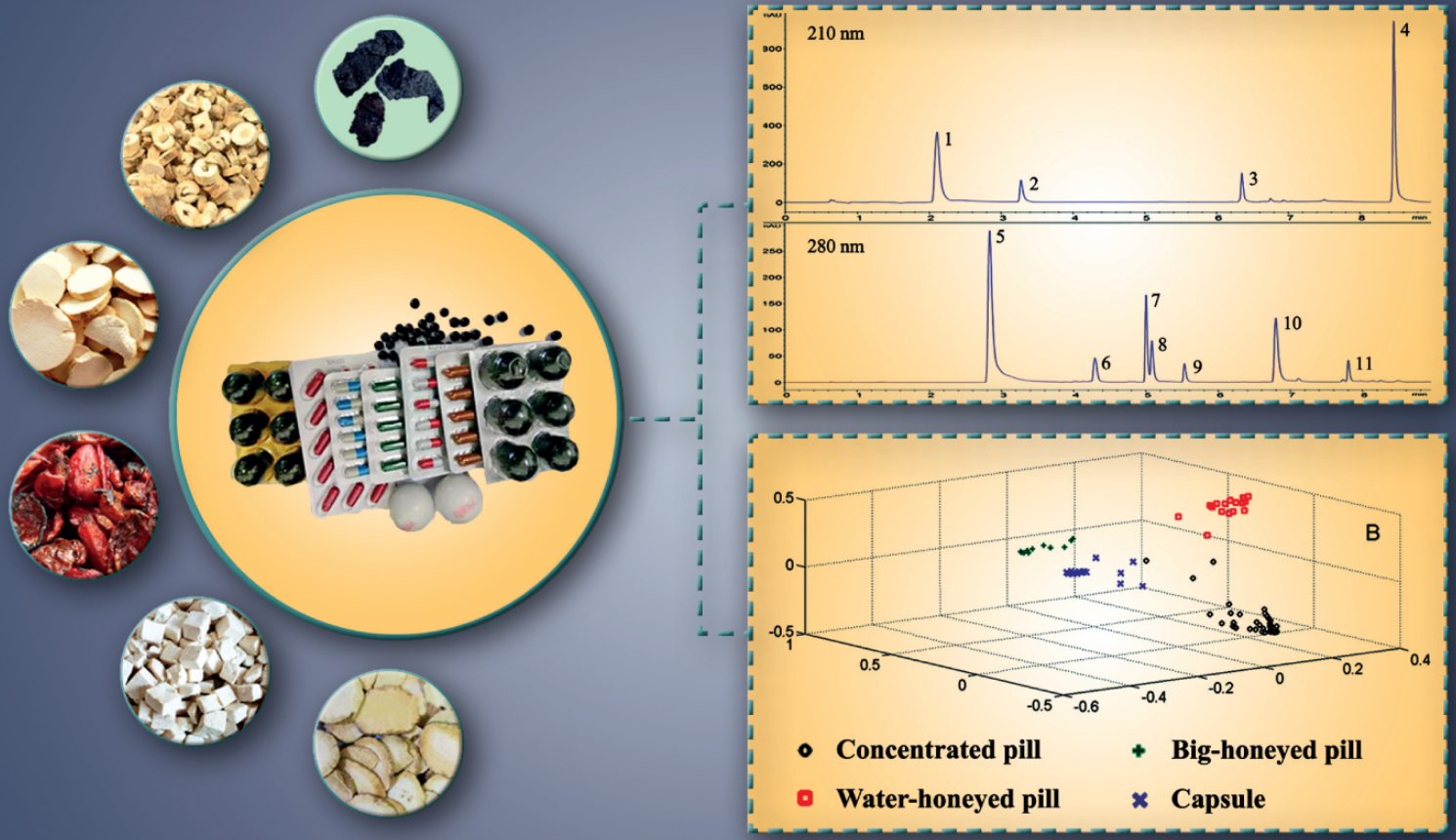

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Research Article

A reversed-phase compatible thin-layer chromatography autography for the detection of acetylcholinesterase inhibitors

A dual readout autographic assay to detect acetylcholinesterase inhibitors present in complex matrices adsorbed on reversed-phase or normal-phase thin-layer chromatography plates is described. Enzyme gel entrapment with an amphiphilic copolymer was used for assay development. The effects of substrate and enzyme concentrations, pH, incubation time, and incubation temperature on the sensitivity and the detection limit of the assay were evaluated. Experimental design and response surface methodology were used to optimize conditions with a minimum number of experiments. The assay allowed the detection of 0.01% w/w of physostigmine in both a spiked *Sonchus oleraceus* L. extract chromatographed on normal phase and a spiked *Pimenta racemosa* (Mill.) J.W. Moore leaf essential oil chromatographed on reversed phase. Finally, the reversed-phase thin-layer chromatography assay was applied to reveal the presence of an inhibitor in the *Cymbopogon citratus* (DC.) Stapf essential oil. The developed assay is able to detect acetylcholinesterase inhibitors present in complex matrixes that were chromatographed in normal phase or reversed-phase thin-layer chromatography. The detection limit for physostigmine on both normal and reversed phase was of 1×10^{-4} μg. The results can be read by a change in color and/or a change in fluorescence.

Keywords: Acetilcholinesterase / Alzheimer's disease / Bioautography / Inhibitors / Poloxamers
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1 Introduction

Acetylcholineterase (AChE) is the enzyme responsible for the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of acetylcholine (ACh) [1]. The inhibition of AChE serves as a strategy for the treatment of Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis and Parkinson's disease [2]. Alzheimer's disease is one of the most common neurodegenerative disorders and a prevalent cause of dementia with aging [1].

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Abbreviations: ACh, acetylcholine; **AChE**, acetylcholinesterase; **AChEA**, acetylcholinesterase amount; **AD**, Alzheimer's disease; **IA**, indoxyl acetate; **IAA**, indoxyl acetate amount; **IOD_{UV}**, integrated optical intensity under UV_{365n} light; **IOD_{vis}**, integrated optical intensity under visible light; **NS_{UV}**, number of detected spots under UV_{365n} light; **NS_{VIS}**, number of detected spots under visible light; **PRO**, *Pimenta racemosa* (Mill.) J.W. Moore leaf essential oil; **RSM**, response surface method; **SOE**, *Sonchus oleraceus* L. extract

One of the most studied characteristic features of AD is the decrease in the number of cholinergic neurons, with a resulting loss of the choline acetyltransferase, and consequently the diminution in the levels of the neurotransmitter acetylcholine (ACh) [3]. Therefore, AChE inhibition has become an important target for the design of drugs. There are a few AChE inhibitors approved for symptomatic treatment of AD: tacrine, donepezil, rivastigmine, and galantamine. These compounds have been reported to have several adverse effects and problems associated with bioavailability [4].

Besides neurobiology, AChE activity assays are applicable in toxicology, QC, food safety, environmental monitoring, and agricultural industries, playing an important role in diagnosis after potential exposure to organophosphorus, carbamate pesticides, and nerve agents [5, 6].

Natural products have been the main source of AChE inhibitors [2, 7]. Due to the complexity of natural extracts, TLC coupled with biological detection is one of the most employed screening methods. Such combination facilitates the location of compounds that are AChE inhibitors, and their follow-up during purification and isolation [8].

There are several autographic assays available for the screening of AChE inhibitors. The most employed are di-

*These authors contributed equally to this work.

rect methods wherein an enzyme suspension is sprayed on the TLC surface [9, 10]. Efforts have been made to develop new TLC assays by modifying conditions of reported substrate/detection systems [11, 12] or by employing alternative substrate/detection systems [13]. Recently we have immobilized AChE in an agar matrix as part of a strategy for the direct assignment of molecular formulas of active compounds from TLC–autography [14]. Each of the reported methods faces its own difficulties such as lack of color reproducibility, low spot definition, or the presence of false positives. Assays based on diazotization give false-positive results in presence of phenols, amines, tanning agents, cumarins, cannabinols, and flavonols [15] whereas assays based on Ellman's method give false-positive results in the presence of aldehydes and amines [16]. The development of false-positive assays has solved in part this problem; however, there is a necessity for new autographic assays based in other substrate/chromogen systems that can complement the existing assays.

One common limitation of the reported bioautographic assays is their lack of compatibility with RP-TLC. Complementary to normal-phase chromatography, RP chromatography is a key tool for the purification of small molecules from complex mixtures. The hydrophobicity of RP-TLC plate surfaces hampers the deposition of a homogeneous aqueous layer containing the enzyme and the reagents required for the assay. Additionally, the repulsion between the hydrophilic and the hydrophobic layers at the interface makes inefficient the diffusion of compounds.

In this paper, we report an autographic assay for the detection of AChE inhibitors employing RP-TLC as support layer. The method involves the use of a fluorogenic substrate/chromogen to reveal enzyme activity, and an amphiphilic copolymer to immobilize AChE over the nonpolar support.

2 Materials and methods

2.1 Materials

Acetylcholinesterase from *Electrophorus electricus* Type VI-S (lyophilized powder), indoxyl acetate (IA), and physostigmine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pluronic® F-127 (poloxamer 407) was donated by BASF Argentina (Buenos Aires, Argentina). Aluminum-backed silica gel 60 F254 TLC layers were purchased from Merck (Darmstadt, Germany). *Pimenta racemosa* (Mill.) J.W. Moore leaf, and *Cymbopogon citratus* (DC.) Stapf essential oils were purchased from EUMA (Buenos Aires, Argentina). Aerial parts of *Sonchus oleraceus* L (SO) were collected in Pergamino, Buenos Aires, and identified by Dr. Oscar Micheloni (National University of Northwest of Buenos Aires, Argentina). Voucher specimens were deposited at the Herbarium of the National University of Rosario.

2.2 *Sonchus oleraceus* L. extract preparation

The plant material was oven dried (at 65°C for 72 h) and milled. The powdered plant material (200 g) was extracted in refluxing methanol (4 L) for 45 min, three times. The filtered extracts were combined and evaporated to dryness under reduced pressure by rotary evaporation. The obtained *S. oleraceus* crude extract (51 g) was suspended in H_2O/MeOH (90:10, 1 L) and extracted with hexane (2×500 mL). The MeOH was removed from the aqueous phase by rotary evaporation and the resulting aqueous fraction was extracted with dichloromethane (3×500 mL). Then, the resulting aqueous fraction was extracted with butanol (3×500 mL). The butanol fraction was concentrated by rotary evaporation to obtain 2 g of dry extract (final yield 4%).

2.3 Solutions

Phosphate buffer (100 mM) was prepared in distilled water and adjusted with HCl to pH 8.00 or pH 6.00 as required. Indoxyl acetate stock solution (227 mM) was prepared in ethanol. AChE stock solution (132.5 U/mL) was prepared in phosphate buffer (pH 8.00 or 6.00) and stored at –20°C. Pluronic® 20% w/w was prepared in phosphate buffer (pH 8.00 or 6.00) and kept at 4°C until used. Physostigmine (0.1 mg/mL) stock solution was prepared in ethanol. Spiked *Sonchus oleraceus* extract was produced by adding physostigmine stock solution to 25 mg/mL ethanol solution of *S. oleraceus* extract (final concentration of physostigmine, 0.01% w/w). Spiked *Pimenta racemosa* essential oil extract was produced by adding physostigmine stock solution to 25 mg/mL ethanol solution of *Pimenta racemosa* essential oil solution (final concentration of physostigmine, 0.01% w/w).

2.4 TLC assay for screening and optimization phases

Six serial ten-fold dilutions were freshly prepared by dilution of the physostigmine stock solution. 10 μL of each diluted solution were spotted (10, 1, 0.1, 1×10^{-2}, 1×10^{-3}, and 1×10^{-4} μg of physostigmine), using an analytical syringe, onto 2×14 cm RP-TLC plates or Silica gel TLC plates. Detection was carried out without prior chromatographic separation.

2.5 Factors for Plackett–Burman design

The analyzed factors were: indoxyl acetate amount (IAA), acetylcholinesterase amount (AChEA), pH, incubation time, and incubation temperature. Each of these factors was evaluated at two levels. Each factor range was selected based on prior knowledge about the system under study. Enzyme concentration ranged between 0.25 U/mL, which is half of the value reported by Yang et al. [12] in the AChE autography that

uses the lowest enzyme concentration, and four times this reported minimum value (2 U/mL). Incubation time ranged between 15 min, the minimum incubation timed reported [10] and 60 min. The pH range evaluated is the range used in the report of indoxyl acetate as AChE substrate [17] and also takes in consideration that AChE activity decreases around 50% when the pH values are below 6 or they are higher than 8 [18]. The effect of incubation temperature was evaluated between 25 and 40°C. Such range surrounds 37°C, the human temperature usually employed in AChE assays and was selected taking into consideration that AChE activity decreases around 40–55% at temperatures below 25°C or higher than 40°C [18]. The evaluated substrate concentration ranged between 1 and 5 mM, starting with the lowest amount usually employed in AChE autographic assays [10].

2.6 AChE autographic assay and chromatographic conditions

The analysis of pure samples of physostigmine absorbed o TLC plates, and the analysis of the different extracts previously chromatographed on TLC was carried out using the following typical procedure: At 4°C 202 μL of AChE solution (132.5 U/mL) were added to 13.8 mL of Pluronic ® (20% w/w, pH 6) and mixed carefully by inversion. Then 314 μL of indoxyl acetate (IA) solution was added. Immediately after mixing, the final solution was distributed over the TLC plate (64 cm^2). After gelation at room temperature, the plate was incubated in a stove at 37°C in a closed and humid Petri dish in the dark. Fluorescence and absorbance were observed at 60 min under UV_{365nm} and white light respectively. Final concentrations: IA (3.75 mM) and AChE (2 U/mL).

For the analysis of physostigmine in a spiked *Pimenta racemosa* essential oil on RP-TLC, the plate was spotted with 10 μL of spiked *Pimenta racemosa* essential oil solution (25 mg/mL). Additionally, 0.025 μg of physostigmine standard were seeded in one side lane, as positive control for bioactivity in absence of matrix, and 10 μL of *Pimenta racemosa* essential oil solution (25 mg/mL) were seeded in the other side lane as control for the lack of bioactivity of the matrix. The RP-TLC plate was developed with methanol containing 0.01% v/v of triethylamine, which places physostigmine in a central part of the TLC (Rf = 0.56) together with other components from the matrix.

For the analysis physostigmine in a spiked *Sonchus oleraceus* extract on normal phase TLC, the Silica gel plate was spotted with 10 μL of spiked *Sonchus oleraceus* extract (25 mg/mL, final quantity of physostigmine in plate: 0.025 μg). Additionally, 0.025 μg of physostigmine standard were seeded in one side lane and 10 μL of 25 mg/mL spiked *Sonchus oleraceus* extract solution were seeded in the other side lane, and the plate was developed with dichloromethane/methanol (60:40).

For the analysis of *Cymbopogon citratus* (DC.) Stapf essential oil, a RP-TLC plate was spotted with 10 μL of essential oil solution (25 mg/mL). The RP-TLC plate was developed with methanol containing 0.01% v/v of triethylamine.

2.7 Image analysis

The optical density of each spot was measured using GelPro software. The analysis of 1-D gels was carried out on monochrome images of the TLC plates, generated from the color images by extracting intensity. TLC lanes were defined manually and the spots were automatically detected using the "find bright bands in dark background" function of the software for visible light detection (measured in grey level/$pixel^2$) and, using the "find dark bands in bright background" function for UV_{365nm} light detection (measured in optical density/$pixel^2$).

Design Expert trial version 7.0.3 (Stat-Ease, Minneapolis, MN, USA) was used to perform experimental design, polynomial fitting, ANOVA results and desirability calculations. GelPro 3.0 (Media Cybernetics, Silver Spring, MD, USA) was used to measure signals for inhibition spots.

3 Results and discussion

The difficulties experienced to obtain a reproducible homogeneous layer of buffered enzyme suspension onto the hydrophobic surface of RP-TLC plates by direct spray, made necessary to search for another strategy for enzyme application. Enzyme immobilization by gel entrapment has been applied for the development of xanthine oxidase [19–21], β-glucosidase [22], tyrosinase [23], and AChE [14] autographic assays. This strategy increases enzyme stability, decreases operational difficulties and improves reproducibility. It facilitates to obtain uniform enzyme coverage of the TLC surface and to know the exact final concentration of enzyme per unit area. In these reported assays, the corresponding enzyme was immobilized by entrapment in agar gel. The hydrophilicity of this gel makes difficult its application onto RP-TLC plates; however, gels with amphiphilic properties may be a better choice. Poloxamers are non-ionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)). The presence of these blocks gives rise to molecules with the ability to form temperature dependent micelles. Micelles formation allows solubilization of hydrophobic compounds, making these copolymers attractive for delivering poorly water-soluble compounds. Water solutions of Poloxamer 407 with concentrations of 20% w/v or above, show interesting thermoreversible gelation behavior [24, 25]. They are sols below room temperature and get converted to gels at body temperature (37.2°C) [26]. In addition, it was reported that a poloxamer showed a positive effect in preserving enzymes from alkaline stress and aggregation, and in increasing its thermal stability [27].

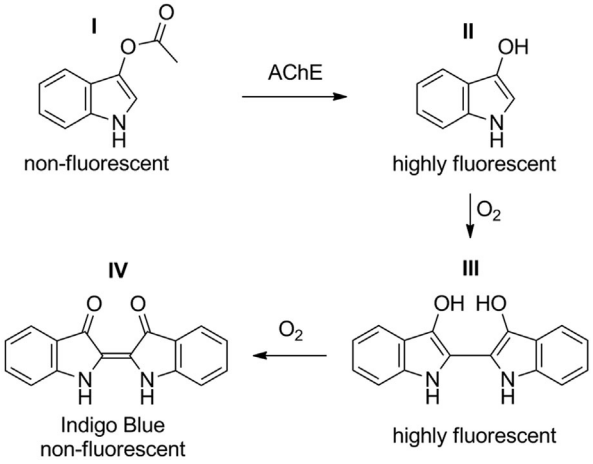

Figure 1. Reaction of AChE with IA (I) to the subsequence formation of fluorescents (II and III) and blue (IV) products.

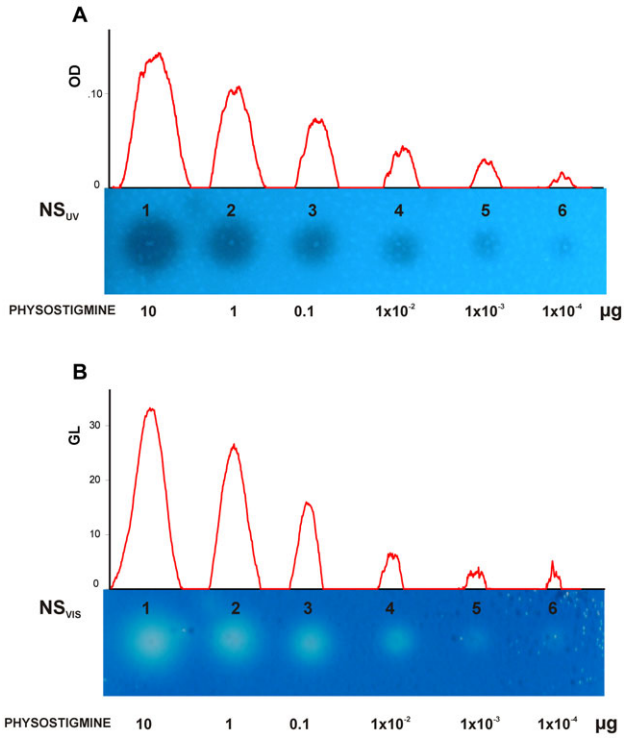

Figure 2. TLC layer spotted with decreasing amounts of physostigmine and stained with the AChE assay. (A) TLC observed under UV_{365nm} light and optical density of the spots as measured by the Gel-Pro software and (B) TLC observed under visible light and grey level of the spots as measured by the Gel-Pro software.

Fluorogenic substrates are generally several orders of magnitude more sensitive to measurement than chromogenic ones, in this way a fluorometric method can measure extremely small concentrations of inhibitors [16]. The proposed autography relies on the cleavage by AChE of the non-fluorescent indoxyl acetate (IA, I) to form the highly fluorescent 3-hydroxyindole (II) that is then oxidized to indigo white (III), also highly fluorescent and, subsequently, to the blue non-fluorescent product indigo blue (IV) [17] (Fig. 1).

Since IA does not interact with thiols, it has been recommended as an interesting alternative to Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) [28]. IA presents high stability toward spontaneous hydrolysis, and enough difference between the λ_{exc} and λ_{em} to allow the monitoring of product formation without substrate interference [6]. IA has been used for the development of a cholinesterase-based dipstick assay for the detection of organophosphate and carbamate pesticides [29].

To check proper functioning of immobilized AChE, we performed tube assays in absence and in presence of the poloxamer 407. The results showed that the gel does not affect formation of the fluorescent products (II and III) and the subsequent indigo blue. What is more, the presence of the poloxamer, prevented precipitation of products generating a homogeneous color distribution.

Therefore, substrate and enzyme were solubilized in poloxamer solution and distributed on a RP-TLC layer. Again, both fluorescence and blue color were observed. As the enzymatic reaction proceeds, fluorescence decreases whereas absorbance increases. This characteristic could make possible a dual readout of the autography under visible light and under UV_{365nm} light, facilitating the detection of inhibitors and decreasing the chances of interferences by the matrix.

To estimate the assay sensitivity and detection limit, four response indicators were defined: IOD_{UV}, IOD_{VIS}, NS_{UV}, and NS_{VIS}. The responses IOD_{UV} and IOD_{VIS} were defined as the integrated optical intensity (IOD) obtained for 10 µg of physostigmine under visible and UV_{365nm} light, respectively. The responses NS_{UV} and NS_{VIS} were established as the number of detected spots present in a plate by applying physostigmine in decreasing amounts spotted onto a TLC plate. An example is shown in Fig. 2.

To identify the design variables that have large effects on the detection limit and sensitivity of the assay, a Plackett–Burman design was built. The analyzed factors were: indoxyl acetate amount (IAA), acetylcholinesterase amount (AChEA), pH, incubation time, and incubation temperature. Each of these factors was evaluated at two levels (Supporting information, Table S1). The factor ranges were selected based on prior knowledge about the system under study (see Section 2). After performing the experiments, the plates were photographed at different incubation times and the defined experimental responses were evaluated.

An ANOVA test was applied to the data corresponding to the design shown in Supporting Information Table S1, using the effect of the dummy variables to obtain an estimate of standard errors in the coefficients. Three factors were significant ($p < 0.05$): indoxyl acetate amount, acetylcholinesterase amount, and incubation time. For this reason, in the following optimization stage the incubation temperature and the pH, which have no significant influence on the responses, were set at 37°C and 6.00, respectively.

A systematic optimization procedure was carried out using a response surface method (RSM) to estimate the values of the most significant factors (IAA, AChEA, and incubation time) leading to the best compromise between maximum IOD_{UV}, IOD_{VIS}, NS_{UV}, and NS_{VIS}. A central composite design was employed for applying the RSM, consisting in 20 experiments ($2^6 = 8$ two-level two-factor points, $2\times3 = 6$ axial points and a sextuple central point). The factors were combined in the following ranges: IAA 1–4 mM, AChEA 1.3–2.5 U/mL, and incubation time 30–60 min (Supporting information, Table S2). All experiments were performed in random order to minimize the effects of uncontrolled factors that may introduce bias on the measurements.

The first step was carried out to find a suitable approximation to the true relationship between the factors and each of the responses. Then, the responses for all the 20 experiments were fitted to polynomial models, using backward elimination to estimate the best models. The most common forms are low-order polynomials (first or second order). The construction of response surface models was an iterative process. Once an approximate model was obtained, the goodness-of-fit was used to determine if the solution was satisfactory. The results indicated that quadratic models adequately explained the behavior of the responses NS_{VIS}, NS_{UV}, IOD_{VIS}, and IOD_{UV}. For this optimization design, partial ANOVA showed good statistical indicators (i.e. a non-significant lack of fit, a reasonable adequate precision, and adequate R^2, model and coefficient SDs).

The relationship between the responses and the independent variables were shown through response surface plots that are the theoretical three-dimensional plots. Although responses were modeled as a function of all factors, incubation time proved to be the least significant factor. For illustrative purposes, the response surface plots were built as function of IAA and AChEA whereas time was fixed at 60 min. The visualization of the predicted model equations was obtained by the response surface plot and contour plot (Supporting information, Fig. S1).

Responses NS_{VIS}, NS_{UV}, IOD_{VIS}, and IOD_{UV} presented maximum values, in the design region, at around 2 U/mL of enzyme and 4 mM of substrate, respectively.

According to the surface plot obtained for each response, both IOD_{uv} and IOD_{vis} depend on the IAA and AChEA but in different manner. IOD_{vis} is more sensitive to the AChEA than to the IAA (Supporting information, Fig. S1 left, top). On the contrary, IOD_{uv} is more sensitive to the changes on the IAA than to the changes on AChEA (Supporting information, Fig. S1 right, top). Concerning the response number of detected spots, NS_{vis} increases with IAA, whereas NS_{uv} increases with IAA only at AChEA lower than 1.90 U/mL. For both responses, the effect of AChEA is less marked.

To simultaneously optimize these responses, a desirability function was employed. First a function for each individual response was created (d_i) and then a global function D was maximized choosing the best conditions of the design variables. The function D ranges from 0 (value totally undesirable) to 1 (all responses are in a desirable range simultaneously).

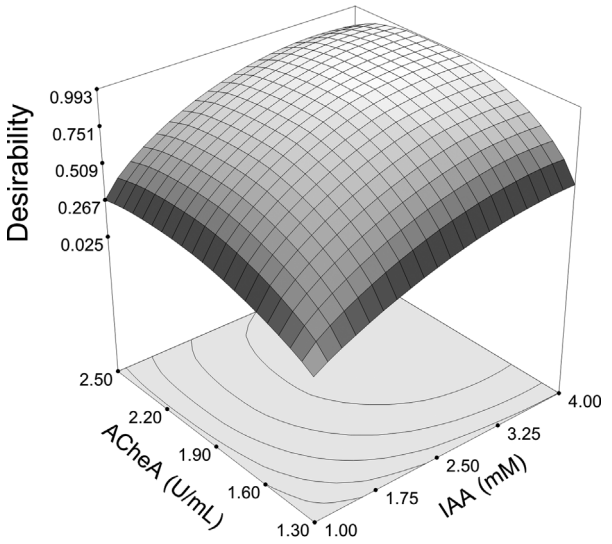

Figure 3. Response surface plots for the global desirability function.

In our case, the four responses (NS_{VIS}, NS_{UV}, IOD_{VIS}, and IOD_{UV}) were optimized simultaneously. The criterion for the optimization was that each response was maximal. Accordingly, the optimization process was carried out and the global desirability function was obtained based on the three independent variables, IAA, AChEA, and incubation time. After adequate models were found for each of these responses, a response surface for the global desirability function was built as a function of the influencing factors IAA and AChEA, whereas a fixed time of 60 min was maintained (Fig. 3).

The experimental conditions of a maximum in the desirability function ($D = 0.993$) were: 3.63 mM IAA, 2.09 U/mL AChEA and 60 min of incubation (incubation temperature and pH were set at 37°C and 6.00, respectively). The predicted values for the individual responses corresponding to this desirability value were: $NS_{VIS} = 5.87$, $NS_{UV} = 6.31$, $IOD_{VIS} = 347395$ Gray Level × pixel^2 and $IOD_{UV} = 814.11$ Optical Density × pixel^2.

The optimal conditions for the assay were verified by independent additional experiments in triplicate, using the amounts of enzyme and substrate indicated by the maximum desirability function (3.63 mM IAA, 2.09 U/mL AChEA, and 60 min of incubation).

The experimental results were in agreement with the predicted values, and the differences found are within the range of the SD indicating that the experimentally obtained values are consistent with the solutions proposed by the Design expert program (Supporting information, Table S3).

Natural extracts contain numerous components that might make difficult the visualization of inhibitors. To test the applicability of the assay for the detection of active compounds in a complex matrix the presence of physostigmine was investigated using the inactive *Pimenta racemosa* (Mill.) J.W. Moore leaf essential oil (PRO) spiked with physostigmine, at 0.01% w/w. This natural mixture was used as matrix because it showed, by TLC analysis, the presence of

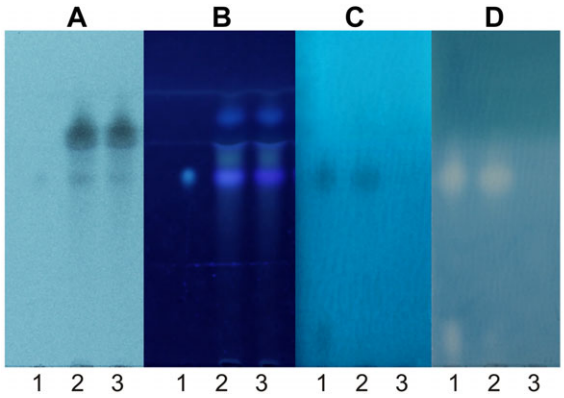

Figure 4. Autography for detection of AChE inhibitors on RP-TLC. From left to right, physostigmine 0.025 μg (1), 250 μg of 0.01% physostigmine spiked *Pimenta racemosa* leaf essential oil (2) and 250 μg of *Pimenta racemosa* leaf essential oil (3): (A) UV_{254nm} light detection; (B) UV_{365nm} light detection; (C) AChE autography visualized under UV_{365nm} light; (D) AChE autography visualized under visible light. Elution was carried out with methanol containing 0.01% of triethylamine.

a variety of components, including spots with similar chromatographic behavior to physostigmine. PRO, spiked PRO and physostigmine were chromatographed in parallel. Once developed, the eluent was removed from the TLC plate at room temperature under air current and the plate was stained using the optimal conditions found for the AChE inhibition assay.

As shown in Fig. 4, the presence of physostigmine in the spiked extract passed undetected when the TLC plate is analyzed under UV_{365nm} and UV_{254nm} (Fig. 4a and b) before biological evaluation. When the autography was applied, an inhibition spot was clearly observed at Rf = 0.53 (methanol containing 0.01% v/v of triethylamine) corresponding to physostigmine, indicating that the conditions found allow the detection of 0.01% w/w physostigmine in a complex matrix (Fig. 4c and d). It is interesting to note that the other components of the oil did not generate false positives, interfered with the color reaction nor generated fluorescence quenching.

The applicability of the assay in complex mixtures, employing Silica gel as support (normal phase), was tested using as complex matrix an inactive *Sonchus oleraceus* L. extract (SOE) that was spiked with physostigmine, at 0.01% w/w (Supporting information, Fig. S2a and b). This natural mixture was used as matrix because it showed, by TLC analysis, the presence of a variety of components, including spots with similar chromatographic behavior to physostigmine. SOE, spiked SOE, and physostigmine were chromatographed and, after solvent removal, the poloxamer solution containing AChE and indoxyl acetate, was distributed over the plate. The autography exhibited the presence of one inhibition halo with Rf = 0.47 in dichloromethane/methanol (60:40), corresponding to physostigmine (Supporting information, Fig. S2c and d).

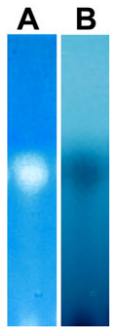

Figure 5. Autography for detection of AChE inhibitors on RF-TLC of *Cymbopogon citratus* essential oil. (A) AChE autography visualized under visible light; (B) AChE autography visualized under UV_{365nm} light. Elution was carried out with methanol containing 0.01% of triethylamine.

These results showed that the assay is also compatible with normal phase TLC, taking the advantage of monitoring the enzymatic activity both under visible and UV light. The sensitivity in Silica gel-TLC was below 1×10^{-4} μg making this test more sensitive than some of the reported methods for Silica gel plates coupled to other AChE activity detection systems (detection limit 1×10^{-2} μg) [9,10]. However, the observed sensitivity is still lower than that of the method reported by Yang et al. [12]. This assay is a modified version of Marston's method [9] wherein the consumption of enzyme is reduced by 85% and the detection limit for physostigmine is decreased to 1×10^{-5} μg.

The assay was then applied to the essential oil of *Cymbopogon citratus* (DC.) Stapf (lemongrass). This essential oil was used to demonstrate the application of the autographic assay because it had previously presented acetylcholinesterase inhibitory activity [30, 31]. Initially the sample was spotted onto a RP-TLC plate and then analyzed for activity after the development of the chromatogram. Using this method, inhibition of the enzyme activity by the essential oil was observed with clear enzyme inhibition zone, with Rf = 0.5 (Fig. 5).

4 Concluding remarks

The RP-TLC AChE autography described herein is rapid and simple to use. The optimized experiment presents a background with intense blue color under visible light and with intense fluorescence under UV_{365nm} light. It shows good background-spot contrast and very good sensitivity, both under UV_{365nm} and under visible light (halos were visible up to 10^{-4} μg of physostigmine).

When 1-D TLC experiments are carried out, the activity of several plant extracts can be analyzed at the same time. This is especially useful for the identification of bioactive compounds present in other complex mixtures such as chemically engineered extracts [32, 33].

Finally, poloxamers could be applicable to other autographic assays both in RP-TLC or Silica gel TLC. The thermal reversibility of the gels generated with these polymers

allows enzyme solution storage, and the temperature change required for gel formation is around ten degrees: poloxamer 407 water solutions are liquid below 4°C and form a gel at 14°C. Such characteristics make these block copolymers particularly suited to develop assays with enzymes that are temperature sensitive.

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The authors have declared no conflict of interest.

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