

Simultaneous Spectrophotometric Determination of Trigonelline, Diosgenin and Nicotinic Acid in Dosage Forms Prepared from Fenugreek Seed Extract

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Abstract

Mathematical algorithms offer a useful method for quantitative analysis of compounds in multi-component mixtures to overcome the overlapping problems occurred in UV spectrophotometry. The aim of this study is to develop a method for simultaneous determination of bioactive compounds in herbal dosage forms produced from fenugreek extract. A UV- spectrophotometric method based on mathematical algorithm was used to simultaneous determination of trigonelline (TRG), diosgenin (DI), and nicotinic acid (NA). The maximum absorbance (λ_{max}) was determined to be 232.65 nm, 296.23 nm, and 262.60 nm for TRG, DI, and NA, respectively. The calibration curves showed good linearity for all analytes in the concentration range of 1–20 $\mu\text{g/mL}$ ($R^2=0.9995$, 0.9997, 0.9994 for TRG, DI and NA, respectively). The Intra- and inter-day precisions were in the range of 1.1-10.7% and 1.2-8.2%, respectively. The accuracy of the method was 96.0% for TRG, 92.9% for DI, and 104.2% for NA. The limits of detection (LOD) and quantification (LOQ) were found to be 0.91 and 3.06 $\mu\text{g/mL}$ for TRG, 0.99, and 3.30 $\mu\text{g/mL}$ for DI and 0.33 and 1.10 $\mu\text{g/mL}$ for NA. The validated method was applied for determination of the analytes in the tablet, capsule and thin film dosage forms prepared from the fenugreek seed extract. The mean recovery percentages of the analytes were in the range of 90.0-97.4%, 85.6-105.4%, and 90.0-99.0% for tablet, capsule, and film dosage forms, respectively. Generally, the validated method could be a good candidate for routine spectrophotometric determination of the analytes without any necessity for pre-analysis extraction.

Keywords: Fenugreek; Simultaneous determination; Spectrophotometry; Herbal Dosage Forms; Trigonelline; Diosgenin; Nicotinic Acid.

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Introduction

Bioactive compounds in medicinal plants can be considered as initial candidates for new drug discovery (1). Phytotherapy is a definite part of primary health care, so ingredient assay in herbal product seems to be necessary (2). The herbal ingredients have been used in the traditional and modern medicines, nutraceuticals, and dietary supplements. Efficacy and safety of herbal products directly depend on the chemistry of medicinal herb. Phytochemical stability of herbal medicines is provital to their effectiveness (3).

Trigonella foenum-graecum L., commonly known as fenugreek (Shanbalileh in Persian), is an annual plant belongs to Fabaceae family. There is a long history for the usage of the leaves and seeds of fenugreek in Iranian traditional medicine as general tonic and anti-diabetic remedy (4). The seeds of the plant have hypoglycemic, anti-oxidant, anti-inflammatory (5, 6), and anti-nociceptive

effects (7, 8). Fenugreek dermal patch has been effective for management of inguinal hernia post-operative pain (9). Polyphenol compounds (e.g., isovitexin and rhaponticin), alkaloids (e.g., trigonelline; TRG), and sapogenin (e.g., diosgenin; DI) seem to be the major bioactive ingredients in fenugreek seeds (10). In this study, TRG and DI have been selected for simultaneous determination due to known pharmacological effects and significant amount in the plant seeds.

TRG is a pyridine alkaloid (Figure.1) and a vitamin B3 (nicotinic acid; NA) derivative (11) which possess many therapeutic properties such as neuroprotective, anti-diabetic, hypolipidemic, anti-cancer, and anti-mutagenic activity (12). DI is a steroid sapogenin present in various plants e.g. *Costus speciosus*, *Smilax menispermoides*, *Aletris*, *Trigonella*, and many species of *Dioscorea* (13). It has estrogenic activity (14) and also is a precursor of the progesterone synthesis so that it has been previously administered as contraceptive pills (15).

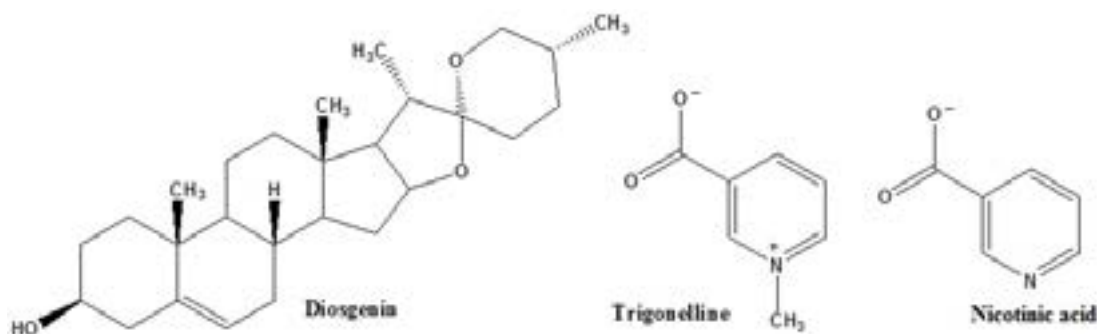


Figure 1. Chemical structures of trigonelline, diosgenin and nicotinic acid

Herbal pharmacologic biomarkers were frequently used for quality control of medicinal plants.

Development of validated and reliable analytical methods for identity and purity of herbal ingredient has not been progressed with the rapidly increasing number of ingredients in the market. Lack of validated analytical methodologies in tune with complex ingredient chemistry suggests the much needed research in this area. Among the various experimental techniques, chromatographic methods are commonly used for quantitative and quantitative analysis which is a part of medicinal herbs quality control. In general,

these methods are difficult to optimize and they are time-consuming and costly; therefore, the present study aims to develop a simple, sensitive, accurate, time-saving, and inexpensive UV-spectrophotometric method for simultaneous determination of TRG, DI, and NA in various herbal dosage forms.

Experimental

Chemical and Instruments

Standard TRG and DI were purchased from Sigma Company (USA); and standard NA was prepared from Merck Company (Germany). All used solvents were of high

purity and analytical grade. A Lambda™ 25 UV-Vis spectrophotometer (PerkinElmer, Germany) was used to measure the absorbance of standard and sample solutions.

Calibration curves

A primary standard solution with a concentration of 100 µg mL⁻¹ of each analyte was prepared; TRG and NA were dissolved in deionized water, while DI was dissolved in 2 mL methanol and then was made to the volume with deionized water. For determination of maximum wavelengths (λ_{\max}) of standards, the solutions were separately scanned in the range of 200 - 400 nm against blank. Different aliquots were then taken from the stock solution and diluted with deionized water to prepare a serial concentration in the range of 1–20 µg mL⁻¹. The calibration curves were constructed by plotting absorbance vs concentration.

Method validation

Accuracy and precision

A mixed aqueous solution of analytes (10 µg mL⁻¹ of each analyte) was scanned in the range of 200-400 nm using UV spectrophotometer. The absorbance of each individual compound and a mixture of three analytes were measured at three determined λ_{\max} (232.65 nm, 296.23 nm and 262.60 nm); then, the concentration of all analytes were calculated by using the following equations.

$$A_{mi\lambda 1} = \varepsilon_{TRG\lambda 1} C_{TRG} + \varepsilon_{DI\lambda 1} C_{DI} + \varepsilon_{NA\lambda 1} C_{NA}$$

$$A_{mi\lambda 2} = \varepsilon_{TRG\lambda 2} C_{TRG} + \varepsilon_{DI\lambda 2} C_{DI} + \varepsilon_{NA\lambda 2} C_{NA}$$

$$A_{mi\lambda 3} = \varepsilon_{TRG\lambda 3} C_{TRG} + \varepsilon_{DI\lambda 3} C_{DI} + \varepsilon_{NA\lambda 3} C_{NA}$$

Where A_{mi} is the absorbance of the mixture at three wavelength of $\lambda_{1(232.65)}$, $\lambda_{2(296.23)}$, $\lambda_{3(262.60)}$.

Intra- and inter-day precision and accuracy were investigated by analysis of six mixed solution of analytes in concentrations of 1, 2, 4, 8, 10, and 20 µg/mL (three independent replicates). Precision levels were expressed as the relative standard deviation (RSD%) (n = 18).

Recovery Studies

Recovery percentage of the method was calculated based on the following formula

with measuring the concentration of three analytes in spiked and non-spiked samples:

$$\text{Recovery} = (X_s - X_{ns}) / X_{ad} \times 100$$

Where X_s is the mean result of spiked samples, X_{ns} is the mean result of non-spiked samples and X_{ad} is the amount of added analyte (10 µg/mL). The recovery percentage of each analyte for dosage forms was carried out in three replicates (totally n = 9) and the results were reported as Mean ± SD.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were estimated as $3.3 \times SD_b / \text{Slope}$ and $10 \times SD_b / \text{Slope}$ respectively, where SD_b is the standard deviation of the blank samples (n = 25).

Preparation of dosage forms

The validated method was applied for simultaneous determination of three analytes in three herbal dosage forms including the tablet, capsule and bucoadhesive thin film containing *Trigonella foenum-graecum* seed extract. The dosage forms were prepared based on the thesis of Pharm.D students of Kerman University of Medical Sciences (16-18).

Estimation of the analytes in the real samples

Ten tablets and capsules content were separately weighed, powdered, and extracted with 80% ethanol by sonication method for 30 min. The solutions were filtered through whatman filter paper, and the filtrates were diluted to 100 µg/mL and the samples were analyzed using proposed method. Ten thin films were extracted with warm water by sonication method for 30 min. The extract was centrifuged at 5000 rpm for 15 min. The supernatant was diluted to prepare a 100 µg/mL concentration and analyzed by proposed method.

Result and Discussion

Many analytical techniques including UV-spectrophotometry (19, 20), GC-MS (21, 22), HPLC (23-25), and HPTLC (26, 27) have been developed and reported for individual determination of TRG, DI, and NA; but simultaneous analysis of these phytochemicals

is of high importance for evaluating the quality of herbs and herbal products.

UV-spectrophotometry as a simple analytical method usually requires specific low-overlapped spectra of chemicals for simultaneous analysis of multi-component mixture (28). Spectrophotometry based on mathematical algorithm helps to overcome the spectral overlapping problems (29). In the current work, the λ_{\max} of TRG, DI, and NA was determined to be 232.65 nm, 296.23 nm, and 262.60 nm, respectively (Figure.2a). TRG and NA showed no absorbance at 296.23 nm. Hence, this wavelength is selective for DI determination

by zero-order spectrophotometry. However, there were no other wavelengths at which TRG and NA can be determined selectively due to high level of overlapping among the spectra. The same problem was found for the second derivative spectra of these compounds. A third derivative spectrum was provided for simultaneous determination of TRG at presence of DI at 253.8. But due to overlapping of NA spectrum with the spectra of the other two, a mathematical algorithm system of three variables was used to calculate the analytes concentration in a multi-component mixture (30) (Figure. 2b).

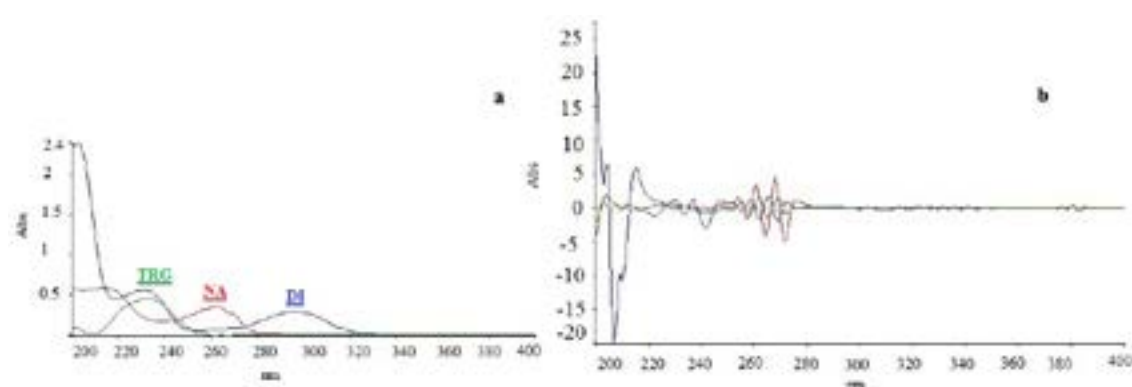


Figure 2. Zero-order (a) and Third-derivative (b) UV spectra of standard TRG, DI and NA at concentration of 10 $\mu\text{g/mL}$.

Calibration curve

The method was linear in the concentration range of 1-20 $\mu\text{g/mL}$ of the analytes with an acceptable regression coefficient (R^2 equivalent to 0.9995, 0.9997, and 0.9994 for TRG, DI, and NA respectively). The values of standard deviation for slope and intercept are very low and near to zero which indicates high precision of the method. The results are summarized in Table 1.

Accuracy and precision

The mean recoveries and CVs are illustrated in Tables 1 and 2. The results showed an acceptable accuracy and precision over the entire examined concentration range. The validity of the proposed method was more assessed by recovery study *via* standard addition test as explained in the experimental section. The mean recovery percentages of the analytes were in the range of 90.0-97.4%,

85.6-105.4%, and 90.0-99.0% for tablet, capsule, and film dosage forms respectively. Intra-day precision (as repeatability) and inter-day (as intermediate precision) were in the range of 2.3-10.7%, 1.1-7.9%, and 4.6-8.2% for TRG, DI, and NA respectively. The results have been shown in Table 2.

LOD and LOQ

LOD and LOQ of the analytes were determined as 0.91 and 3.06 $\mu\text{g/mL}$ for TRG, 0.99, and 3.30 $\mu\text{g/mL}$ for DI, and 0.33 and 1.10 $\mu\text{g/mL}$ for NA (Table 1).

Determination of the analytes in the herbal dosage forms

The results of determination of the analytes in herbal tablet, capsule, and thin film are shown in Table 3. The concentration of TRG, DI, and NA in different formulations respectively ranged 6.00-7.31 $\mu\text{g/mL}$, 1.60-3.60, and 2.00-6.90 $\mu\text{g/mL}$.

Table 1. Linearity, accuracy, LOD and LOQ parameters of the spectrophotometry method.

| Parameters | TRG | DI | NA |
|---|---------------------|---------------------|----------------------|
| Slope \pm SD | 0.0430 \pm 0.0001 | 0.0285 \pm 0.0003 | 0.0045 \pm 0.0003 |
| Confidence limits of slope (95%) | 0.0427;0.0426 | 0.0285;0.0285 | 0.0045;0.0044 |
| Intercept \pm SD | 0.020 \pm 0.0002 | 0.0003 \pm 0.0002 | -0.0127 \pm 0.0001 |
| Confidence limits of intercept (95%) | 0.0201;0.0199 | -0.0002;-0.0004 | -0.0143;-0.0146 |
| Correlation coefficient (R ²) | 0.9995 | 0.9997 | 0.9994 |
| Accuracy% | 96.0 | 92.9 | 104.2 |
| LOD (μ g/mL) | 0.91 | 0.99 | 0.33 |
| LOQ (μ g/mL) | 3.06 | 3.30 | 1.10 |

Table 2. Precision of the spectrophotometry method at two intra- and inter-day levels for each analyte (1- 20 μ g/mL) at the presence of the other two analytes (4 μ g/mL).

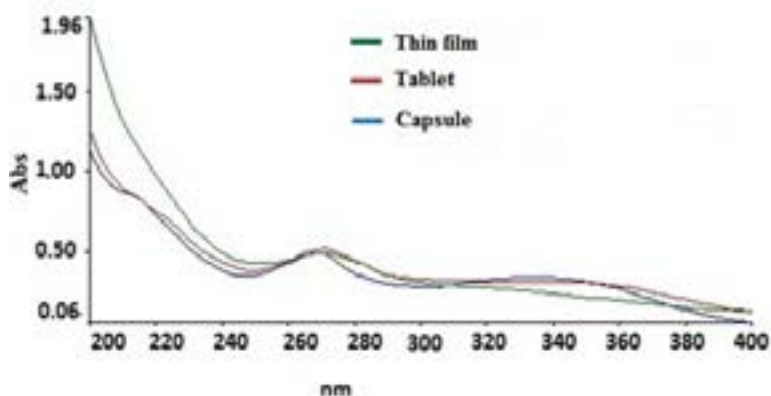
| Conc. (μ g/mL) | Intra-day (RSD%) | | | Inter-day (RSD%) | | |
|---------------------|------------------|-----|-----|------------------|-----|-----|
| | TRG | DI | NA | TRG | DI | NA |
| 1 | 10.7 | 3.6 | 5.8 | 7.5 | 7.9 | 5.5 |
| 2 | 7.7 | 6.1 | 7.9 | 6.9 | 6.8 | 8.0 |
| 4 | 2.6 | 2.5 | 6.7 | 1.2 | 2.4 | 4.6 |
| 8 | 2.9 | 1.1 | 8.2 | 3.1 | 2.8 | 8.2 |
| 10 | 2.6 | 1.2 | 6.8 | 3.8 | 1.8 | 6.7 |
| 20 | 2.3 | 2.4 | 6.6 | 2.3 | 2.5 | 5.3 |

Table 3. Determination of TRG, DI, NA in herbal dosage forms (100 μ g/mL) (n=9)

| Dosage Forms | TRG | | DI | | NA | |
|--------------|-----------------------------|-----------------|-----------------------------|-----------------|-----------------------------|-----------------|
| | Mean \pm SD (μ g/mL) | Recovery (%) | Mean \pm SD (μ g/mL) | Recovery (%) | Mean \pm SD (μ g/mL) | Recovery (%) |
| Tablet | 6.10 \pm 1.10 | 97.4 \pm 2.6 | 3.60 \pm 0.39 | 101.5 \pm 2.1 | 2.30 \pm 1.46 | 90.0 \pm 1.1 |
| Capsule | 7.31 \pm 1.37 | 101.2 \pm 3.3 | 3.30 \pm 0.09 | 85.6 \pm 3.9 | 6.90 \pm 0.25 | 105.4 \pm 2.5 |
| Thin film | 6.00 \pm 1.42 | 99.0 \pm 1.7 | 1.60 \pm 0.25 | 90.0 \pm 2.3 | 2.00 \pm 1.48 | 91.7 \pm 1.0 |

The log P of DI is about 6.34 and is very lipophilic compound and the low amount of DI in the thin film dosage form must be due to

its low solubility in aqueous medium utilized in exaction process to prepare the thin film sample (Figure 3).

**Figure 3.** UV-absorption spectra of 100 μ g/mL of the table, capsule and thin film dosage forms of fenugreek extract.

Laila *et al.* in 2014 suggested an HPTLC method for the simultaneous determination of quercetin and DI in fenugreek extract (31). A sensitive and reproducible TLC method has been reported by Trivedi *et al.* 2007 for quantitative analysis of DI. They reported DI value in the range of 0.53% (w/w) in the fenugreek seed powders, 0.087% (w/w) in the fenugreek leaf powder, 0.015 and 1.27% (w/w) in the fenugreek stem powder and extract, respectively, and 0.586% (w/w) in a formulation containing fenugreek seed powder (32). In current study, DI was determined in the range of 1.6% (w/w) in the thin film, an aqueous extract of fenugreek seeds, and 3.3-3.6 % (w/w) in the tablet and capsule which contain containing ethanolic extract of plant seeds. Chopra *et al.*, 2007 applied a spectrophotometric and HPTLC method for the determination of TRG in pharmaceutical formulations (vaginal tablets and bioadhesive vaginal gels). As this report indicated, the sample recoveries from all formulations were accorded with their respective label claims (19). A rapid liquid chromatography–mass spectrometry method has been reported for the simultaneous quantification of TRG and NA in coffee by Perrone *et al.*, 2008 (33). Content of TRG was found 1.03% (w/w) in the green coffee samples.

Conclusion

Overall, a precise, accurate, and linear UV spectrophotometry method based on mathematical algorithms was developed for simultaneous determination of trigonelline, diosgenin, and nicotinic acid in herbal dosage forms prepared from fenugreek seed extract. The method was simple as well as low-cost which could be a good candidate for routine UV spectrophotometry determination of these analytes without any necessity for intensive pre-analysis extraction of the samples.

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References

- (1) Katiyar C, Gupta A, Kanjilal S and Katiyar S. Drug discovery from plant sources: An integrated approach. *AYU* (2012) 33: 10-9.
- (2) Mehta P, Shah R, Lohidasan S and Mahadik K. Pharmacokinetic profile of phytoconstituent (s) isolated from medicinal plants—A comprehensive review. *eJTCM*. (2015) 5: 207-27.
- (3) Govindaraghavan S and Sucher NJ. Quality assessment of medicinal herbs and their extracts: Criteria and prerequisites for consistent safety and efficacy of herbal medicines. *Epilepsy Behav.* (2015) 52: 363-71.
- (4) Rashidi AA, Mirhashemi SM, Taghizadeh M and Sarkhail P. Iranian medicinal plants for diabetes mellitus: a systematic review. *Pak. J. Biol. Sci.* (2013) 16: 401-11.
- (5) Shariffar F, Khazaeli P and Alli N. *In-vivo* evaluation of anti-inflammatory activity of topical preparations from Fenugreek (*Trigonella foenum-graecum* L.) seeds in a cream base. *IJPS*. (2009) 5: 157-62.
- (6) Pournamdari M, Mandegary A, Shariffar F, Zarei G, Zareshahi R, Asadi A and Mehdipour M. Anti-Inflammatory Subfractions Separated from Acidified Chloroform Fraction of Fenugreek Seeds (*Trigonella foenum-graecum* L.). *J. Diet. Suppl.* (2018) 15: 98-107.
- (7) Marzouk M, Soliman A and Omar T. Hypoglycemic and antioxidative effects of fenugreek and termis seeds powder in streptozotocin-diabetic rats. *Eur. Rev. Med. Pharmacol. Sci.* (2013) 17: 559-65.
- (8) Mandegary A, Pournamdari M, Shariffar F, Pournourmohammadi S, Fardiar R and Shooli S. Alkaloid and flavonoid rich fractions of fenugreek seeds (*Trigonella foenum-graecum* L.) with antinociceptive and anti-inflammatory effects. *Food Chem. Toxicol.* (2012) 50: 2503-7.
- (9) Ansari M, Sadeghi P, Mahdavi H, Fattahi-Dolatabadi M, Mohamadi N, Asadi A and Shariffar F. Fenugreek dermal patch, a new natural topical antinociceptive medication for relieving the postherniotomy pain, a double-blind placebo controlled trial. *J. Complement Integr. Med.* (2019) 16: 1-8.
- (10) Wani SA and Kumar P. Fenugreek: A review on its nutraceutical properties and utilization in various food products. *JSSAS* (2018) 17: 97-106.
- (11) Zeiger E and Tice R. Trigonelline; Review of Toxicological Literature. Research Triangle Park, North Carolina: National Institute of Environmental Health Sciences and Integrated Laboratory Systems (1997) 1-27.

- (12) Mohamadi N, Shariffar F, Pournamdari M and Ansari M. A Review on Biosynthesis, Analytical Techniques, and Pharmacological Activities of Trigonelline as a Plant Alkaloid. *J. Diet Suppl.* (2018) 15: 207-22.
- (13) Patel K, Gadewar M, Tahilyani V and Patel DK. A review on pharmacological and analytical aspects of diosmetin: a concise report. *Chin. J. Integr Med.* (2013) 19: 792-800.
- (14) Liu M-J, Wang Z, Ju Y, Wong RN-S and Wu Q-Y. Diosgenin induces cell cycle arrest and apoptosis in human leukemia K562 cells with the disruption of Ca²⁺ homeostasis. *Cancer Chemother. Pharmacol.* (2005) 55: 79-90.
- (15) Jiming X. Application for Synthesis of Steroid Drugs from Diosgenin. *J. Ankaung Uni.* (2014) 3: 002.
- (16) Malekoladi A. Formulation & physico-chemical characterization of fenugreek aqueous extract oral thin film: School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran (2017) 21-3.
- (17) Gilani A. Formulation & physico-chemical characterization of anti nociceptive & anti-inflammatory of oral capsule of standardized extract of fenugreek seeds (*Trigonella foenum-graecum* L.): School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran (2016) 25-7.
- (18) Rahbari Moghadam B. Formulation and physicochemical characterization of aniniceptive and anti inflammatory of oral sustained release tablet of standardized extract of fenugreek seeds (*Trigonella foenum-graecum* L.): School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran (2017) 20-2.
- (19) Chopra S, Motwani SK, Ahmad FJ and Khar RK. Simple, sensitive, selective and validated spectrophotometric methods for the estimation of a biomarker trigonelline from polyherbal gels. *Spectrochimica Acta Part A: Spectrochim Acta A Mol Biomol. Spectrosc.* (2007) 68: 516-522.
- (20) Wang J, Yang K-d and Chen J. Determination of diosgenin by spectrophotometry. *Chin. J. Anal. Lab.* (2004) 23: 73-5.
- (21) Chang Y-r. GC-MS determination of diosgenin in rats plasma. *Chin. J. Pharm. Anal.* (2006) 26: 177-180.
- (22) Mathur M and Kamal R. Studies on trigonelline from *Moringa oleifera* and its *in vitro* regulation by feeding precursor in cell cultures. *Rev. Bras Farmacogn.* (2012) 22: 994-1001.
- (23) Oncina R, Botia J, Del Río J and Ortuño A. Bioproduction of diosgenin in callus cultures of *Trigonella foenum-graecum* L. *Food Chem.* (2000) 70: 489-492.
- (24) Yi T, Fan L-L, Chen H-L, Zhu G-Y, Suen H-M, Tang Y-N, Zhu L, Chu C, Zhao Z-Z and Chen H-B. Comparative analysis of diosgenin in *Dioscorea* species and related medicinal plants by UPLC-DAD-MS. *BMC Biochem.* (2014) 15: 1.
- (25) Arai K, Terashima H, Aizawa S-i, Taga A, Yamamoto A, Tsutsumiuchi K and Kodama S. Simultaneous Determination of Trigonelline, Caffeine, Chlorogenic Acid and Their Related Compounds in Instant Coffee Samples by HPLC Using an Acidic Mobile Phase Containing Octanesulfonate. *Anal. Sci.* (2014) 31: 831-35.
- (26) Pazhanichamy K, Bhuvanewari K, Kunthavai B, Eevera T and Rajendran K. Isolation, characterization and quantification of diosgenin from *Costus igneus*. *Jpc-J Planar Chromat.* (2012) 25: 566-70.
- (27) Kumbhalkar B, Rajopadhye A and Upadhye A. Development and validation of HPTLC-densitometric method for estimation of trigonelline in leaves of *Abrus precatorius* L. and its formulation. *Acta Chromatogr.* (2012) 24: 511-18.
- (28) Hasanjani HRA and Sohrabi MR. Artificial Neural Networks (ANN) for the simultaneous spectrophotometric determination of fluoxetine and sertraline in pharmaceutical formulations and biological fluid. *Iran. J. Pharm. Res.* (2017) 16: 478-89.
- (29) Souri E, Jalalizadeh H, Farsam H, Rezwani H and Amanlou M. Simultaneous determination of anthocyanoside and Beta-carotene by third-derivative ultraviolet Spectrophotometry. *Daru* (2005) 13: 11-6.
- (30) Chaydhary J, Jain A and Saini V. Simultaneous estimation of multicomponents formulations by UV-Visible spectroscopy: an overview. *IRJP* (2011) 2: 81-3.
- (31) Laila O, Murtaza I, Abdin M, Ahmad S, Ganai NA and Jehangir M. Development and validation of HPTLC method for simultaneous estimation of diosgenin and quercetin in fenugreek seeds (*Trigonella foenum-graceum*). *ISRN Chromatogr.* (2014) 2014: 1-8.
- (32) Trivedi PD, Pundarikakshudu K, Rathnam S and Shah KS. A validated quantitative thin-layer chromatographic method for estimation of diosgenin in various plant samples, extract, and market formulation. *J. AOAC Int.* (2007) 90: 358-63.
- (33) Perrone D, Donangelo CM and Farah A. Fast simultaneous analysis of caffeine, trigonelline, nicotinic acid and sucrose in coffee by liquid chromatography-mass spectrometry. *Food Chem.* (2008) 110: 1030-35.