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Multiresponse Optimisation Applied to the Development of a TLC Autography for the Detection of Tyrosinase Inhibitors

Paula García and Ricardo L. E. Furlan*

ABSTRACT:
Introduction – Autographic methods are useful tools to detect bioactive compounds in complex matrixes. Experimental design and optimisation techniques were implemented for the development of an autographic assay suitable for the detection of tyrosinase inhibitors.
Objectives – To develop an autographic assay to detect tyrosinase inhibitors using gel entrapped enzyme, experimental design and response surface methodology (RSM) to optimise conditions with a minimum number of experiments.
Methods – Gel entrapment was used for the assay and the effects of four factors on the sensitivity and the detection limit for known inhibitors of the enzyme were evaluated. The factors were: tyrosinase amount (TA), L-tyrosine amount (LTA), incubation time and incubation temperature.
Results – The assay allowed the detection of kojic acid in an extract of *Calamagrostis viridiflavescens* (Poir.) Steud spiked with 0.1% w/w.
Conclusion – The developed assay is able to detect tyrosinase inhibitors present in complex matrixes in a reproducible way. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: Response surface methodology; TLC-bioautography; tyrosinase inhibition; dermatological disorders; herbal drugs

Introduction

Polyphenol oxidase (PPO) or tyrosinase (EC 1.14.18.1) is a copper bifunctional enzyme widely distributed in microorganisms, plants and animals which catalyses the production of melanin and other pigments by oxidation of L-tyrosine (Sánchez-Ferrer *et al.*, 1995).

Tyrosinase is responsible for the colouring of skin and hair in mammals. Various dermatological disorders such as melasma, age spots and sites of actinic damage, arise from the accumulation of an excessive level of epidermic pigmentation (Kim and Uyama, 2005). Additionally, tyrosinase is responsible for enzymatic browning of fruits and vegetables during senescence or post-harvest handling (Martinez and Whitaker, 1995). In insects tyrosinase is involved in wound healing, parasite encapsulation and sclerotisation, playing an important role in developmental and defensive functions (Sugumaran, 1991). Consequently, the discovery of novel inhibitors of tyrosinase becomes attractive due to their potential applications for improving quality of foods, insect pest control and prevention and treatment of melanin-related health problems in humans.

The TLC bioautographic methods are a useful tool to detect new bioactive compounds from natural sources (Marston, 2011; Cheng and Wu, 2013). Different enzyme-based autographic assays have been developed, including one assay for the detection of tyrosinase inhibitors (Wangthong *et al.*, 2007). In this assay, enzyme and substrate solutions are sprayed onto the TLC surface to produce a brownish purple colour except in those areas where an inhibitor is present.

When performing this test, enzyme stability may be a problem: drying out of the enzyme solution on the silica surface will result in reduced enzymatic activity, thereby decreasing the contrast of the assay (Wangthong *et al.*, 2007). One approach to increase enzyme stability in bioautographic assays is enzyme immobilisation by gel entrapment (Ramallo *et al.*, 2006; Salazar and Furlan, 2007). It is known that high operational stability can be obtained when tyrosinase is immobilised by entrapment with different polymers (Munjal and Sawhney, 2002).

During a bioassay development, selection of the most appropriate conditions can define the assay convenience. The implementation of response surface methodology (RSM) can facilitate finding the optimum response and understanding how a response changes when different design variables are adjusted (Vera Candioti *et al.*, 2014). In this work we report a bioautographic assay to detect tyrosinase inhibitors using gel entrapped enzyme. Experimental design and RSM were used to optimise conditions with a minimum number of experiments.

Experimental

Materials

Mushroom tyrosinase was purchased from Sigma-Aldrich (St Louis, MO, USA), kojic acid was purchased from MAGEL SA (Buenos Aires, Argentina).

* Correspondence to: R. L. E. Furlan. Facultad de Ciencias Bioquímicas y Farmacéuticas. Universidad Nacional de Rosario. Suipacha 531, S2002LRK Rosario, Argentina.
Email: rfurlan@fbioyf.unr.edu.ar

Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario. CONICET, Suipacha 531, S2002LRK, Rosario, Argentina

Aluminum-backed silica gel 60 F254 TLC layers were purchased from Merck (Darmstadt, Germany). Agar was purchased from Britania (Buenos Aires, Argentina). Plant material of *Calamagrostis viridiflavescens* (Poir.) Steud was collected in Pergamino, Buenos Aires province, Argentina, during November 2005. Voucher specimens were deposited at the herbarium of the Universidad Nacional de Rosario.

Preparation of plant extract

The plant material was oven dried (at 65°C for 72 h) and milled to a fine powder. 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.

Preparation of tyrosinase stock solution

Tyrosinase stock solution of 1881 U/mL was prepared suspending 10 mg of lyophilised enzyme (1881 U/mg) in 10 mL 20 mM phosphate buffer pH 6.8.

Preparation of L-tyrosine stock solution

L-Tyrosine stock solution of 2.5 μmol/mL was prepared by dissolving 4.53 mg of L-tyrosine powder in 10 mL of 20 mM phosphate buffer pH 6.8.

Typical assay procedure

Agar was dissolved at 80°C in phosphate buffer (20 mM, pH 6.8). The solution was allowed to cool down to 55°C and L-tyrosine solution (2.5 μmol/mL) was added and mixed by rotation. After further cooling to 35°C, tyrosinase solution (1881 U/mL) was added and the solution thus obtained was mixed by rotation to produce a final concentration of agar of 12 mg/mL and the final concentrations of enzyme and substrate required for each experiment. This staining solution was immediately poured directly on a TLC plate and allowed to reach room temperature to produce a colourless gel.

The TLC assay for linear regression curves

500, 250, 125, 62.5, 31.25 and 15.625 ng of kojic acid were spotted onto a 2 × 14 cm silica gel layer, from a methanol solution using an analytical syringe. Detection was carried out without prior chromatographic separation.

The TLC assay for detection of kojic acid in a complex mixture

Separations were performed on 4.5 × 10 cm silica gel layers. Elution was carried out with ethyl acetate:methanol:water (74:15:11). Prior to the addition of the tyrosinase staining solution, the eluent was removed from the plate at room temperature under air current (20 min). The amounts of samples loaded were: 100 μg of extract/spot, 0.1 μg of kojic acid/spot and 100 μg of spiked extract/spot (0.1% of kojic acid). In all cases, 10 μL of a methanol extract solution were manually applied to the TLC plate using a 10 μL microsyringe, as 0.5 cm bands.

Image analysis

Optical density of the spots was measured using the software GelPro. The analysis of one-dimensional gels was carried out on monochrome images of the TLC plates generated from the colour images by extracting intensity. Lanes were defined manually and spots were automatically detected using the 'find bright bands in dark background' function of the software.

Software

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

Results and Discussion

Several enzyme-based TLC autographic assays have been reported to detect inhibitors of acetyl cholinesterase (Rhee *et al*., 2001; Marston *et al*., 2002; Yang *et al*., 2011), xanthine oxidase (Ramallo *et al*., 2006), α- and β-glucosidase (Salazar and Furlan, 2007; Simões-Pires *et al*., 2009), lipase (Hassan, 2011), and tyrosinase (Wangthong *et al*., 2007). In most cases, an enzyme suspension is directly applied onto the TLC surface in order to expose an inhibitor that is present in a complex mixture. Direct spray of enzyme suspension onto TLC plates may cause problems in reproducibility mainly due to enzyme instability, heterogeneity of both enzyme and substrate coverage, decreased resolution on inhibition spots mostly due to aqueous buffer sprayed on a polar surface, and operational difficulties when spraying small volumes of reagents with conventional sprayers. In order to overcome these problems, in this work the enzyme was immobilised by entrapment in agar. This strategy facilitates uniform enzyme coverage of the TLC plate surface and allows knowing exactly the final concentration of enzyme per unit area.

Enzyme gel entrapment

In order to determine the effectiveness of the immobilised tyrosinase for a bioautographic assay, 53 μL of 1881 U/ mL enzyme solution and 600 μL of 2.5 μmol substrate solution were added to a 12 mg/mL agar solution in phosphate buffer (pH 6.8) previously melted at 80°C and cooled down to 35°C, to reach a final concentration of 25 U/mL of tyrosinase and 375 nmol/mL of L-tyrosine. This staining solution was poured onto a TLC plate and allowed to reach room temperature to produce a colourless gel (4 U/cm^2 of tyrosinase and 60 nmol/cm^2 of L-tyrosine). Within 2 min, the gel turns into a brown 'skin like' colour due to tyrosinase catalysed oxidation of L-tyrosine. When, the staining solution was layered onto a TLC plate spotted with 0.50, 0.20, 0.10 and 0.02 μg of kojic acid, white spots were observed against the brown coloured background, indicating that agar entrapment is appropriate for the assay.

Experimental responses definition

In order to estimate the assay sensitivity and detection limit, five response indicators were defined: IOD1, IOD2, IOD3, SFL and DT. Responses IOD1, IOD2 and IOD3 were defined as the integrated optical intensity (IOD) obtained for 500, 250 and 125 ng of kojic acid respectively (Fig. 1). The SFL (slope of the fitted line) was established as the slope of a linear regression curve obtained by applying kojic acid in decreasing amounts onto the TLC plate, and DT (detection threshold) was defined as the amount of inhibitor for which the signal obtained was equal to zero (Fig. 2).

Screening phase

In order to identify the design variables that have large effects on the detection limit and sensitivity of the assay, a Placket–Burman design was built. The analysed factors were: tyrosinase amount (TA), L-tyrosine amount (LTA), incubation time and incubation temperature. Each of these factors was evaluated at two levels (Table 1). The factor ranges were selected based on prior knowledge about the system under study.

An ANOVA test was applied to the experimental data corresponding to the design of Table 1, using the effect of the dummy variables to obtain an estimate of standard errors in the coefficients. Two significant factors were found (values of $p < 0.05$): tyrosinase amount and L-tyrosine amount. During the optimisation of these two factors in the following section, incubation temperature was set at 20°C and incubation time was set at 20 min.

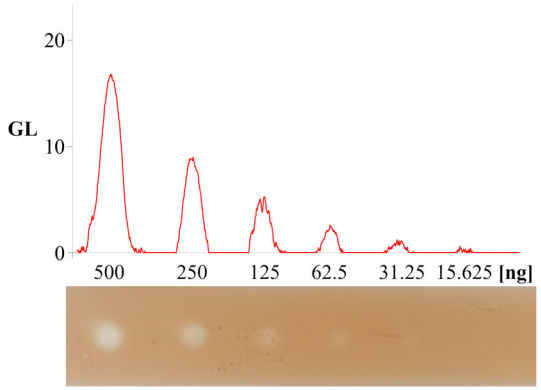
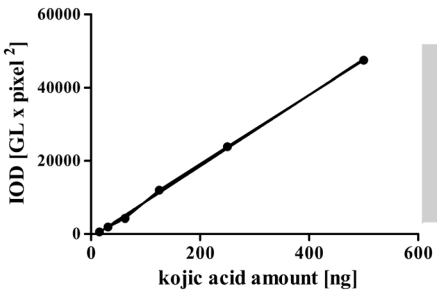


Figure 1. (a) Optical density of the spots shown on the above layer as measured by Gel-Pro software. (b) TLC layer spotted with decreasing amounts of kojic acid and stained with the tyrosinase assay.

Response surface design

Once it was established that TA and LTA had a significant influence on the five analysed responses, a systematic optimisation procedure was carried out using a response surface method (RSM). The objective of this procedure was to estimate the values of the most important factors leading to the best compromise between maximum SFL, IOD1, IOD2 and IOD3, and minimum DT. A central composite design (CCD) was employed for applying the RSM, consisting of 13 experiments ($2^2 = 4$ two-level two-factor points, $2 \times 2 = 4$ axial points and a quintuplicate central point), which were



kojic acid amount [ng]	IOD [GL x pixel^2]
500	47404
250	23808
125	11947
62.5	4232
31.25	1947
15.625	564

Figure 2. Plot and table to obtain SFL and DT parameters from a linear regression curve.

Table 1. Plackett–Burman design built for factor selection

Experiments	Factors[a]				Responses[b]				
	TA (U/cm^2)	LTA (nmol/cm^2)	Temperature (°C)	Time (min)	SFL (GL × pixel^2/ng)	DT (ng)	IOD3 (GL × pixel^2)	IOD2 (GL × pixel^2)	IOD1 (GL × pixel^2)
1	4	60	40	40	87.29	15.07	7834.8	22543.0	41734
2	8	60	20	20	103.86	20.62	9537.6	23957.0	49984
3	4	100	40	20	49.37	7.50	5663.6	12659.0	24022
4	8	60	40	40	96.50	7.38	10477.0	24833.0	47049
5	8	100	20	40	91.49	113.00	4509.5	7416.8	37111
6	8	100	40	20	44.56	42.76	4661.5	7737.3	20871
7	8	100	20	40	64.90	85.26	4108.3	8398.0	27682
8	4	60	20	20	44.61	13.58	4661.5	10839.0	20871
9	4	100	40	40	49.77	23.41	4975.3	10733.0	23990
10	4	60	20	40	81.67	11.74	8957.7	20613.0	39393
11	4	100	20	20	49.27	11.58	4752.0	12901.0	23694
12	8	60	40	20	86.87	7.11	9677.3	20676.0	42663

[a]TA, tyrosinase amount; LTA, L-tyrosine amount.
[b]SFL, slope of the fitted line; DT, detection threshold; IOD1, IOD2 and IOD3, integrated optical intensity obtained for 500, 250 and 125 ng of kojic acid respectively.

Table 2. Central composite design used for the optimisation of the evaluated responses

Experiments	Factors[a]		Responses[b]				
	TA (U/cm^2)	LTA ($nmol/cm^2$)	SFL (grey level × $pixel^2$/ng)	DT ng	IOD3 (grey level × $pixel^2$)	IOD2 (grey level × $pixel^2$)	IOD1 (grey level × $pixel^2$)
1	6.00	27.6	71.61	14.76	8331.1	15010	35510
2	8.00	40.0	93.23	11.70	10187.0	22653	45410
3	6.00	112.0	97.31	4.26	11933.0	24148	48114
4	4.00	40.0	64.03	11.73	7132.8	16546	30723
5	6.00	70.0	87.15	66.96	10502.0	21630	42783
6	8.00	100.0	93.51	51.36	11550.0	22339	46485
7	3.17	70.0	50.77	9.59	5432.5	12876	24695
8	8.83	70.0	106.11	10.98	11653.0	24682	50055
9	6.00	70.0	97.52	6.07	11288.0	23556	48347
10	6.00	70.0	92.47	4.62	13458.0	26446	49262
11	6.00	70.0	90.52	9.39	10818.0	22274	44143
12	4.00	100.0	57.85	3.89	6820.8	14420	28650
13	6.00	70.0	97.47	10.43	11947.0	23808	47404

[a]TA, tyrosinase amount; LTA, L-tyrosine amount.
[b]SFL, slope of the fitted line; DT, detection threshold; IOD1, IOD2 and IOD3, integrated optical intensity obtained for 500, 250 and 125 ng of kojic acid respectively.

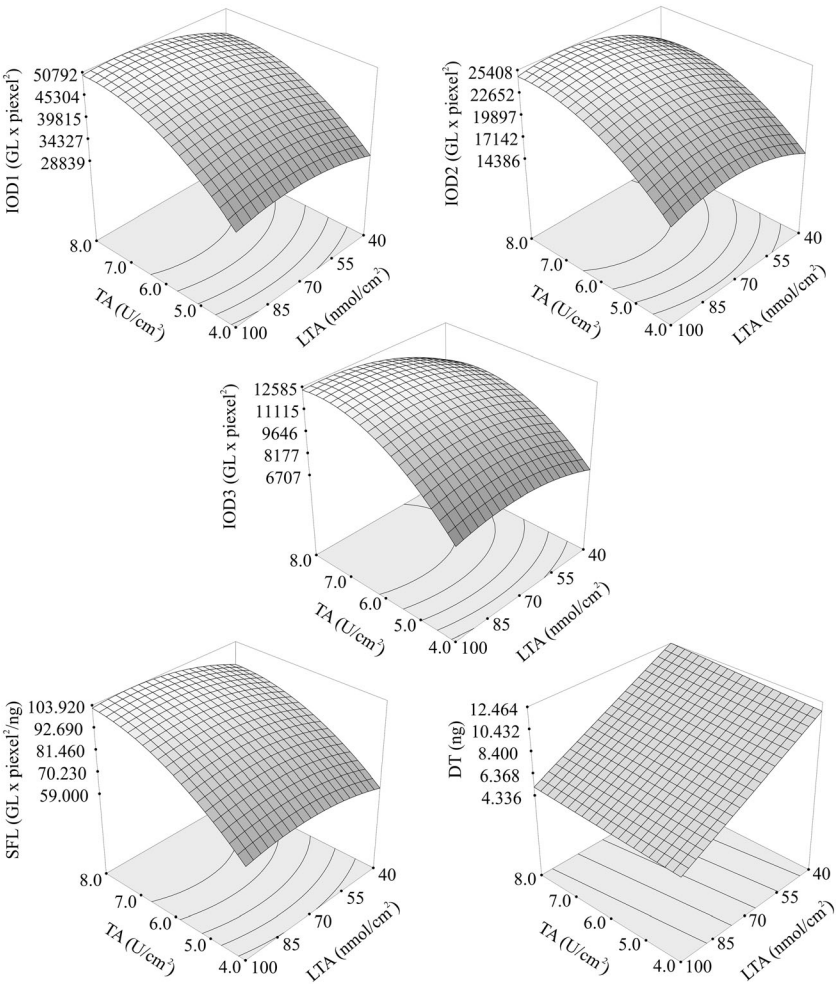


Figure 3. Response surface plots for the slope of the fitted line (SLF), integrated optical intensity for 500 ng of kojic acid (IOD1), integrated optical intensity for 250 ng of kojic acid (IOD2), integrated optical intensity for 125 ng of kojic acid (IOD3) and the detection threshold (DT), as indicated.

combinations of the factors in the following ranges: tyrosinase amount, 4–8 (U/cm^2) and L-tyrosine amount, 40–100 (nmol/cm^2) (Table 2). All experiments were performed in random order to minimise 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 13 experiments were fitted to polynomial models, using backward elimination in order 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 determined whether the solution was satisfactory. The results indicated that a linear model explained better the behaviour of DT, whereas quadratics models were appropriate for the SLF, IOD1, IOD2 and IOD3. Partial ANOVA results for this optimisation design showed good statistical indicators (i.e. non-significant lack of fit, reasonable adequate precision and adequate R^2 and model and coefficient standard deviations).

The visualisation of the predicted model equations was obtained by the response surface plot and contour plot (Fig. 3). The response surface plot is the theoretical three-dimensional plot showing the relationship between the response and the independent variables.

Responses SLF, IOD1, IOD2 and IOD3 presented maximum values in the design region where enzyme and substrate amounts were high. In contrast, DT presented its minimum value in the design region where enzyme amount was low and substrate amount was high.

In order to simultaneously optimise all 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 maximised choosing the best conditions of the designed variables. The function D ranges from 0 (value totally undesirable) to 1 (all responses are in a desirable range simultaneously).

In our case, the five responses (DT, SLF, IOD1, IOD2 and IOD3) were optimised simultaneously. Table 3 shows the criteria that were followed for the optimisation of individual responses: it was considered desirable that all responses were maximal except for DT, in which the minimum value was desirable.

According to the restrictions and conditions defined, the optimisation process was carried out and the global desirability function was obtained based on the two independent variables, TA and LTA. After the optimisation procedure was carried out, and 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 TA and LTA (Fig. 4)

Table 3. Optimisation criteria for individual responses

Factors/responses	Goal	Lower limit	Upper limit
TA (U/cm^2)	Is in range	4	8
LTA (nmol/cm^2)	Is in range	40	100
DT (ng)	Minimum	3.894	14.758
FLS (GL × pixel^2/ng)	Maximum	50.76	106.11
IOD1 (GL × pixel^2)	Maximum	24695	50055
IOD2 (GL × pixel^2)	Maximum	12876	26446
IOD3 (GL × pixel^2)	Maximum	5432.5	13458

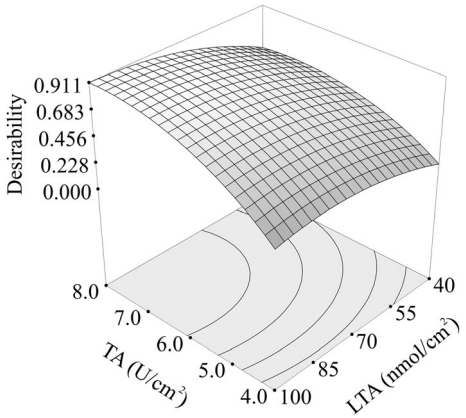


Figure 4. Response surface plots for the global desirability function.

The experimental conditions of a maximum in the desirability function (D = 0.911) were: 99.4 nmol/cm^2 LTA and 7.76 U/cm^2 TA (incubation temperature and incubation time were set at 20°C and 20 min, respectively). The predicted values for the individual responses corresponding to this desirability value were: DT = 5.154 ng, FLS = 102.172 grey level × pixel^2/ng, IOD1 = 50149.8 gray level × pixel^2, IOD2 = 24736.18 gray level × pixel^2, IOD3 = 12459.84 gray level × pixel^2.

Experimental verification

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 (99.4 nmol/cm^2 of L-tyrosine and 7.76 U/cm^2 of tyrosinase). The experimental results were in agreement with the predicted values, and the differences found are within the range of the standard deviation, indicating that the experimentally obtained values are consistent with the solutions proposed by the design expert programme (Table 4).

Applicability of the test to complex mixtures

In order to test the applicability the assay for the localisation of active compounds in a complex matrix, the presence/absence of kojic acid was investigated using an inactive crude plant extract spiked with this inhibitor. A dichloromethane extract from *Calamagrostis viridiflavescens* (Poir.) Steud was used as matrix because it showed, by TLC analysis, the presence of a variety of components, including spots with similar chromatographic behaviour to kojic acid. Accordingly, 0.1 μg of kojic acid reference standard, 100 μg of extract, and 100 μg of extract spiked with 0.1 μg of kojic acid (0.1% w/w) were chromatographed. Once developed, the eluent was removed from TLC plates at room temperature under air

Table 4. Summary predicted values and experimental values

Response	Predicted value	Experimental value
DL (ng)	5.15	7.50
FLS (GL × pixel^2/ng)	102.17	102.10
IOD1 (GL × pixel^2)	50149.80	50568.30
IOD2 (GL × pixel^2)	24736.18	23894.00
IOD3 (GL × pixel^2)	12459.84	12733.00

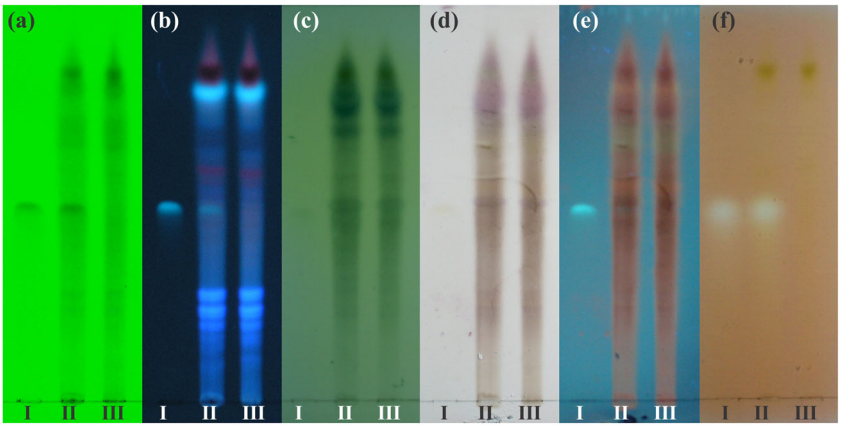


Figure 5. Bioautography for detection of tyrosinase inhibitors. From left to right, kojic acid 0.1 μg (I), 100 μg dichloromethane fraction *C. viridiflavescens* with 0.1 μg kojic acid (II) and 100 μg of dichloromethane fraction of *C. viridiflavescens* (III): (a) 254 nm UV light detection; (b) 365 nm UV light detection (c); phosphomolybdic acid stain; (d) *p*-anisaldehyde sulphuric acid stain; (e) *p*-anisaldehyde sulphuric acid under 365 nm UV light; (f) autography for tyrosinase inhibitor detection. Elution was carried out with ethyl acetate:methanol:water (74:15:11)

current and the plates were stained using the optimum conditions found for the tyrosinase inhibition assay.

As shown in Fig. 5 the presence of kojic acid in the spiked crude extract is hardly detected when the TLC plate is analysed under UV light or when the plate is sprayed with some common derivatising reagents (Fig. 5a-e, lane II). When the assay is applied, one inhibition spot is observed at R_f = 0.5 corresponding to kojic acid, indicating that the conditions found allow the detection of 0.1% w/w kojic acid in a complex matrix (Fig. 5f).

For the first time, experimental design and RSM were used to optimise conditions during development of a bioautographic assay. The use of RSM methodology allowed the determination of the optimal conditions where independent variables were studied simultaneously.

In a previous report (Ramallo *et al*., 2006) we introduced the use of enzyme immobilisation by gel entrapment as a simple technique to increase enzyme stability on TLC layers. In this tyrosinase assay, gel entrapment decreased operational difficulties and improved reproducibility, facilitating uniform enzyme coverage of the TLC plate surface. The observed bioactivity spots conserved the shape of the chromatographic spots and showed more defined borders than those produced with the previously reported method (Wangthong *et al*., 2007), allowing image analysis and facilitating the readout of the assay.

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