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Simultaneous determination of three steroidal glycoalkaloids in *Solanum xanthocarpum* by high performance thin layer chromatography

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ABSTRACT

A new high-performance thin-layer chromatographic (HPTLC) method has been developed for the simultaneous quantitation of three bioactive steroidal glycoalkaloid (SGA) markers, solasonine (SN), solamargine (SM) and khasianine (KN) in the plant *Solanum xanthocarpum*. Extraction efficiency of targeted SGAs from plant matrix using methanol and acidified methanol were studied using percolation, ultrasonication and microwave techniques. The separation was achieved on silica gel $60F_{254}$ TLC plates using chloroform–methanol–water as mobile phase. The quantitation of SGAs was carried out using the densitometric reflection/absorption mode at 520 nm after post chromatographic derivatization using Dragendorff's reagent. The method was validated for peak purity, precision, accuracy, robustness, limit of detection (LOD) and quantitation (LOQ). Method specificity was confirmed using retention factor (R_f), Vis spectral correlation and electrospray ionization mass spectra (ESI-MS) of marker compounds in the sample track.

1. Introduction

Steroidal glycoalkaloids (SGAs) are the secondary metabolites produced by solanaceous plants and possesses potential food safety concerns [1,2]. Some of the solanaceous plants are being used in Indian-system of medicine after processing with specific purification processes [3]. *Solanum xanthocarpum* Schrad & Wendl (Syn. *S. virginianum*/*S. surattense*; Family – Solanaceae), commonly known as '*Kantakari*' is prickly diffuse bright green perennial herb which grow mostly in dry places as a weed on roadsides and waste lands of India and other South-East Asian countries [4,5]. *S. xanthocarpum* is an important ingredient of generic Ayurvedic formulation "*Dashamularishta*", and its each part is used in several others medicinal formulations such as *Kantakari ghrta*, *Kantakari avaleha*, *Kantakari kalpa*, *Vyaghri taila*, *Vyaghriharitaki*, *Kanakasava*, *Nidigdhadi* and *Kvatha*. Its decoction is used in "*Chyawanprash – A Rejuvenating Ayurvedic tonic*" as mucolytic and expectorant agent to cure the *Kaphaj Dosha* related to allergic bronchitis, bronchial asthma, common cold, anthelmintic, antipyretic, laxative, anti-inflammatory and antiasthmatic [6–8].

Antispasmodic, antitumor, cardiotonic, hypotensive, antianaphylactic, cytotoxic [9], hypoglycemic [10], bronchodilator [11], activities have also been reported. Several secondary metabolites such as steroidal alkaloids like solanocarpine [12], solanocarpidine, solasonine (SN) [7] solamargine (SM) [13] and steroidal glycosides [14] are reported from the plant. Biological investigations of SM and SN showed significant cytotoxicity against the several human cell lines and skin tumor [15,16]. Human intake of high doses of other solanaceae steroidal glycoalkaloids has led to acute intoxication, in severe cases coma and death [1,2,17], but no adverse effect of *S. xanthocarpum* is reported so far.

Absence of chromophore in SGAs makes their detection a major challenging problem in the assay of a biological sample [18,19]. Detection after derivatization on a TLC plate is a simple and rapid option for such compounds. Among various analytical techniques, high-performance thin-layer chromatography (HPTLC) in particular appears to be suitable for phytomolecules of varying nature and provides a rational approach in the authentication and quality assessment of crude medicinal herbs and their formulations [20–22]. Earlier reports on quality analysis of *S. xanthocarpum* involve complex sample preparation steps, require long analysis time, use of gradient elution or involve tedious pre-chromatographic derivatization to form an ion-paring complex followed by LC–UV detection [23]. Acid dye based TLC method provide the quantitation of only one alkaloid and also involves addition

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step of ion pair complex formation of solasodine prior to detection [24].

Our continued interest on the development of rapid HPTLC method for quality assessment of medicinal plants [20–22] led us to develop an analytical procedure for quality assessment of *S. xanthocarpum*. The objective of present study was to optimize, develop and validate a rapid, sensitive and accurate HPTLC method for the simultaneous determination of three potentially toxic steroidal glycoalkaloids in aerial part of *S. xanthocarpum*.

2. Materials and methods

2.1. Plant materials

The aerial parts of wildly grown *S. xanthocarpum* plants were collected in the month of June 2009, at the flowering stage from the research farm of CIMAP, Lucknow. Voucher specimen (CIMAP No. 12470) is deposited in the Botany and Pharmacognosy Department of the Institute. The plant material was powdered, packed in airtight container and stored at 20 °C until analysis.

2.2. Chemicals and reference compounds

All reagents and solvents used were either of analytical or HPLC grade (E. Merck Ltd., Mumbai, India). Prior to use, the solvents were filtered through a 0.45 μm membrane (Millipore, Billerica, MA, USA). Pre-coated TLC silica gel $60F_{254}$ aluminum plates were purchased from Merck (Darmstadt, Germany). The sorbents silica gels, used for vacuum liquid chromatography (VLC) and flash chromatography were procured from Qualigens Fine Chemicals, Mumbai, India. The standard compounds, solasonine (SN), solamargine (SM) and khasianine (KN) were isolated (purity >99% using area normalization method of HPLC–MS) and characterized by spectral analysis in our laboratory.

2.3. Apparatus

Vario system, TLC Scanner, winCATS-III, Reprostar-3, twin trough chamber, immersion device-III, TLC plate heater (Camag, Muttenz, Switzerland) were used for digital image scanning, method development and validation. Ultrasonic extraction was performed in ultrasonic bath (Oscar Micro clean-109, Mumbai, India). Microwave assisted extraction (MAE) experiments were performed with microwave oven (Whirlpool, New Delhi, India) with programmable heating power and pulse radiations (controlled temperature). Shimadzu (Japan) LC–MS was used for ESI-MS spectra which consisted of LC-20AD solvent delivery pumps, a DGU-$20A_5$ degasser, a CTO-20A column oven and a SPD-M 20A photodiode array detector and a mass spectrometer LCMS-2010EV. The 300 MHz NMR (Avance, Bruker, Switzerland) was used to record spectra in CD_3OD with tetramethyl silane (TMS) as internal standard.

2.4. Extraction and isolation of SGAs from S. xanthocarpum

Air dried and finely powdered aerial parts of *S. xanthocarpum* (1.3 kg) were exhaustively extracted with 3 × 10 l of methanol in a percolator at room temperature (25 ± 2 °C). The pooled extract was evaporated to dryness in vacuum at 40 °C. From the material thus obtained the SGAs were extracted with 10× 200 ml of 2% aqueous HCl solution as water-soluble hydrochloride salts. The combined acidic extract was defatted with 3× 500 ml hexane. The defatted acidic extract was basified under cooling with 10% sodium hydroxide solution up to pH 12 and extracted with *n*-butanol saturated with water (4× 500 ml). The pooled butanol extract was washed with water and evaporated under vacuum to afford a dark brown residue (9.7 g).

Table 1
Extraction efficiency of different solvents for chemical marker (SGAs) from the aerial parts of *S. xanthocarpum* on plant dry weight basis.

Techniques[a]/solvents	Amount of compound quantified (%, w/w)		
	Solasonine (SN)	Solamargine (SM)	Khasianine (KN)
Cold percolation			
Methanol	0.100 ± 0.005	0.209 ± 0.009	0.076 ± 0.004
Acidified methanol	0.108 ± 0.005	0.229 ± 0.012	0.082 ± 0.005
Hot extraction			
Methanol	0.108 ± 0.005	0.237 ± 0.011	0.082 ± 0.004
Acidified methanol	0.115 ± 0.007	0.338 ± 0.017	0.085 ± 0.005
Soxhlet extraction			
Methanol	0.090 ± 0.006	0.368 ± 0.013	0.085 ± 0.004
Acidified methanol	0.110 ± 0.009	0.442 ± 0.022	0.090 ± 0.005
Ultrasonic extraction			
Methanol	0.102 ± 0.010	0.257 ± 0.012	0.085 ± 0.005
Acidified methanol	0.166 ± 0.007	0.443 ± 0.010	0.088 ± 0.005
Microwave extraction			
Methanol	0.105 ± 0.006	0.241 ± 0.011	0.082 ± 0.006
Acidified methanol	0.168 ± 0.008	0.449 ± 0.020	0.110 ± 0.007

[a] The experimental conditions are described in Section 2.5.

For the isolation of SGAs, 7.4 g of the butanol extract was subjected to vacuum liquid chromatography (VLC, 7.5 cm × 7.5 cm, Silica gel H, 125 g). Gradient elution was carried out with $CHCl_3$, $CHCl_3$:MeOH and MeOH in increasing polarity. On the basis of TLC monitoring of a total of 296 fractions using Merck (Darmstadt) silica gel $60F_{254}$ plates and visualization with Dragendorff's reagent, 3 g material from fractions 257–280 was selected for further fractionation by flash chromatography using glass column (3 cm × 23 cm) packed with silica gel (230–400 mesh). After four subsequent fractionation steps 16 mg of KN, 60 mg of SM and 20 mg of SN were obtained. The structure elucidation of SN, SM and KN (Fig. 1) was carried out with the help of ^{1}H NMR, ^{13}C NMR, 2-dimensional NMR experiments (HSQCGP and HMBC) and mass (ESI-MS) spectroscopic data, which were in complete agreement with the reported data [25].

2.5. Standard stock solutions and sample preparation

Standard stock solutions of compounds SN and SM were prepared as 5.0 mg/ml and of KN 15 mg/ml in methanol. Working stocks for calibration studies were prepared by dilution using Hamilton syringe (Bonaduz, Switzerland). The dried and milled aerial parts of *S. xanthocarpum* were extracted with acidified methanol (1% AcOH) by cold percolation (3× 15 ml, 10 h extraction time at room temperature); hot extraction (3× 15 ml, 30 min extraction time at 50 °C); soxhlet (50 ml, 2 h extraction time); ultrasonication (3× 5 ml, 15 min extraction time); microwave (3× 5 ml, at 650 W, 50 °C for 3 min extraction time) assisted extraction separately (1.0 g plant in each case). The extracts were evaporated to dryness and re-dissolved in 1.0 ml of methanol and centrifuged at 10,000 rpm for 10 min. The supernatants were pre-filtered with 0.45 μm and used for HPTLC analysis. The result of extraction efficiency of solvents and suitability of extraction techniques are summarized in Table 1.

2.6. Chromatographic procedure

Chromatography was performed on preactivated HPTLC plates (10cm × 10 cm or 20cm × 10 cm). The plates were washed with methanol and activated at 120 °C for 20 min. Standard and sample solutions were applied in the form of band at 15 mm from both the lower and left edge with 20 mm space between two bands using a 100 μl syringe (Hamilton, Bonaduz, Switzerland). Linear ascending development was carried out in pre-saturated (optimized to

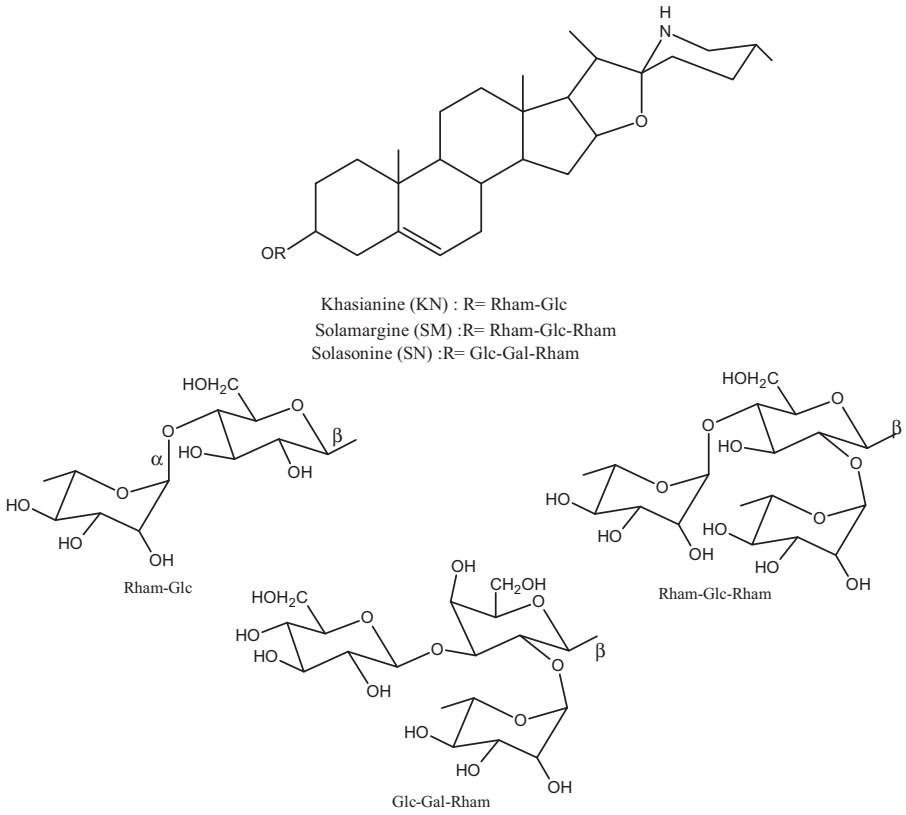

Fig. 1. Chemical structures of steroidal glyco-alkaloids.

4 min for better resolution) vertical twin trough glass chambers (10 cm × 10 cm or 20 cm × 10 cm) saturated with the mobile phase. The mobile phase selection and optimization was carried out using the Vario System wherein different compositions consisting of different ratios of solvents of varying polarity were tried. Finally, a mobile phase consisting of organic layer of chloroform–methanol: water (70:30:10, v/v/v) was used as mobile phase and HPTLC separation of the three SGAs was achieved without interference of sample matrix components under laboratory conditions (temperature 25 ± 3 °C and relative humidity 35–40%). After development, plates were dried and the components were visualized by Dragendorff's reagent using the immersion device (dipping time 2 s, dipping speed 5 cm/s) followed by air drying for 30 min. The plates were scanned and quantified densitometrically at 520 nm (maximum wavelength in the spectra of the derivative). TLC Scanner-III controlled by winCATS 1.4.2.8121 software (Camag) was used for quantitative evaluation. The densitometry scanning was performed in the reflectance/absorbance mode, slit width 6.00 mm × 0.45 mm, scanning speed 20 mm/s and data resolution 10 μm/step. Savitsky-Golay-7 was used for data filtering and the lowest slope for baseline correction in order to integrate the area. A tungsten lamp was used for recording of characteristic derivatized bands of standards SN, SM, KN and sample tracks in the range 400–800 nm. Reprostar 3 with cabinet cover and mounted digital camera (Canon PowerShot G5 with Neck Strap NS-DC2, Canon, Japan) was used for imaging and archiving the chromatograms. Quantitation was performed using linear regression equations of respective compounds.

3. Results and discussion

3.1. Optimization of extractability of SGAs from sample matrix

Different extracts of *S. xanthocarpum* through various methodologies viz. cold percolation, soxhlet, hot extraction, ultra sonication and micro-wave were studied to evaluate the extraction efficiency of SGAs from the plant. During optimization, the operating conditions for sonication (250 W, 40 kHz for 15, 30, 45 and 60 min) and microwave extraction (90, 160, 350, 500, 650, 750 W at 50 °C for 3 min extraction time) were studied. Ultrasonic (3× 15 ml, 15 min) and microwave (3× 15 ml at 650 W and 50 °C for 3 min) extraction were found optimum. Acidified methanol as extraction solvent and microwave at 650 W were found to be the most suitable for sample preparation.

The extracts were concentrated under vacuum and re-dissolved in methanol for HPTLC analysis. All the sample and standard tracks were scanned at 520 nm after derivatization with Dragendorff's reagent (Fig. 2). It is clearly evident that no interfering compound eluted in the sample tracks to affect the quantitation of the targeted markers SN, SM and KN (Fig. 2) but the extraction efficiency varied. For both qualitative and quantitative analysis, visible range after derivatization was most suitable for scanning of spots (Fig. 2) as it ruled out the possibility of merging of closet compounds. The spectra of SN, SM and KN in both standard and sample track were highly correlated (Table 2) and confirm the mode of scanning to be effective and sensitive. Further, the specificity of respective SGA in the sample track was ensured by ESI-MS spectra matching. Characteristic peaks of mass adduct ions $[M+H]^+$ and/or $[M+Na]^+$ were found in respective SGA of standard and sample track, further confirming the specificity of SGAs analysis by HPTLC separation and densitometric quantitation (S1).

3.2. Method validation

Method validation was performed on the parameters such as linearity, limit of sensitivities, specificity, precision, accuracy, recovery and robustness as per ICH [26] and IUPAC [27] guidelines.

All data were evaluated using standard statistical packages for Windows and GraphPad Prism 4.0 (GraphPad Software, Inc., USA).

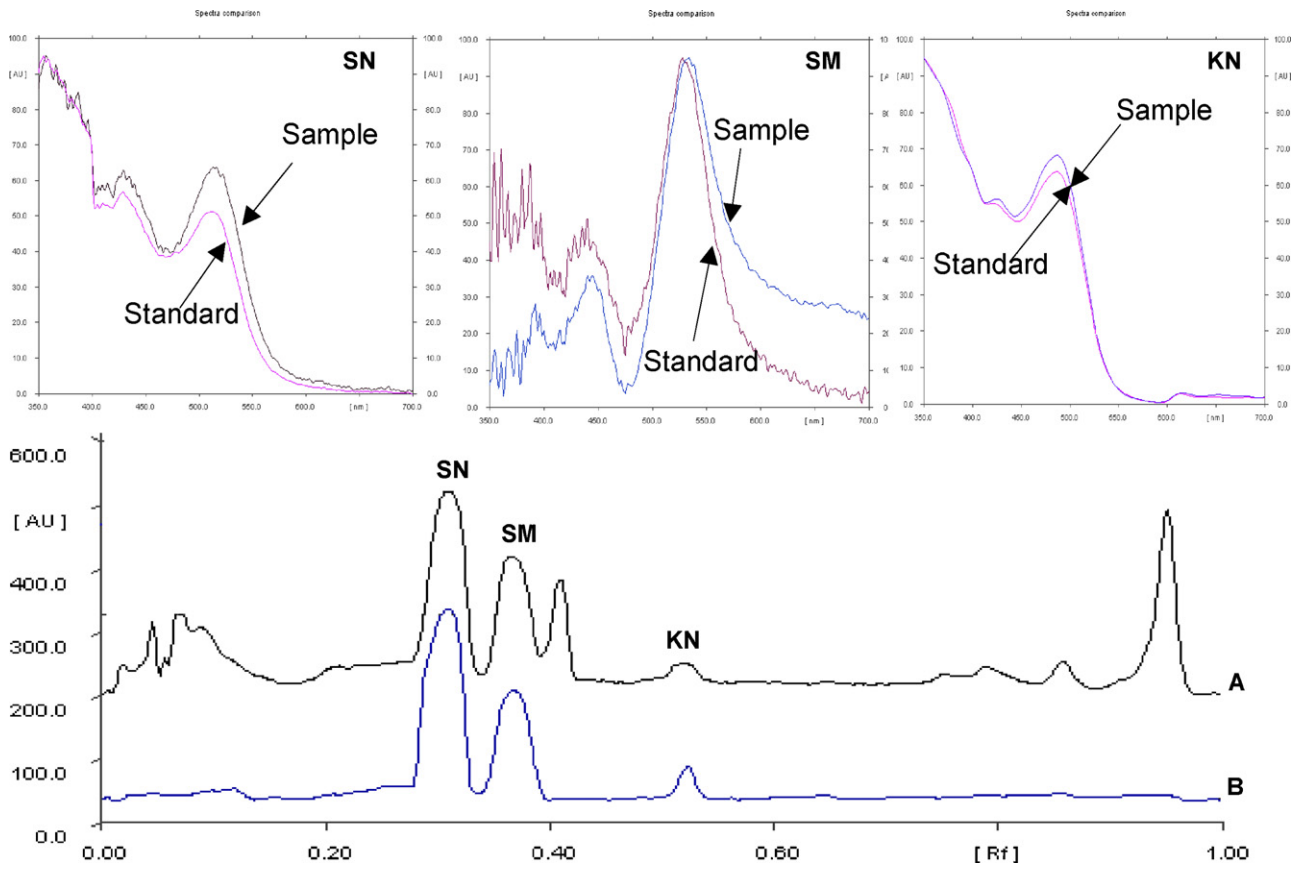

Fig. 2. Track A: HPTLC chromatogram of acidified methanolic extract of aerial part of *Solanum xanthaocarpum* (Ext) and Track B: model mixture of three chemical markers solasonine (SN), solamargine (SM) and khasianine (KN) [Concentration: Ext 100 mg/ml; SN, SM 0.5 mg/ml and KN 5.0 mg/ml; sample loading: 5 μl/band; inset showing the comparison of visible spectra of respective compounds in sample and standard track].

Statistical significance was considered at 95% probability level ($p < 0.05$). Pearson's coefficient (r^2) was used to evaluate the significance of correlations.

Computing and comparing HPTLC standard curves with reference compounds and spiked samples curves was in compliance with ICH validation requirements [21,22].

3.2.1. Calibration curves, limits of detection/quantification (LOD/LOQ)

Working stock solutions of SN and SM were prepared by dilution with methanol to give the compound concentrations 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml whereas KN was prepared as 3.0, 6.0, 9.0, 12.0 and 15.0 mg/ml. Standard solutions of each compound were

Table 2
Analytical characteristics of the validated HPTLC method for the quantitation of SGAs in *S. xanthocarpum*.

Parameters	SN	SM	KN
R_f value	0.31 ± 0.04	0.37 ± 0.03	0.52 ± 0.03
Densitometric linear relationship[a]			
Working concentration range	2–10 μg/band	2–10 μg/band	6–30 μg/band
Regression equation	$y = 1965x - 404$	$y = 305.25x + 238.5$	$y = 575.2x - 471.2$
Correlation coefficient (r^2)	0.9954	0.9926	0.9823
Accuracy (%)			
Repeatability (RSD %; $n = 6$) at 6 μg/band (SN & SM), 18 μg/band (KN)	1.26	1.70	0.52
Precision			
Intra-day (RSD %) ($n = 3$)	1.03	0.64	0.07
Inter-day (RSD %) ($n = 3$)	0.82	1.92	0.28
Specificity			
Peak purity[b]			
R (s,m)			
Standard track	0.9997	0.9822	0.9999
Sample track	0.9980	0.9654	0.9998
R (m,e)[c]			
Standard track	0.9990	0.9893	0.9998
Sample track	0.9825	0.9611	0.9998

[a] Digital scanning at 520 nm after post derivatization with Dragendorff reagent and statistical relationship were established considering five data point each in triplicate; X, amount of compound (μg/band); Y peak area counts.
[b] Correlation of spectrum at start of peak with spectrum at the centre of peak at 520 nm scanning.
[c] Correlation of spectrum at centre of peak with spectrum at the end of peak at 520 nm scanning.

Table 3
Results of recovery study.

SGA content in plant sample (μg absolute)	Spiked amount (μg absolute)	Theoretical value (μg)	Experimental value (μg)	Percentage recovery	Recoveries (sverage ± RSD)
SN/4.02	2	6.02	5.84	97.01	94.74 ± 2.10
		6.02	5.59	92.86	
		6.02	5.68	94.35	
	4	8.02	6.66	83.04	83.04 ± 0.12
		8.02	6.67	83.17	
		8.02	6.65	82.92	
SM/20.55	2	22.55	23.55	104.43	105.66 ± 1.09
		22.55	23.91	106.03	
		22.55	24.02	106.52	
	4	24.55	24.95	101.63	101.02 ± 0.61
		24.55	24.80	101.02	
		24.55	24.65	100.41	
KN/1.98	12	13.98	13.48	96.42	93.71 ± 2.48
		13.98	12.80	91.56	
		13.98	13.02	93.13	
	18	19.98	19.24	96.30	94.51 ± 1.63
		19.98	18.60	93.09	
		19.98	18.81	94.14	

spotted on HPTLC plate to give absolute amounts of 2, 4, 6, 8, and 10 μg/band for SN and SM and 6, 12, 18, 24 and 30 μg/band for KN. Each concentration was spotted thrice. The calibration curves were prepared by the least-squares method using absolute amount (μg/band) as independent variable (X) and the peak area of SGA standards as dependent variable (Y). Regression analyses test of the compound was performed by GraphPAD Prism 4.0. The selection of working concentration range of respective SGA was based on their response. The curves (Table 2) confirm linear relationship ($r^2 \geq 0.98$) between the working concentration and the peak areas. Linearity has been checked for three consecutive days for the same concentration range (five data point in triplicate) from different stock solutions ($p \geq 0.287$). The intercept was not statistically significant ($p \geq 0.05$). A statistical residual analysis was also performed for each point of concentration range and corresponding to the difference between estimated and mean concentrations. Residual plot analysis demonstrates that residuals were randomly distributed around the zero value. This further confirms the choice of the linear model.

LOD and LOQ were determined using the linear regression equations: LOD = $3S_{y,x}/b$ and LOQ = $10S_{y,x}/b$, where $S_{y,x}$ is the standard deviation of the Y-value distribution around the regression line and b is the slope of the calibration curve. LODs for SN, SM and KN were 0.75, 0.95 and 4.41 μg/band, respectively; and LOQs determined in the same way were 2.49, 3.16 and 14.71 μg/band, respectively.

3.2.2. Specificity

The method specificity was assessed by comparing the R_f (SN 0.31, SM 0.37, KN 0.52) and absorption spectra of reference compounds in sample and standard tracks. On comparison of spectra at peak start, peak apex and peak end positions of the band, an acceptable correlation (r^2 = 0.96–0.99) was obtained between standards and sample overlay spectra which confirms the purity of SN, SM and KN peaks in sample track. Further, to confirm the specificity, silica was scraped from the centre of band corresponding to SN, SM, and KN in both standard and sample tracks and ESI-mass spectra (S1) were obtained by direct introduction into mass detector. In HPLC–MS experiments the HPTLC plate was dried and the zones of interest were scraped and dissolved in 100 μl of methanol in glass micro tubes. After centrifugation at 10,000 rpm for 3 min, the supernatant of 20 μl of analytes were eluted isocratically at a flow rate of 0.5 ml/min using methanol–water (80:20, v/v) with the restricted coil thermostated at 28 °C. Mass spectrometric detection conditions were as follows: curved desolvation line (CDL) temperature and heat block temperature: 250 and 480 °C, respectively; probe voltage +4.5 kV; detector voltage 1.5 kV; CDL voltage – 20 V and Q-array Bios – 50 V. Nebulizing gas was nitrogen at a flow rate of 1.5 l/min. Mass spectra of compounds SN, SM and KN from sample track were stored in the library of LCsolution software and matched with those of standard track. The spectra similarity of Target (Spl track) with reference compound (Std track) of TLC was found between 58 and 88% (S1). Checking the method specificity with off line MS was introduced for the first time in SGAs determination by planar chromatography.

3.2.3. Precision and accuracy

The repeatability of measurement (n = 5) of peak area of all SGA standards was expressed in terms of percent coefficient of variation (RSD %). The intra- and inter-day variations were also evaluated at 6 μg/band for SN and SM; 18 μg/band for KN. Due to the poor response of KN, higher amount per band was applied for the evaluation. The results shown in Table 2 depict the method to be accurate and precise for the analysis of chemical test markers in *S. xanthocarpum*.

3.2.4. Recovery

The accuracy of quantitation in terms of recovery was assessed. For this purpose, plant sample with known amounts of SN, SM and KN was extracted with methanol (as per procedure of sample preparation described above) and applied in triplicate on the plate. The sample was spiked with two different amounts of SN, SM and KN to reach a final content within the working range of linear calibration. The recovery results were within acceptable limits (Table 3).

3.2.5. Robustness

To test the robustness of the method deliberately small changes in the chromatographic parameters which may affect the performance of the method i.e. mobile phase composition, chamber saturation time, delay between spotting and plate development and delay in digital scanning after derivatization, were made and only negligible changes in the peak areas were found. The time gap between derivatization and scanning greatly affected the quantitation when the analysis was performed within 30 min of derivatization. After 30 min of derivatization time, it remained stable for 2 days under laboratory conditions. Quantitation was not significantly affected by changing scanning wavelength ±5 nm.

Table 4
Percent content of steroidal glycoalkaloids in the aerial parts of *S. xanthocarpum* (calculated on plant dry weight basis).

S. no.	Locations	SGAs amount quantified (%, w/w)		
		SN	SM	KN
1	CIMAP, Lucknow	0.080 ± 0.006	0.416 ± 0.052	0.038 ± 0.007
2	BKT, Lucknow	0.077 ± 0.007	0.396 ± 0.054	0.034 ± 0.006
3	Amausi, Lucknow	0.074 ± 0.007	0.389 ± 0.054	0.024 ± 0.006

3.3. Quantitative evaluation of SGAs

As seen in the inset of Fig. 2, the SGAs in *S. xanthocarpum* samples show characteristic VIS-spectra after densitometry of HPTLC plates. The same figure shows that acceptable separation was achieved under the specified conditions. The most important advantages of HPTLC as a screening technique for phytomolecules are its low cost, methodological simplicity and high sample throughput [21,22]. Method validation data show that this procedure is sensitive, selective, and reproducible. The applicability of the developed method was demonstrated. For the quantitative evaluation of SGAs, microwave assisted extraction of aerial part of *S. xanthocarpum* was done in 1% acidified methanol. Plant samples from three locations around the vicinity of CIMAP, Lucknow research farm were collected. The analysis was performed in triplicate and results are summarized in Table 4. The amount of marker was source dependent and varied. Several factors such as age, plant health, and environmental condition affect the content of plant secondary metabolites. These parameters are beyond control in wildly grown plants. The specificity of measurement of SGAs was ensured by corresponding R_f, visible spectra and ESI-MS spectral matching of respective compound in *S. xanthaocarpum* samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.09.025.

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