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# Validated high-performance thin-layer chromatography method for steviol glycosides in *Stevia rebaudiana*

Vikas Jaitak, A.P. Gupta, V.K. Kaul*, P.S. Ahuja

*Natural Plant Products Division, Institute of Himalayan Bioresource Technology (CSIR), Palampur 176061, H.P., India*

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ABSTRACT

A high-performance thin-layer chromatographic (HPTLC) method was developed and validated as per ICH (International Conferences on Harmonization) guidelines for simultaneous quantification of three steviol glycosides, i.e. steviolbioside, stevioside and rebaudioside-A in *Stevia rebaudiana* leaves. For achieving good separation, mobile phase of ethyl acetate–ethanol–water (80:20:12, v/v/v) on pre-coated silica gel 60 $F_{254}$ HPTLC plates were used. The densitometric quantification of steviol glycosides was carried out at $\lambda = 510$ nm in reflection–absorption mode after spraying with acetic anhydride:sulphuric acid:ethanol reagent. The calibration curves were linear in the range of 160–960 ng/spot for steviolbioside, 1–6 μg/spot for stevioside and 0.5–3 μg/spot for rebaudioside-A with good correlation coefficients (0.998–0.999). The method was found to be reproducible for quantitative analysis of steviol glycosides in *S. rebaudiana* leaves collected from ten different locations and will serve as a quality control indicator to monitor the commercial production of stevioside and its allied molecules during different stages of its processing.


## 1. Introduction

*Stevia rebaudiana* (Bertoni) family Asteraceae is a herbaceous perennial plant indigenous to Paraguay and Brazil where its leaves are used by the local Guarani Indians as natural sweetener for hundreds of years. About 150 stevia species are known, among them *S. rebaudiana* is the only one with significant sweet tasting properties [1]. This plant is of world wide importance today because its leaves are used as non-nutritive high potency sweetener primarily in Japan, Korea, China and South America. The consumption of stevia extract in Japan and Korea is about 200 and 115 tons/year, respectively [2]. The water extract of *S. rebaudiana* has beneficial effects on human health, including hypoglycemic [3], hypotensive effects [4] and as source of antioxidant [5]. Its leaves contain nine sweet glycosides. They possess an ent-kaurene diterpene steviol skeleton (ent-13-hydroxy kaur-16-en-19-oic acid). Generally dominant are stevioside (6–10%), rebaudioside-A (2–4%) while other minor glycosides are present up to 1–2% in the leaves [6]. Many analytical methods have earlier been reported in literature for the separation and quantification of diterpene glycosides from the leaves of *S. rebaudiana*. Mizukami et al. [7] quantified stevioside by enzymatic hydrolysis followed by a chemical method, Sakaguchi and Kan [8] quantified total glycosides by gas chromatography after acid hydrolysis. Fullas et al. [9] have separated and identified six glycosides by overpressure thin-layer chromatography. Dacome et al. [10] quantified sugar and steviol derivatives by densitometry. Nikolova-Damyanova et al. [11] compared HPLC and high-performance thin-layer chromatographic (HPTLC) technique for quantification of stevioside and rebaudioside-A and reported only three parameters of validation, i.e. accuracy, reproducibility and resolution, while the present paper covers whole validation part as per ICH (International Conferences on Harmonization) guidelines using three steviol glycosides. Quantification of stevioside and rebaudioside-A has been reported by Tanaka [12]. HPLC methods have also been reported for the quantification of steviol glycosides by using hydrophilic (OH) columns [13] and by size exclusion chromatography [14,15]. Makapugay et al. [16] determined eight steviol glycosides by HPLC and Mauri et al. [17] have used capillary electrophoresis method to quantify steviolbioside and rebaudioside-A. Due to high sample throughput at low operating cost and short analysis time HPTLC is a method of choice for quantification [18,19]. In continuation to our work on quantification of secondary metabolites in medicinal plants by HPTLC [20–24], the present study, aims to develop a validated HPTLC method for quantification of three steviol glycosides, i.e. steviolbioside, stevioside and rebaudioside-A (Fig. 1) in leaves and to investigate their variability when grown at different locations under different environmental conditions. The methodology can be used for

* Corresponding author at: Department of Natural Plant Products, Institute of Himalayan Bioresource Technology, Post Bag # 06, Palampur 176061, H.P., India. Tel.: +91 1894 230426; fax: +91 1894 230433.
*E-mail address:* vkaul2002@yahoo.co.in (V.K. Kaul).

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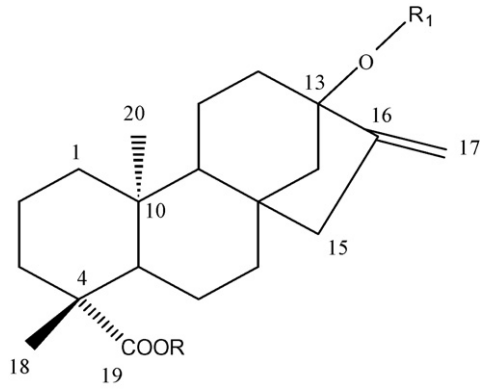


**Fig. 1.** Structure of steviol glycosides. Steviol-derived sweeteners from S. rebaudiana.

| Sr. no. | Diterpene glycosides | R | R1 |
|---|---|---|---|
| 1. | Steviolbioside | H | β-Glc″-β-Glc″ |
| 2. | Stevioside | β-Glc″ ″ | β-Glc″-β-Glc″<br>β-Glc′ –β-Glc″<br>\|<br>β-Glc‴ |
| 3. | Rebaudioside-A | β-Glc″ ″ | |

Glc, glucose.

selection of plants yielding high level of steviosides by screening large number of samples and monitoring steviosides at different stages of plant development before the commercial production of steviosides.

## 2. Experimental

### 2.1. Material

The plant material of *S. rebaudiana* was collected from ten different locations in India during August–September 2006. All samples were collected at flowering stage. The samples were authenticated by our biodiversity department and voucher specimens were deposited in our herbarium section (# IHBT-PLP 12609). The samples were stored at 25 °C. The HPTLC plates (20 cm × 10 cm) (E. Merck, Darmastadt, Germany) were used without any pretreatment. All chemicals and solvents used were of analytical grade (E. Merck, Ltd, Worli, and Mumbai, India). Ethanol was used from Bengal Chemicals, Calcutta.

### 2.2. Isolation of steviol glycosides

Air dried leaf powder (1 kg) of *S. rebaudiana* was extracted with MeOH–$H_2O$ 80:20 (v/v) for 12 h at room temperature. The percolation was repeated three times. The combined percolations were evaporated to dryness and fractionated with hexane, chloroform, ethyl acetate and butanol. All fractions were dried with anhydrous $Na_2SO_4$ and concentrated under reduced pressure at 50 ± 5 °C yielding hexane (30.0 g), chloroform (10.0 g), ethyl acetate (10.5 g) and butanol (150.2 g) extracts, respectively.

Butanol extract (150.2 g) was subjected to column chromatography over silica gel (60–120 mesh) using a gradient elution of $CHCl_3$:MeOH with increasing proportion of MeOH, i.e. 05%, 10%, 20% and 30% in chloroform to give four fractions (i–iv). Fraction (iii) was re-chromatographed over silica gel using gradient elution of 5–20% MeOH in chloroform yielding pure steviolbioside (100 mg) having m.p 188–192 °C. Fraction (iv) was re-chromatographed over silica gel using gradient elution of 5–30% MeOH in chloroform yielding pure stevioside (8 g) having m.p. 196–198 °C and rebaudioside-A (400 mg) having m.p. 242–244 °C. Structures of all these compounds 1–3 were characterized by NMR spectroscopy and also by comparison with the reported literature data [25,26]. These compounds were used as reference standards during the course of present work.

### 2.3. Extraction and analysis of samples

The samples were prepared by extraction of accurately weighing 100 mg of powdered dry leaf plant material with MeOH (10 ml × 3 ml) by sonication at 50 ± 2 °C for 45 min. These were filtered through filter paper and dried under reduced pressure in rota-vapor at 50 ± 5 °C. The extracts were defatted with hexane (5 ml × 3 ml) in a conical flask. The hexane solution was removed and the residue was dried under reduced pressure. From this residue 20 mg/ml concentration was made with MeOH. Five μl of the solution (20 mg/ml) was applied on TLC plate followed by development of bands which were visualized by spraying agent. The analysis was carried out in triplicate from the plant leaf samples collected from ten different locations in India. The stability of sample solutions was 10 days at 4 °C.

### 2.4. HPTLC procedure

A Camag HPTLC system equipped with an automatic TLC sampler ATS4, Automated developing chamber (ADC2), TLC scanner 3, and integrated software win CATS version 1.4.2 was used for the analysis. HPTLC was performed on a pre-coated silica gel HPTLC 60 $F_{254}$ (20 cm × 10 cm) plate of 0.20 mm layer thickness. Chromatography was carried in ADC which was pre-saturated with 20 ml mobile phase ethyl acetate–ethanol–water (80:20:12, v/v/v) for 30 min at room temperature (25 ± 2 °C) and 50 ± 5% relative humidity. The samples and standards were applied on the plate as 6 mm wide bands with an automatic TLC sampler (ATS4) under a flow of $N_2$ gas, 10 mm from the bottom, 10 mm from the side, space between two bands was 6 mm of the plate, and application speed 150 nm/s. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust).

The length of chromatogram run was 9 cm from the base. After that, TLC plates were dried in a current of air with the help of air dryer in a wooden chamber with adequate ventilation. Bands were visualized by spraying with acetic anhydride:sulphuric acid:ethanol (01:01:10, v/v/v) followed by heating on Camag HPTLC plate heater at 110 °C for 2 min. Quantitative evaluation of the plate was performed after 20 min in reflection–absorption mode at 510 nm, slit width 6 mm × 0.3 mm, scanning speed 20 mm/s and data resolution 100 μm/step. A Camag video documentation system in conjunction with the Reprostar 3 was used for imaging and archiving the thin-layer chromatograms. The object was captured by means of a high sensitivity digital camera DXA252, CAMAG. A special digitizing board (frame grabber) assisted in rapid processing via the personal computer system. Image acquisition processing and archiving were controlled via win CATS software.

### 2.5. Calibration curve of three steviol glycosides

The standard solutions were prepared separately (an accurately weighed amount of steviolbioside (4 mg/50 ml), stevioside (10 mg/10 ml) and rebaudioside-A (4 mg/10 ml) in MeOH) and overspotted through software. For these compounds, a calibration curve of standard compounds (1–3) was established over six analyte levels in duplicate by applying 2–20 μl of steviolbio-

**Table 1**
Statistical analysis for the calibration curves of steviol glycosides (no. of data points, $n = 6$)

| Steviol glycosides | $R_F$ | Equation $y = ax + b$ | $r$ | R.S.D. (%) | LOD (ng) | LOQ (ng) |
|---|---|---|---|---|---|---|
| Steviolbioside | 0.55–0.57 | −2434 + 3.885$xX$ | 0.99908 | 2.88 | 120 | 160 |
| Stevioside | 0.30–0.31 | 852.8 + 1662$xX$ | 0.99828 | 3.06 | 180 | 1000 |
| Rebaudioside-A | 0.18–0.20 | 1677 + 3501$xX$ | 0.99903 | 2.07 | 80 | 500 |

For each curve the equation is $y = ax + b$, where $y$ is the peak area, $x$ is the concentration of the analyte, $a$ is the slope, $b$ is the intercept, $r$ the correlation coefficient, R.S.D. the relative standard deviation of peak area.

side, stevioside and rebaudioside-A on HPTLC plate of the working solution. The calibration curves were plotted between amounts of analyte versus average response (peak area).

### 2.6. Method validation

The HPTLC method developed was validated for the following parameters.

#### 2.6.1. Sensitivity

The sensitivity of the method was determined with respect to LOD, LOQ. The standard solutions were spotted in the range from 160 to 960 ng/spot for steviolbioside, 1–6 μg/spot for stevioside and 0.5–3 μg/spot for rebaudioside-A ($n = 6$). The limit of detection and quantification were calculated based on calibration curve and experimentally verified as per the ICH [27] guidelines.

#### 2.6.2. Specificity

Specificity of the method was ascertained by analyzing the standard and sample solutions. The bands of steviolbioside, stevioside and rebaudioside-A in the samples were confirmed by comparing their $R_F$ values and overlaid spectra of the spotted bands with standards.

#### 2.6.3. Accuracy

The accuracy of the analytical procedure was evaluated using the recovery test. This involved the addition of known quantities of the reference standard compounds taken from stock solution to one of the pre-analyzed sample. The known standards were diluted based on the percentage of three glycosides present in the pre-analyzed sample. Three concentration levels were tested (low, middle and high). At each level, samples were prepared in triplicate and analyzed according to previously described procedure. Accuracy was expressed as percentage (observed concentration × 100/theoretical concentration.

#### 2.6.4. Precision

The ICH guideline breaks precision into two parts:

*2.6.4.1. Repeatability.* Repeatability expresses the precision of the method under the same operating conditions over a short interval of time. It is also termed intra day precision.

*2.6.4.2. Intermediate precision.* Intermediate precision express the precision variation within laboratory in different days, different analysts or different equipments and is expressed as %R.S.D.

#### 2.6.5. Robustness

Robustness is a measure of the method which remains unaffected by small variations in the method conditions and is an indication of the method reliability. For robustness different parameters, i.e. developing TLC distance, mobile phase composition, humidity, temperature, chamber dimensions and chamber saturation were studied [28,29].

## 3. Results and discussion

### 3.1. Sensitivity

Under the proposed experimental conditions, the lowest amount of compounds which could be detected were found to be 120, 180, 80 ng/spot and the lowest amount of compound which could be quantified was found to be 160, 1000, 500 ng/spot for steviolbioside, stevioside and rebaudioside-A, respectively. The calibration curve was found to be linear in the range of 160–960 ng/spot for steviolbioside, 1–6 μg/spot for stevioside and 0.5–3 μg/spot for rebaudioside-A with good correlation coefficient (0.998–0.999) in Table 1.

### 3.2. Specificity

The bands of steviolbioside, stevioside and rebaudioside-A in the samples were confirmed by comparing $R_F$ values and overlaid spectra of the spotted bands with standards. The peak purity of steviolbioside, stevioside and rebaudioside-A were confirmed by comparing the spectra at three different levels viz. peak(s) apex and peak end position, i.e. for steviolbioside r(s,m)-0.999455 and r(m,e)-0.999964, stevioside r(s,m)-0.998877 and r(m,e)-0.999340, rebaudioside-A r(s,m)-0.999840 and r(m,e)-0.998680, respectively performed through win CATS software.

### 3.3. Accuracy

The percentage mean recovery values for steviolbioside, stevioside and rebaudioside-A were (93.92%, 95.93%, 98.84%), (96.62%, 96.61%, 95.48%) and (101.12%, 99.42%, 94.94%) for three compounds from lowest to highest level spiked, i.e. 50%, 100%, 150%, respectively (Table 2).

### 3.4. Precision

#### 3.4.1. Repeatability (intra day precision)

For repeatability, six samples of same concentration were prepared as per method and analyzed by proposed method to determine variation arising from method and expressed as %R.S.D. Percentage R.S.D. of method precision was in the range of 1.63–3.24%.

#### 3.4.2. Inter day precision (intermediate precision)

Inter day precision or intermediate precision express within laboratory variations in different days. The %R.S.D. varies from 1.68–3.43%.

### 3.5. Robustness

The standard and test solutions were spotted on HPTLC plates and several slightly different combinations of the three solvents were used to assess robustness. The modified mobile phase of ethyl acetate–ethanol–water (80:20:12), afforded good resolution with

**Table 2**
Recovery analysis

| Compounds | Original (mean ng) | Added (ng) | Detected (mean ng) ($n = 3$) | Mean recovery (%) ($n = 3$) | R.S.D. (%) |
|---|---|---|---|---|---|
| Steviolbioside | | | | | |
| 50% | 170.25 | 85.12 | 239.84 | 93.92 | 0.72 |
| 100% | | 170.25 | 326.66 | 95.93 | 0.33 |
| 150% | | 255.37 | 420.63 | 98.84 | 1.15 |
| Stevioside | | | | | |
| 50% | 2429.73 | 1214.86 | 3513.00 | 96.62 | 1.60 |
| 100% | | 2429.73 | 4681.33 | 96.61 | 1.08 |
| 150% | | 3644.59 | 5786.00 | 95.48 | 0.81 |
| Rebaudioside-A | | | | | |
| 50% | 690.97 | 345.48 | 1047.67 | 101.12 | 3.96 |
| 100% | | 690.97 | 1374.00 | 99.42 | 1.90 |
| 150% | | 1036.45 | 1639.00 | 94.94 | 1.25 |

**Table 3**
Amount of steviol glycosides present in *S. rebaudiana* from different locations in India

| Sr. No. | Location | Steviolbioside | | Stevioside | | Rebaudioside-A | |
|---|---|---|---|---|---|---|---|
| | | Average ($n = 3$) (%) | R.S.D. (%) | Average ($n = 3$) (%) | R.S.D. (%) | Average ($n = 3$) (%) | R.S.D. (%) |
| 1. | Sangrur, India | 0.330 | 2.25 | 5.798 | 2.13 | 1.286 | 0.74 |
| 2. | Dharamsala, India | 0.348 | 5.49 | 4.249 | 2.59 | 2.378 | 4.52 |
| 3. | Baroda, India | 0.434 | 1.85 | 4.483 | 1.66 | 2.062 | 0.88 |
| 4. | Karimnagar, India | 0.477 | 0.72 | 5.266 | 1.84 | 1.370 | 0.98 |
| 5. | Ghimtoli, India | 0.881 | 0.98 | 3.348 | 1.03 | 1.302 | 0.20 |
| 6. | Chhatisgarh, India | 0.340 | 3.42 | 4.846 | 1.68 | 1.380 | 1.69 |
| 7. | Nagpur, India | 1.77 | 0.46 | 4.486 | 0.40 | 1.622 | 0.51 |
| 8. | Ahmedabad, India | 0.399 | 1.42 | 5.384 | 0.23 | 2.062 | 2.85 |
| 9. | Gujrat, India | 0.500 | 1.96 | 6.238 | 2.18 | 2.172 | 3.98 |
| 10. | IHBT (Palampur), India | 0.380 | 0.38 | 6.754 | 1.26 | 1.492 | 0.62 |

$R_F$ 0.55, 0.30, 0.18 for steviolbioside, stevioside and rebaudioside-A, respectively.

### 3.6. Estimation of steviol glycosides in different samples

The proposed HPTLC method has been used for the quantification of three steviol glycosides present in the extracts of *S. rebaudiana* collected from ten different locations in India. For optimization, various combinations of mobile phases were used. Mobile phase of ethyl acetate–ethanol–water (80:20:12, v/v/v) showed highest selectivity for resolution of steviol glycosides. A typical 3-D overlaid chromatogram of steviolbioside, stevioside and rebaudioside-A with sample is shown in Fig. 2. Bands of all the three compounds and samples collected from ten different locations were well separated on HPTLC plate. The calibration curves were linear in the range of 160–960 ng/spot for steviolbioside, 1–6 μg/spot for stevioside and 0.5–3 μg/spot for rebaudioside-A with correlation coefficient 0.998–0.999. The proposed HPTLC method used for the extraction of steviol glycosides in *S. rebaudiana* afforded 94–101% recovery.

The results showed difference in the amounts of three steviol glycosides present in the same species when grown under different geographical locations (Table 3). In general, the percentage of steviolbioside is lowest followed by rebaudioside-A and stevioside. However, in the Nagpur sample percentage of steviolbioside was found to be higher than that of rebaudioside-A.

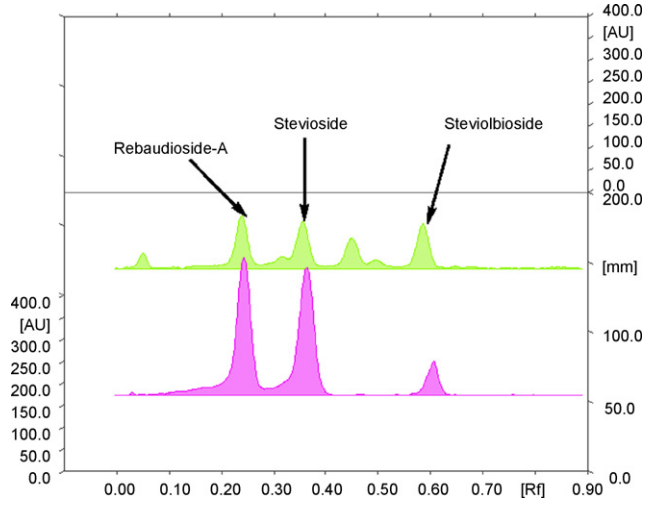


**Fig. 2.** 3-D overlay chromatogram of standard and sample for specificity.

## 4. Conclusion

In the present study an HPTLC method was developed for the quantification of steviolbioside, stevioside and rebaudioside-A in the leaves of *S. rebaudiana* plants traditionally used as substitute for sugar. The method is simple, cost effective, eco-friendly and easily adaptable for simultaneous screening and quantitative determination of steviol glycosides as compared to other analytical techniques. The method was validated and found to be selective, linear, repeatable and accurate within the established ranges. The method will be suitable for the quality control for the production of steviol glycosides in *S. rebaudiana* leaves.

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## References


[1] D.D. Soejarto, C.M. Compodra, P.J. Medon, S.K. Komath, A.D. Kinghorn, Econ. Bot. 37 (1983) 71–79.
[2] A.D. Kinghorn, C.D. Wu, D.D. Soejarto, Stevioside, in: O' Brien Nabors (Ed.), Alternative Sweeteners, third ed., Dekker, New York, 2000, pp. 167–183 (revised and expanded).
[3] P.B. Jeppesen, S. Gregersen, K.K. Alstrupp, K. Hermansenn, Phytomedicine 9 (2002) 9–14.
[4] P. Chan, B. Tomlinson, Y. Chen, J. Liu, M. Hsieh, J. Cheng, Br. J. Clin. Pharmacol. 50 (2000) 215–220.
[5] Y. Xi, T. Yamaguchi, M. Sato, M. Takeuchi, Nippon Shokuhin Kagaku Kaishi 45 (1998) 317–322.
[6] K.C. Phillips, in: T.H. Grenby (Ed.), Developments in Sweeteners, 3, Elsevier, London, 1989, pp. 1–43.
[7] H. Mizukami, K. Shiiba, H. Ohashi, Phytochemistry 21 (1982) 1927–1930.
[8] M. Sakaguchi, M. Kan, Ciencia e Cultura 34 (1982) 235–248.
[9] F. Fullas, J. Kim, C.M. Compadre, A.D. Kinghorn, J. Chromatogr. 464 (1989) 213–219.
[10] A.A. Dacome, C.C. da Silva, C.E.M. da Costa, J.D. Fontana, J. Adelman, S.C. Costa, Process Biochem. 40 (2005) 3587–3594.
[11] B. Nikolova-Damyanova, V. Bankova, S. Popov, Phytochem. anal. 15 (1994) 81–85.
[12] O. Tanaka, Trends Anal. Chem. 1 (1982) 246–248.
[13] Y. Hashimoto, M. Moriyasu, S. Nakamura, S. Ishiguro, M. Komuro, J. Chromatogr. 161 (1978) 403–405.
[14] M.S. Ahmed, R.H. Dobberstein, J. Chromatogr. 236 (1982) 523–526.
[15] M.S. Ahmed, R.H. Dobberstein, J. Chromatogr. 245 (1982) 373–376.
[16] H.C. Makapugay, N.P.O. Nanayakkara, A.D. Kinghorn, J. Chromatogr. 283 (1984) 390–395.
[17] P. Mauri, G. Catalano, C. Gardana, P. Pietta, Electrophoresis 17 (1996) 367–371.
[18] X. Di, K.K.C. Chan, H.W. Leung, C.W. Huie, J. Chromatogr. A 1018 (2003) 85–95.
[19] T. Larsen, J. Axelsen, H.W. Ravn, J. Chromatogr. A 1026 (2004) 301–304.
[20] N.P. Singh, A.P. Gupta, A.K. Sinha, P.S. Ahuja, J. Chromatogr. A 1077 (2005) 202–206.
[21] P. Bhandari, A.P. Gupta, B. Singh, V.K. Kaul, J. Sep. Sci. 28 (2005) 2288–2292.
[22] N. Mishra, A.P. Gupta, B. Singh, V.K. Kaul, P.S. Ahuja, J. Liq. Chromatogr. Rel. Tech. 28 (2005) 1–13.
[23] N. Singh, A.P. Gupta, B. Singh, V.K. Kaul, Chromatographia 63 (2005) 209–213.
[24] P. Bhandari, A.P. Gupta, B. Singh, V.K. Kaul, J. Sep. Sci. 30 (2007) 2092–2096.
[25] K. Yamasaki, H. Kohda, T. Kobayashi, R. Kasai, O. Tanaka, Tetrahedron Lett. 13 (1976) 1005–1008.
[26] H. Kohda, R. Kasai, K. Yamasaki, K. Murakami, O. Tanaka, Phytochemistry 15 (1976) 981–983.
[27] International Conferences on Harmonization (ICH), ICH harmonized tripartite guideline validation of analytical procedures: text and methodology, Q2 ($R_1$), 2005.
[28] K. Ferenczi-Fodor, Z. Vegh, A. Nagy-Turak, B. Renger, M. Zeller, J. AOAC Int. 84 (2001) 1265–1271.
[29] K. Ferenczi-Fodor, Z. Vegh, in: Sz. Nyiredy (Ed.), Planar Chromatography A, Retrospective view from third millennium, Springer Scientific Publisher, Budapest, 2001, pp. 336–352.