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ARTIGO DE PESQUISA

Evaluation of TLC-densitometry assay validation applied to complex matrix

Avaliação da validação da metodologia CCD-densitometria aplicada a uma matriz complexa

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Abstract

Considering the need to have a fast, cheap and effective method for quality control routine, thin layer chromatography/densitometry has been a chosen method to comply those requests. However, this technique has its limitations when used to determination assays for substances in complex matrices, such as vegetal extracts. Considering this chromatographic technique restriction during the drug development assay, a new procedure was developed to overcome the difficulty in obtaining the validation parameters within the international acceptance criteria, mainly selectivity and repeatability parameters. The developed method was applied in the betulinic acid (BA) quantification from extracts of natural products. All separations were obtained on plates Si_{60} F_{254} with *n*-hexane:ethyl acetate:acetic acid (7:3:0.3) at 560nm, after derivatization with vanillin-sulphuric reagent. The system gave compact spots for BA (Rf. 0.5) and exhibited linearity (R^2= 0.999). The methodology was compared with a validated gas chromatography methodology and no statistical difference was observed.

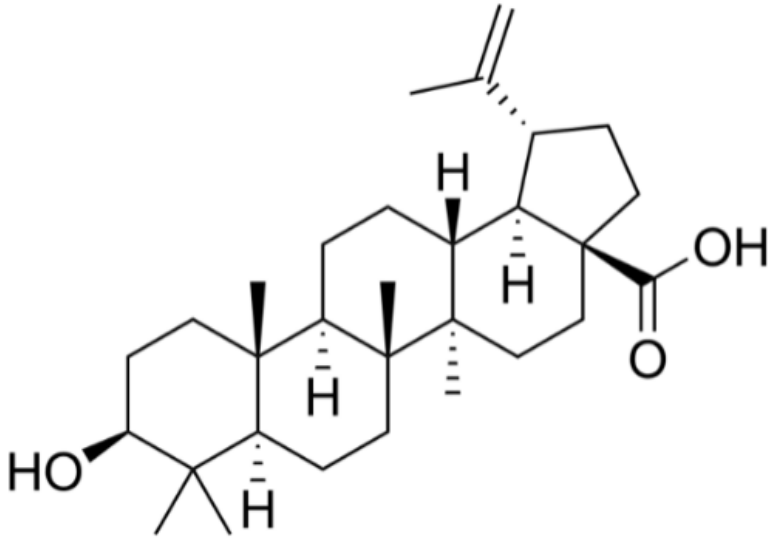
Keywords: TLC/densitometry. Gas chromatography. Validation. Natural extract. Betulinic acid. Quality control.

Introduction

Many analytical techniques have been developed for drugs analyses in complex matrices. Gas chromatography (GC) is described as the best option for assay and purity testing of natural matrices [1-3], while the thin-layer chromatography (TLC) is commonly used for its qualitative analysis [4-6]. However, most of the manuscripts described in the literature since 1993 fail to demonstrate the fitness for purpose to the TLC methods reported [7,8]. While some studies apply inappropriate validation procedures for TLC methods, others compare their results to traditional techniques without the appropriate statistical treatment to meet international acceptance standards [9-12]. Thus, limiting the use of planar chromatography as an analytical technique for the assay of bio samples.

Betulinic acid (BA) is a pentacyclic triterpene widely distributed in different species over the tropics such as in *Morus alba* L. root barks [13], *Davilla rugosa* stalk [14], *Dillenia indica* L. fruits [15], *Licania tomentosa* (Benth) Fritsch fruits and leaves [16-17], *Nelumbo nucifera* Gaertn roots and rizhoma [18] and *Eugenia florida* DC. leaves [19]. A wide range of biological activities is reported for BA, including antiviral [20], antibacterial [21] and anti-inflammatory properties [22-23], and breast cancer [24-27].

FIGURE 1: Betulinic acid structure.



The purpose of this study was to evaluate the parameters to be considered during TLC-densitometry validation for the assay and purity determination of drugs in complex matrices, overcoming the technique limitation describe in the literature, and to compare the method performance with validated GC-FID methodology using statistical tools.

Materials and Methods

Chemical materials

Betulinic Acid 90% and Vanillin 99% were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents of analytical grade were obtained from Merck (Darmstadt, Germany). Silica gel $60F_{254}$ TLC plates of 20 x 10 cm and layer thickness 0.2 mm (Merck, Darmstadt, Germany), were used as stationary phase.

Extraction preparation

The type of extraction technique, part of plant and solvent were chosen and adapted based on literature that presented the best BA yield for each plant [13-18].

Eugenia florida leaves extracts

E. florida leaves were collected at Fiocruz, Rio de Janeiro, Brazil in August 2010. The plant was authentically identified, and a voucher was deposited in the Botanical Garden Herbarium, Rio de Janeiro, Brazil (RB-328.061). Extraction process was established in previous work at Farmanguinhos/Fiocruz. The material was dried at 40°C in hot air oven for 72 h and the dried material was ground to fine power in a knife mill. The dried and powdered leaves (10 g) were placed into a soxhlet apparatus and exhaustively extracted with *n*-hexane followed by ethanol. The fractions were combined and evaporated to dryness by rotary evaporator.

Eugenia florida leaves blank extract

E. florida leaves extract (1.0 g) was subjected to chromatography by using a glass column (50 x 15 cm) filled with Merck silica gel 60 (0.2 mm) and eluted with a gradient of *n*-hexane in ethyl acetate (9.5: 0.5; 9.0:1.0; 8.0:2.0). The fractions of 2 mL each were collected, regrouped (except the fractions from 39 to 66 which contained BA) and evaporated to dryness by using the rotary evaporator.

Dillenia indica L.

D. indica ripe fruits were collected from the Embassy of Portugal, Rio de Janeiro, August 2011, and were deposited in the herbarium of the Rio de Janeiro Botanical Garden – JBRJ, (RB 504.027). The fruits were cut into small pieces the peel and pulp were separated and dried in an oven with air circulation at a temperature 25-30ºC. The dried fruits (10 g each) were extracted by static maceration with methanol for 72 h at room temperature, this step was executed three times, the solvents combined, filtered and evaporated under vacuum at 40-45°C to deliver the crude methanol extract.

Licania tomentosa (Benth) Fritsch

L. tomentosa leaves and fruits were collected at Juliano Moreira Colony - Rio de Janeiro, July 2001, and were deposited in the herbarium of the Rio de Janeiro Botanical Garden – JBRJ, (RB 511.135). The leaves were dried in an electric oven with air circulation at 40 °C and ground. The fruits were separated from their seeds, fragmented and used fresh for the extraction process. The leaves and fruits (30 g each) were extracted by static maceration with methanol and *n*-hexane. The solvents were filtered, combined and evaporated under vacuum to obtain the crude extract.

Nelumbo nucifera (Gaertn)

N. nucifera rhizomes were collected and deposited in the herbarium at Rio de Janeiro, September 2011, and were deposited in the herbarium of the Rio de Janeiro Botanical Garden – JBRJ, (RBv 6300). The rhizomes were dried in an electric oven with air circulation at 40°C and ground. The dried material was extracted separately by static maceration with 70% ethanol by changing the solvent every 72 h until the exhaustion of the plant material, after three changes of solvents.

The solvents were filtered and evaporated under vacuum to afford the dark brown semi-solids extracts of rhizomes.

Sample preparation

A portion of 3 mg of BA standard and the equivalent dried extracts were accurately weighed and transferred into a 50 mL volumetric flask containing 20 mL of methanol. The solution was sonicated for 5 min and diluted to 50 mL with methanol. A 5 mL sample was taken from this solution, transferred to a 50 mL volumetric flask and diluted with methanol. The assay of BA was determined using linear regression equations, defined during the method validation process described below.

GC-FID analysis

Analysis of the products were carried out on an Agilent 6890N (Agilent Technologies, Palo Alto, CA, EUA) gas chromatograph with flame ionization detector (FID), auto sampler in split mode at 5:1, and a capillary column (DB-5, 30 m x 0.32 mm x 1.5 µm). The flow rate of helium carrier gas was 1 mL min^{-1}. Temperature sample introduction: 300°C; oven initial temperature: 200°C (2 min); temperature rise to 350°C at a rate of 15°Cmin^{-1}; isothermal mode for 13 min, detector at 350ºC. Identification was carried out based on retention time values for standards.

TLC analysis

The plates were dried and activated by heating in an oven at 110ºC during 20 min. The samples were applied on analytical TLC plates using Automatic Sampler ATS4 (Camag, Muttenz, Switzerland) at 10 mm from the bottom edge of the plate, with the help of nitrogen gas and a band length of 5 mm. Spots were applied 15 mm apart with application volume of 10 µL by the spraying on samples. The plate was developed in a horizontal degased chamber saturated for 20 min. Mobile phase was *n*-hexane:ethyl acetate:acetic acid (7:3:0.3v/v). The plates were dried, sprayed with vanillin-sulphuric reagent, and kept in a heating plate, producing a derivative which could be viewed and subsequently recorded at 560 nm (Deuterium lamp) in a Scanner III (Camag, Muttenz, Switzerland) for after most quantitative evaluation at Visualizer-DXA 252 Camera (Camag, Muttenz, Switzerland).

Besides the evaluation of figures of merit, the proposed of method validation also considered parameters based on a risk assessment of experimental and environmental conditions that happen in our daily routine work, such as the storage of samples, use of different reagent lots, analysts and equipment, as well as variation in humidity, running distance, plates, chamber saturation time and glassware. The importance of this previous assessment is to reduce or even prevent analytical fails that can occur during validation parameters assays.

The variables that may have critical impact on TLC procedures were sample solution stability, TLC sorbent layer stability before elution and solvent system and relative humidity in TLC plates.

In order to access the solution stability, samples were prepared and stored during 68 h in room temperature (20-25ºC) and eluted immediately after the plotting sampling. To analyze the sorbent layer influence in the BA stability before development, a fresh solution was prepared, spotted and eluted after 0, 2, 4, 6 e 12 h.

Two-dimensional separation was performed to evaluate the analyte during the development with all samples described before.

Different times and temperatures were tested for plate activation: 10, 20 and 30 minutes submitted to 70ºC, 100ºC and 110ºC in hot air oven.

The verification of analytical equipment was done prior to the validation and during the methodology development, considering the procedure specified by the manufacturer.

Validation

The first step to select the validation approach was to determine the purpose of the methodology, including requirements and specifications for the procedure in accordance with international accepted standards [28-31]. Therefore, a quantitative test for BA in samples of natural crude extracts would require tests of accuracy, precision, selectivity, linearity and working range.

Crude extracts, standard solution and blank matrix (extract without BA) were used for the validation assays.

To determine the best solvent or mixture of solvents (named solvent system) to develop the TLC plate, a literature research was performed and the solvent system selection was based on the BA solubility combined with its toxicity, cost, and flammability. Matrix influence over the BA quantification was evaluated by the comparison of the slopes of curves prepared in solvent (standard) and in matrix (*E. florida* leaves blank extract), using Student's *t* test.

Limit of Detection (LoD) was determined by the mean value of the matrix blank readings (n=10) plus 3 standard deviations of the mean, expressed in analyte concentration, and the Limit of Quantitation (LoQ) by the mean value of matrix blank readings (n=10) plus 10 standard deviations of the mean, expressed in analyte concentration.

Calibration curves were constructed with stock solution of BA standard and the blank spiked with BA. The spots in TLC plate were randomly applied from different concentrations by direct independent weighing to avoid variation in band-broadening effects from the different amounts of solution per spot or band. Six concentrations: 17, 34, 67, 100, 133, and 167% of work concentration (6.0 µg $spot^{-1}$) were evaluated. Each of the six standards was prepared in triplicate and analyzed three times.

The effects of random events on the precision of the analytical method to give a first indication of the future transferability of the analytical method was determined by the intermediate precision taking into account various influences within a laboratory, i.e. conducting analyses in two sets of experiments by different laboratory staff members, with different equipment, glassware and reagent lots. The same sample in three different concentrations was analyzed in nine replicates during the same day.

Method accuracy/recovery was determined by the standard addition method. The sample was spiked with known quantities of BA at three different concentrations (80%, 100% and 120%) for the recovery calculation.

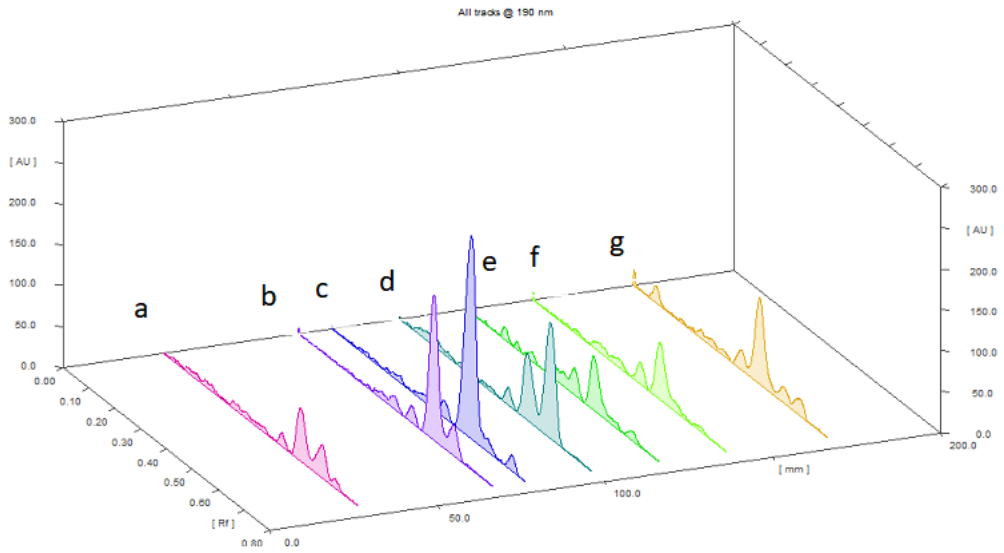
Results and Discussion

TLC Method Development

Elution System

To determine the best solvent or mixture of solvents (named solvent system) to develop the TLC plate, a literature research was performed and the solvent system selection was based on the BA solubility combined with its toxicity, cost, and flammability. Trial runs were performed by changing polarity in each solvent system, but none of the systems mentioned in literature presented the center of spot nearest to the BA separated from it by at least one-half sum of spot diameters, with visual appearance of analyte indistinguishable from the reference material. The ideal strength was achieved with ethyl acetate:*n*-hexane (3:7 v/v) which moved BA to a R_f value of 0.5 and separated it from the nearest neighbor by a difference in R_f value of 0.2, observing more than 95% of spectrum purity (**FIGURE 2**). In order to determine if other compounds from plant matrix could interfere with the measurement of the BA, selectivity was also evaluated with reagent blanks, matrix blanks (without BA) and BA samples from different natural sources and metabolic profiles (*Dillenia indica* L., *Licania tomentosa* (Benth) Fritsch and *Nelumbo nucifera* Gaertn). The results demonstrated the same method selectivity efficiency as shown in *E. florida* (**FIGURE 2**), but the BA yield showed significant differences among the samples (**TABLE 1**).

FIGURE 2: TLC densitogram of BA in the extracts.



Legend: (a) *N. nucifera* rizhome; (b) *E. florida* leaves; (c) Standard BA; (d) *D. indica* ripe fruits; (e) *D. indica* green fruits; (f) *L. tomentosa* fruits; and (g) *L. tomentosa* leaves.

TABLE 1: Samples selection in BA specificity experiments and BA amount quantified.

Source	Extraction Process	Metabolic profile	BA (%)
E. florida	Soxhlet	Extract	8.8 (leaves)
E. florida (leaves)	Soxhlet/ Chromatography column	Matrix blank	(leaves)
D. indica	Static maceration (Methanol-72h)	Extract	6.53 (ripe) 4.49 (green)
L .tomentosa	Static maceration (Methanol/*n*-hexane - 72h)	Extract	2.80 (leaves) 2.29 (fruits)
N. nucifera	Static maceration (Ethanol 70% - 72h)	Extract	3.8 (rhizome)

BA – Betulinic Acid

Solution Stability

The stability in solution of BA standard and sample (extracts) was evaluated on the TLC sorbent layer before, during and after development, during sample-preparation, as well as before and after visualization with sampling at 0, 2, 6, 12, 24, 36, 48 and 68 h through its quantification (**FIGURE 3a**). Degradation was observed during the development on the solution stability sample at 48 and 54 h for BA extract and standard, respectively, as well as on the TLC sorbent layer after elution at 5 h.

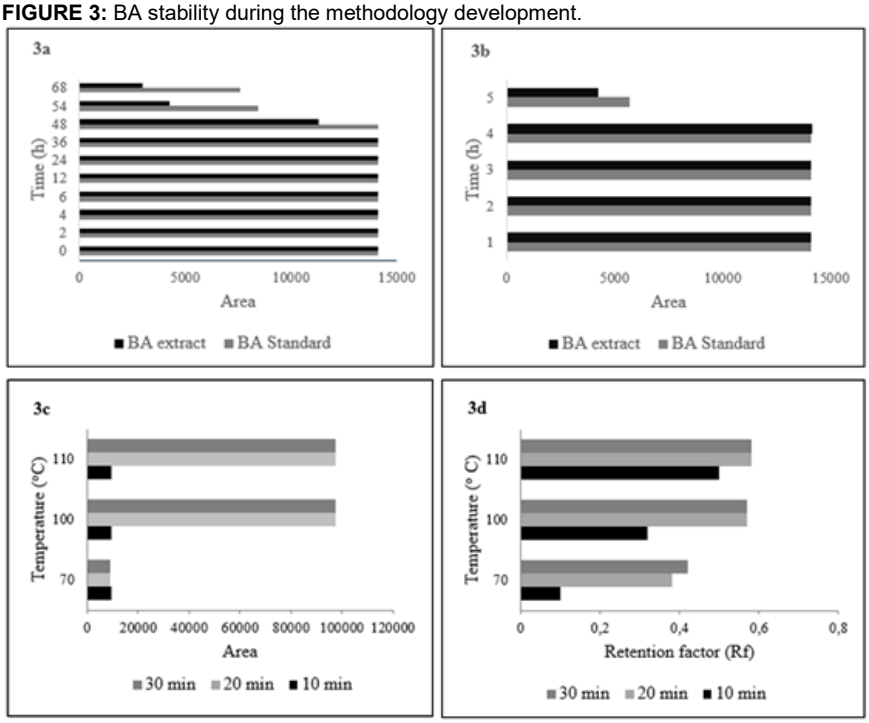
Stability of the samples after the development was also assessed by two-dimension chromatography at 30 and 60 min after elution. No degradation was observed.

TLC plates

Larger spots and different retention factors (R_f) were observed in plates with 10 minutes of previous activation at 70, 100 and 110ºC. The best results were achieved when plates were dried and activated by heating in oven for 20 min at 110°C, ensuring total water withdrawal (**FIGURE 3c**).

The plate humidity affected selectivity (Rf) and this was observed on the quantitative results since broad spots were formed making hard the quantification (**FIGURES 3c and 3d**).

FIGURE 3: BA stability during the methodology development.



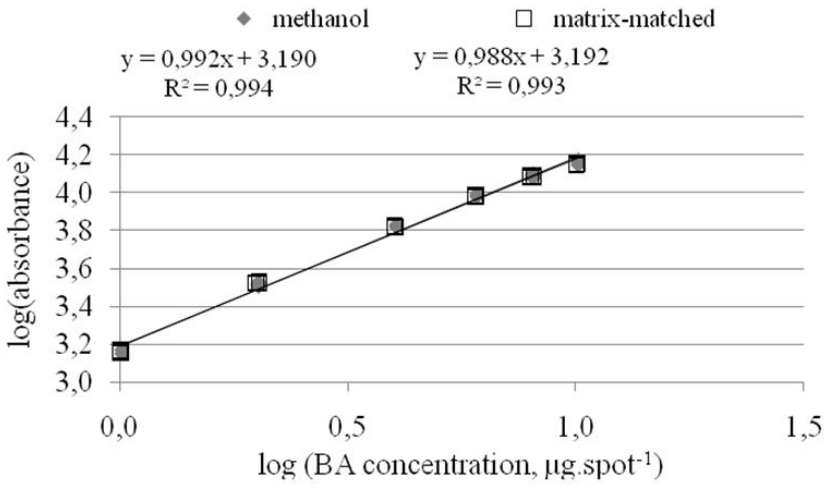
Legend: (3a) In solution, (3b) In TLC sorbent layer before elution, by plate activation influence in the BA TLC performance: (3c) Area and (3d) R_f. BA – Betulinic Acid; TLC – Thin-Layer Chromatography; Rf – Retention factor.

TLC Method Validation

Selectivity

In order to assess the effect of matrix interferents over BA response, the slopes of calibration curves prepared in methanol and in a blank extract (matrix-matched) were compared. Since calibration curves fitted in a polynomial model, each variable was plotted as logarithm to approximate the curves to a linear model (**FIGURE 4**) and allow the comparison by Student's t test. The slopes of the curves did not present significant difference (t=0.026, critical t=1.984, 95%, v=102). Based on these results, the following calibration curves could be prepared in methanol, which made easier the routine of analyses.

FIGURE 4: Comparison of calibration curves prepared in methanol and matrix-matched (blank extract of *E. florida*).



BA – Betulinic Acid

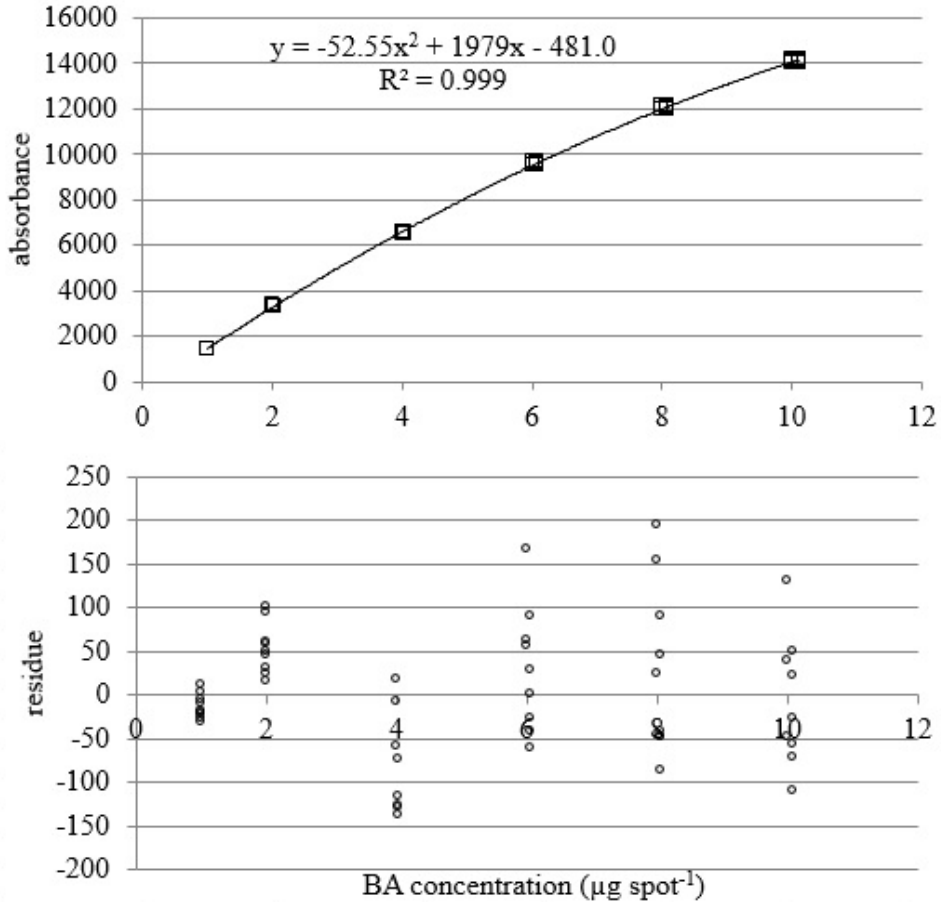
LoD and LoQ

LoD and LoQ of the method were 0.265 and 0.854 µg.spot^{-1}, respectively.

Linearity Range

Calibration curves were constructed by plotting the absorbance against the concentration using polynomial regression model. Data from the curve prepared in blank extract (Figure 5) were tested using Cochran[16] and were considered homoscedastic (C=0.3686, critical C=0.3817, 95%, k=6, v=8). Moreover, y-residues were randomly distributed over the concentration range. Linearity was thus demonstrated between 1 and 10µg.spot^{-1} of BA. Results were similar for the calibration curve prepared in methanol (y=-50.40x^2+1974x-486.1), as discussed in the Selectivity section.

FIGURE 5: Evaluation of the BA calibration curve prepared in blank extract.

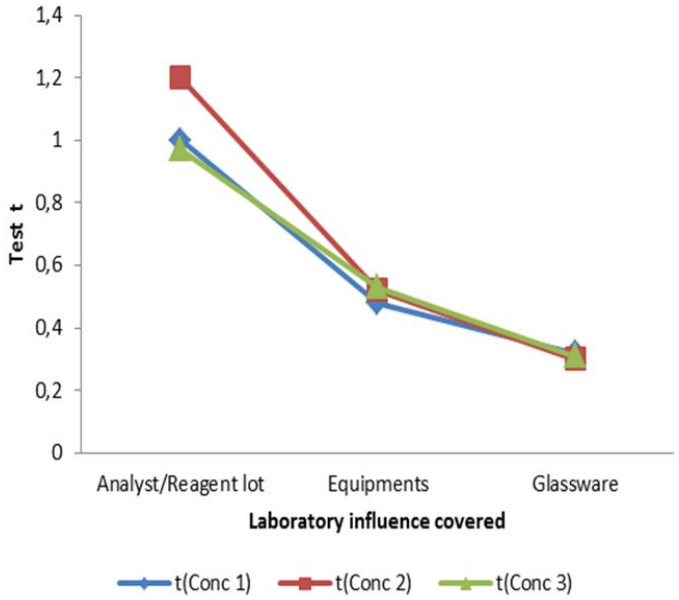


BA – Betulinic Acid

Precision

Results from different analysts/reagents, equipment and glassware were statistically compared by Student's t-test and F-test and no significant difference was observed (**FIGURE 6**).

FIGURE 6: Experimental and environmental conditions that affect the TLC performance on selectivity and quantitative results, compared by *t*-test.



The variability (RSD%) when the method was performed by a single analyst on one piece of equipment over a short timescale was evaluated by the replication of the entire method under the same procedure over three concentration levels presented 0.34; 0.47; 0.73% and 0.56; 0.44; 0.48% as relative standard deviations for standard and sample, respectively. The precision of the method complies international Standards to enable the assay of BA in this intended use.

The efficiency of the separation of BA from other substances in all samples studied was confirmed by two-dimensional separation.

Accuracy/Recovery

As recovery is a vital part of analytical method, the leaves were spiked before the extractions and compared with the non-spiked material and the spiked blank, resulting in values 99.1 to 102.34% (**TABLE 2**).

Results obtained in TLC were compared to those determined with a GC fully validated method by applying Student's *t*-test and *F*-test and no significant (statistical) difference was observed (t=0.37, n=9).

TABLE 2: Recovery studies of BA by TLC and GC.

		Samples			Blank		
Label claim (µL)	Method	Excess BA added (%)	Amount recovery (mg)	% Recovery	Excess BA added (%)	Amount recovery (mg)	% Recovery
900	TLC	80	8.04	100.55	80	8.08	101.02
		100	10.01	100.06	100	9.99	99.98
		120	11.88	99.01	120	12.24	102.03
900	GC	80	8.05	100.57	80	8.01	100.04
		100	10.23	102.34	100	10.01	100.01
		120	12.16	101.34	120	11.99	99.93

Legend: BA – betulinic acid; TLC – Thin-Layer Chromatography; GC – Gas Chromatography;

The results of this study show that the developed method is simple, rapid, sensitive and accurate, and may be of value to the natural product industry and for researchers during the standardization of extracts containing BA in a shorter time comparing to the traditional analytical methodology (**TABLE 3**).

TABLE 3: Comparison of BA methodology validation by TLC and GC.

Parameters	TLC	GC
Working range	1 – 10 µg	10 – 100 µg
Limit of detection	0.27 µg	0.12 µg
Limit of quantification	0.85 µg	0.387 µg
Repeatability (RSD%, *n*=9) Low concentration High concentration	 0.78 0.40	 0.39 0.88
Time per determination (All procedure)	15 min	55 min

Legend: BA – Betulinic Acid; TLC – Thin-Layer Chromatography; GC – Gas Chromatography; RSD – Relative Standard Deviation

Applications

The developed and validated TLC methodology was successfully applied to evaluate the seasonal variation of BA in leaves from *E. florida*, (**TABLE 4**). Each determination was carried out in five replicates and the average results were compared to GC-FID (validated method) data obtained with the same samples. Table 4 showed percentual differences between the techniques results in a range 1.1-9.2%, possibly indicating the matrix complexity. Statistical analysis demonstrated no significant difference between the results from both methods applied ($F_{experimental}$= 1.27 , $F_{critical}$= 2.82 ; $t_{critical}$= 0.23 , $t_{experimental}$= 2.07).

TABLE 4: Comparison of TLC and GC-FID methods applied to BA determination in seasonal evaluation of *E. florida* (leaves).

Month	BA (%)		Results difference (%)
	TLC	GC-FID	
August	18,30	19,20	4,7%
September	12,65	12,37	2,3%
October	17,80	18,89	5,8%
November	22,15	21,39	3,6%
December	18,10	19,69	8,1%
January	16,42	15,12	8,6%
February	18,60	19,90	6,5%
March	10,70	9,80	9,2%
April	19,70	20,64	4,6%
May	21,90	22,15	1,1%
June	26,5	27,62	4,1%
July	23,8	25,34	6,1%

Legend: BA – Betulinic Acid

The critical points detected during the methodology development were sample stability, TLC sorbent stability and plates humidity. The method exhibited linearity in a concentration range of 1.00-10.00 µg.spot^{-1} (R^2= 0.999). The solutions are stable during 48 h and need to be developed in the sorbent until 6 h after

application. No amber glassware was required during the sample preparation and the evaluation of the plates could be carried out until 1 h without degradation.

Conclusions

The method was validated and applied successfully to distinguish BA from the interfering materials in a complex matrix such as the extracts of natural samples. This method can be a cheaper and faster alternative to GC technique and over came all the failures, misconceptions and misleading pointed in the literature for TLC quantitative methods. We believe that this methodology provides a correct and proper validation of a TLC methodology, regarding the suggested protocol involving the environmental and experimental conditions (analyte stability in solution and TLC adsorbent, TLC plate humidity, solvent system selectivity).

Acknowledgements

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