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Method development for simultaneous detection of ferulic acid and vanillin using high-performance thin layer chromatography

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Abstract

Background: A simple, accurate, and reliable high-performance thin-layer chromatography (HPTLC) method was developed for separation and detection of ferulic acid and vanillin.

Methods: Separation of ferulic acid and vanillin was carried out on 20 × 10 cm thin layer chromatography (TLC) plates using mobile phase containing toluene/1, 4-dioxan/acetic acid in the ratio 9:2.5:0.4 (*v/v*). The FA and vanillin were scanned at 320 and 312 nm, respectively. Method was validated for linearity, accuracy, precision, robustness, limit of detection, limit of quantification, and specificity.

Results: Retention factor (Rf) obtained for ferulic acid and vanillin was 0.48 and 0.56, respectively. The correlation coefficients, 0.9975 and 0.9991 with an average recovery of 98.77% and 98.45% obtained for ferulic acid and vanillin respectively by this method were satisfactory.

Conclusion: The optimized method was found to be efficient, precise, accurate, specific, and economic. Therefore, the method would be useful for both qualitative and quantitative routine analysis in pharmaceutical, food industry, and research laboratories.

Keywords: Ferulic acid; Vanillin; High-performance thin layer chromatography (HPTLC); Simultaneous detection

Background

Aromatic compounds are present in natural sources with substantial combinations which are directly responsible for its odor and sensitivity. They can be categorized as volatile organic compounds like aldehydes, alcohols, ketones, esters, lactones, and terpenes (Raisi et al. 2008). They are known to be precursors for the production of numerous products employed in the food, pharmaceutical, and chemical industries and are present at very low concentrations in natural sources. According to US and European legislations, synthetic flavor production is not considered as natural. Alternatively, biotechnology offers microorganisms as production hosts for different types of aromatic compounds in industrial fermentative processes (Lomascolo et al. 1999). The most intensively studied biotransformation using microorganisms is the bio conversion of ferulic acid (FA) to produce natural vanillin (Priefert et al. 2001).

FA is an important precursor of vanillin that is available in abundance in plant cell walls linked to polysaccharide by an ester or ether bonds (Xu et al. 2005). FA is a potent antioxidant because it effectively scavenges free radicals and even possesses antimicrobial properties by preventing the lipid peroxidation caused by microbes (Graf 1992). Moreover, it is used in cosmetics for the photo protection of skin and in protection against various inflammatory diseases.

Vanillin is widely used in food industry as a flavoring agent but also has applications in some fragrances and pharmaceuticals (Priefert et al. 2001). It is also known to possess anti-metastatic, anticancer (Ho et al. 2009) and anti-inflammatory (Wu et al. 2009) activities. It exhibits antimicrobial properties due to its phenolic nature and hence used to develop antimicrobial films used in packaging of bakery products (Rakchoy et al. 2009).

FA and vanillin are generally determined by various chromatographic methods. Different approaches such as UV spectrophotometry (Mabry et al. 1970; Macheix et al. 1990)

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gas chromatography (GC), capillary electrophoresis (CE), high-pressure liquid chromatography (HPLC), thin layer chromatography (TLC), and high-performance thin layer chromatography (HPTLC) are some of the frequently used methods for the detection, qualitative analysis, and quantification (Sharma et al. 2007).

Spectrophotometric methods are used for identification of phenolic acids and are generally carried between a range of 220 to 320 nm (Mabry et al. 1970; Macheix et al. 1990); however, methods such as the Folin Ciocalteu spectrophotometric method results in nonspecific detection of the phenolic compounds and the interference of components such as ascorbic acid in food samples, that behave as reducing agents. Absorption of phenolic compounds is affected by pH, solvents used in the method and the interference of proteins and amino acids (Constantine et al. 2007).

Volatile compounds are directly analyzed by gas chromatography, a technique of unsurpassed separation capacity (Sostaric et al. 2000). GC is a major chromatographic technique employed for the analysis of essential phenolic acids in plants. It deals with high sensitivity and selectivity (Chiou et al. 2007) but requires derivatization step of hydroxyl groups in phenolic compounds. They are modified by various reagents to make more volatile compounds by a process such as methylation, conversion into trimethylsilyl (TMS) derivatives. However, problems such as poor separation and low stability after derivatization state are some of the shortcomings of this method. CE is too employed for analysis of phenolic compounds (Huck et al. 2005; Butehorn et al. 1996). Mostly, the method falls in the field of natural product research, including the analysis of plants, vegetables, herbs, and other plant- or fruit-derived products. It results in oxidation of phenolic compounds by dissolved oxygen and increase in migration time of flavonoids due to the increase in buffer concentrations (Constantine et al. 2007).

TLC methods have the ability to screen phenolic compounds easily (Tilay et al. 2008). The results obtained by TLC method are generally quantified using more multifaceted techniques like HPTLC (Mabinya et al. 2006). However detection of vanillin by spraying with 2, 4-dinitrophenylhydrazine (2, 4-DNPH) is not significant as the peaks are not detected properly. Currently, the main qualitative and quantitative techniques for phenolic compound detection are HPLC (Rao et al. 1999; Zheng et al. 2007). The European pharmacopoeia suggests the development of such analytical method which demands the adequate amount of reagents, solvents, and material (European Pharmacopoeia 2008). HPTLC allows for the simultaneous analysis of large sample size using small quantities of solvents, thus reducing time and cost of the analysis. The sensitivity for phenolic compounds performed by HPTLC is more as compared to HPLC (Prinjaporn et al. 2013). Mobile phase having pH 8 and above can be employed. Sample with turbidity and different combinations of solvent can be directly applied. It facilitates automated application and repeated scanning of the chromatogram with the same or different parameters (Bakshi et al. 2002). Therefore, this technique should be taken into consideration as an alternative to HPLC.

HPTLC is a sophisticated instrumental technique which allows a fast and inexpensive method for analysis. Special advantage of HPTLC includes high sample throughput and low cost per analysis. HPTLC offers a great variety of stationary phases with unique selectivity for mixture components and their separation simultaneously. Processing of standards and samples identically on the same plate directs to better accuracy and precision of method for assessment. HPTLC development is extensive as the mobile phases are fully evaporated before the detection step thus preventing solvent interference in analysis. It minimizes exposure risks and significantly discarding toxic organic effluent problems were reduced thereby reducing possibilities of environment pollution. In response to this, HPTLC-based methods could be considered as a good alternative as they are being explored as an important tool in routine analysis. The HPTLC-developed method is actively used in application of qualitative and quantitative analyses of a wide range of compounds, such as herbal and botanical dietary supplements and nutraceuticals. It helps in identifying compounds present in a given substance; to check starting raw materials (plant extracts, extracts of animal origin, fermentation mixtures) identification of drugs and their metabolites in biological media such as urine, plasma, or gastric fluid (pharmacological, toxicological, pharmacokinetic) (Renger 1993; 1998).

The aim of the present study is to develop rapid, economic, selective HPTLC method for analysis and simultaneous determination of FA and vanillin with proper peak separation and hence can be used for routine high-throughput detection and determination of phenolic compounds. It is a one-step biotransformation process using culture *Pycnoporus cinnabarinus* which undergoes propenoic acid chain degradation of FA to vanillin (Tilay et al. 2008).

Methods

Chemicals/Reagents

Standard FA and vanillin (99% purity), maltose, diammonium tartarate, yeast extract, malt extract, potassium dihydrogen orthophosphate, magnesium sulfate, calcium chloride, thiamine hydrochloride, sodium hydroxide, and agar powder were procured from Hi-Media Laboratory (Mumbai, India). HPLC grade toluene, 1, 4-dioxan, acetic acid, ethyl acetate, and methanol were procured from Hi-Media (Mumbai, India).

Instrumentation

CAMAG TLC system consist of a CAMAG Linomat V (Muttenz, Switzerland) sample applicator and CAMAG

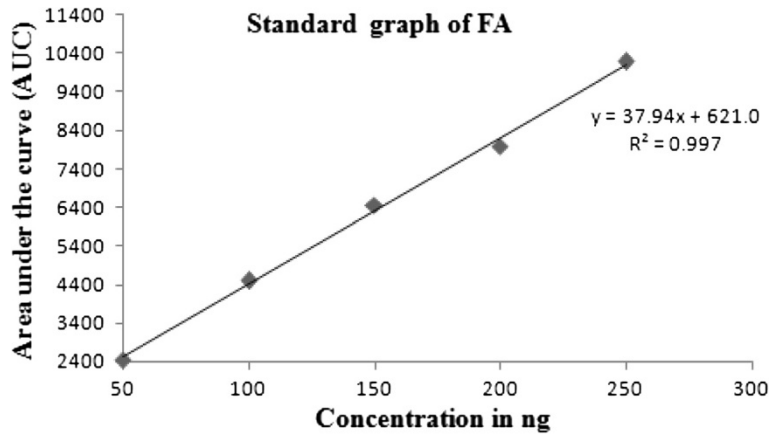

Figure 1 Standard graph of FA by HPTLC method.

TLC Scanner 3 controlled by WinCats software (1.4.3.6336); CAMAG glass twin-trough chambers ($20 \times 10 \times 4$ cm^3); 100-μl Hamilton syringe; Silica gel plates ($G60F_{254}$, 20×10 cm) were procured from E-Merck Pvt. Ltd., Mumbai, India. For extraction of vanillin, Buchi evaporator system was used consisting of Buchi evaporator R-124, Buchi water bath B-480, and Buchi vacuum controller B-721.

Preparation of stock solution

Standard stock solutions were prepared by dissolving 25 mg of vanillin and 25 mg of FA in 25 ml of methanol. Working standard solution was diluted (1:200) from stock solution of FA and vanillin to attain concentration of 5 μg/ml.

Microorganism

Pycnoporus cinnabarinus NCIM 1181 was procured from National Centre for Industrial Microorganism (NCIM), National Chemical Laboratory (NCL), Pune, India. Culture was maintained on potato dextrose agar slants at 4°C.

Media for vanillin production

A production media consisting of maltose 20 g/l, diammonium tartarate 1.8415 g/l, KH_2PO_4 0.2 g/l, $CaCl_2$ $2H_2O$ 0.0132 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, yeast extract 0.5 g/l, and thiamine hydrochloride 2.5 mg/l was adjusted to pH 7 and inoculated using mycelium fragments of *P. cinnabarinus* (Gross et al. 1993). This was further incubated on an incubator shaker (150 rpm) at 37°C for 6 days. After 3 days of growth, sterile solution of FA (0.03 g/100 ml) was added to the media prepared by dissolving FA in 0.1 N NaOH in the form of sodium ferulate.

Extraction of FA and vanillin from the culture media

The broth obtained after fermentation was filtered and the filtrate was acidified to obtain a pH of 1 to 2.80 ml of the above acidified solution was extracted thrice with equal volume of ethyl acetate. The extracts and the residue were redissolved in 50% (*v*/*v*), respectively. The organic phase containing FA and vanillin was concentrated (up to 2 to 3 ml) using a rotary vacuum

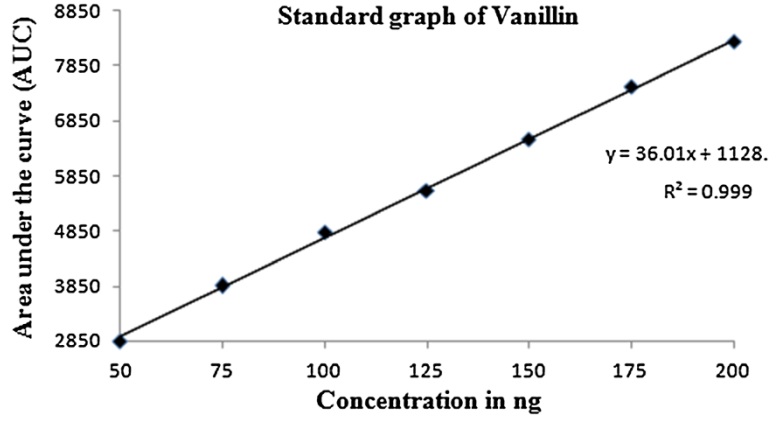

Figure 2 Standard graph of vanillin by HPTLC method.

Table 1 Intra- and inter-day precision ($n = 5$)

Compound	Std amount (ng/band)	Intra-day precision		Inter-day precision	
		SD[a]	%RSD[b]	SD[a]	%RSD[b]
Ferulic acid	100	4.52	0.021	1.64	0.053
	150	2.93	0.015	4.66	0.091
	200	4.51	0.001	4.57	0.050
Vanillin	100	2.09	0.044	2.21	0.040
	150	2.97	0.048	2.77	0.042
	200	1.93	0.022	2.02	0.023

SD, standard deviation ($n = 5$); RSD, percent relative standard deviation ($n = 5$).

evaporator with conditions (55°C, 150 rpm, <80 mbar) followed by reconstitution in 2 ml of methanol 50% (*v*/*v*) (Tilay et al. 2010). This solution was used for quantification of FA and vanillin by HPTLC.

Instrumentation and chromatographic parameters

HPTLC was executed using silica gel $60F_{254}$ plates. A 10 μl of standard working solutions of FA and vanillin were applied to the plates of size 20 × 10 cm with a 5-mm band length. Ascending chromatography silica gel plate development traveled to a distance of 85 mm at temperature of 25°C with toluene/1, 4-dioxan/acetic acid (9:2.5:0.4; *v*/*v*) as mobile phase. After development, the plates were dried and chromatograms were recorded at 320 and 312 nm, respectively using CAMAG TLC Scanner 3. Quantitative evaluation was performed with WinCats software using deuterium lamp having slit width of 5 × 0.45 mm and an application rate of 150 nL/s.

Validation of method

Validation was performed in terms of linearity, specificity, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, and system suitability by ICH guidelines (CPMP/ICH/381/95).

Linearity

Standard solution of FA (50 to 250 ng/band) and vanillin (50 to 200 ng/band) with varying volumes were applied on TLC plates. A plot of peak area against concentrations and its respective standard deviation (SD) and coefficient of correlation was calculated for both the compounds.

Precision

Precision specifies random errors. Results were expressed in percent relative standard deviation (%RSD < 2). Standard solution of FA (100, 150, and 200 ng/band) and of vanillin (100, 150, and 200 ng/band) were applied. Intra-day precision was evaluated by applying each concentration five times on the same day. Inter-day precision was evaluated by applying each concentration five times on three different days with an interval of 24 h.

Recovery

The recovery was used to determine the accuracy of the method. Recovery of FA and vanillin with three different concentration namely, 50, 100, and 150 ng ($n = 3$) was performed. Samples after extraction were spiked with standard concentration and applied. Peak areas of standard added to samples were calculated and average percent recovery was estimated. Further average percent recovery was calculated.

Specificity

Specificity was performed to compare the standard FA and vanillin and extract. It was calculated by comparing the Rf of the peak, peak start, peak apex, and peak end of the standard and extract. The spectral scan of both the standard and extract was compared. Spectrum scan accelerated at 100 nm/s with split dimensions 5 × 0.45 mm, micro. Spectral detection for optimum wavelength was calculated in the range 200 to 700 nm.

Robustness

Modifications in mobile phase concentration, mobile phase volume, mobile phase saturation time, and temperature were examined to check the robustness. Standard solutions of FA (150 ng/band) and vanillin (150 ng/band) were applied thrice and %RSD of each compound was calculated.

Limit of detection and limit of quantification

LOD is the lowest amount of compound that can be detected with signal-to-noise ratio of 3:1 and LOQ is the lowest amount of compound which can be quantified by signal-to-noise ratio 10:1 with adequate precision and

Table 2 Recovery of FA and vanillin

Sample	Amount in sample (ng)	Std added (ng)	Total conc. (ng)	Recovery (ng)	%Recovery	Avg. %Recovery
FA	20.54	50	70.54	69.20 ± 0.30	98.47 ± 0.05	98.77
	20.54	100	120.54	119.23 ± 2.2	99.10 ± 1.89	
	20.54	150	170.54	168.42 ±0.29	98.56 ± 0.17	
Vanillin	37.23	50	87.23	84.08 ± 0.38	96.51 ± 0.34	98.45
	37.23	100	137.23	136.15 ± 0.45	99.19 ± 0.30	
	37.23	150	187.23	186.49 ± 2.30	99.65 ± 1.25	

Mean ± standard deviation ($n = 3$).

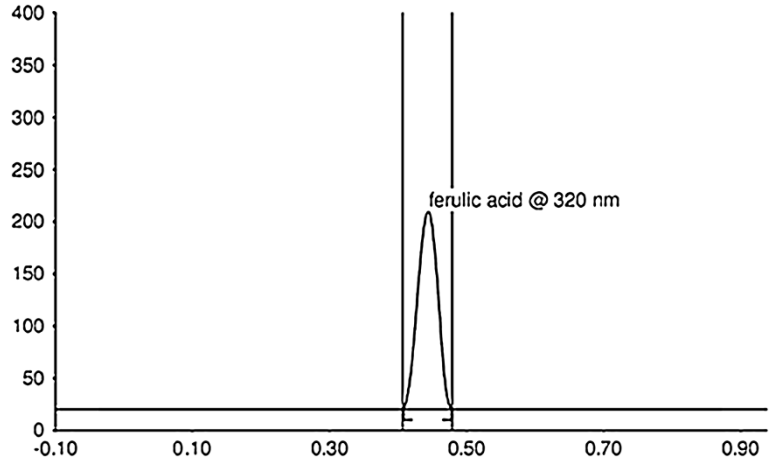

Figure 3 HPTLC chromatogram of standard FA.

specificity. LOD and LOQ of FA and vanillin were calculated.

System suitability

System suitability was executed to check the reproducibility and resolution of the method. Standard solution of both the standards of 150 ng concentration ($n = 5$) was analyzed on the same chromatographic plates. After development, plates were scanned and the peak area of each concentration and their Rf values were calculated.

Results and discussion

A GC method for detection of phenolic compounds is complicated and unsuitable for rapidly analyzing profuse samples and with derivatization step. HPLC-ECD, a new method was developed for the analysis of phenolic compound. However, use of HPLC with ECD is not feasible as a routine analytical method (Takahashi et al. 2013). Even with the HPLC UV detector system, the analysis is time consuming and the quantity of the solvents is the main concern. In the present study, two compounds FA and its bio-transformed product vanillin were quantified using HPTLC. Various mobile phases were screened for proper separation of both the compounds. Well-defined peaks with Rf values 0.48 for FA and 0.56 for vanillin were obtained using toluene/1, 4-dioxan/acetic acid (9:2.5:0.4; *v*/*v*) (Olsson et al. 1974). Experimental conditions should be selected when the compounds to be analyzed moves towards or near to the center of the layer (Rf = 0.5)

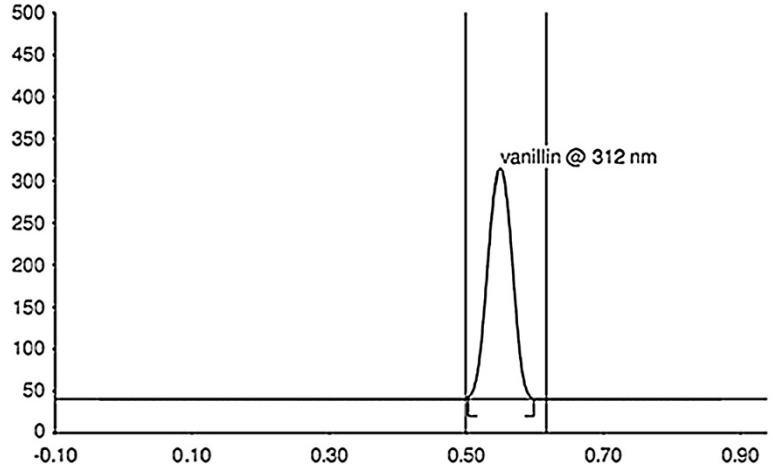

Figure 4 HPTLC chromatogram of standard vanillin.

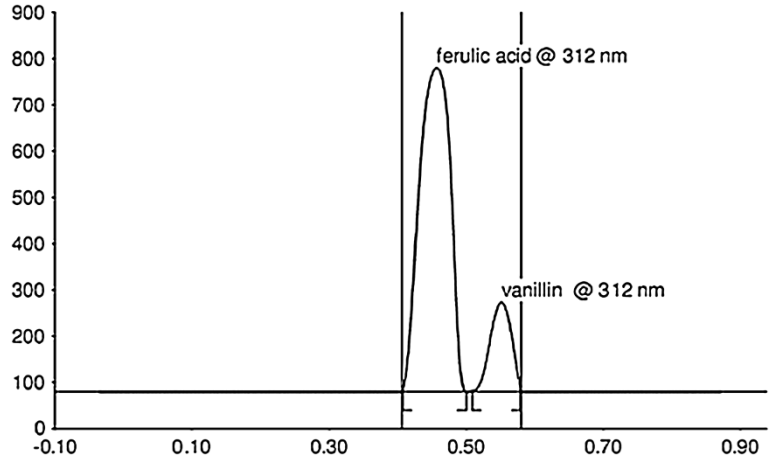

Figure 5 HPTLC chromatogram of FA and vanillin present in crude extract.

(Srivastava 2011). Krishna veni et al. (2013) used methanol/water/glacial acetic acid (20:5:2; *v*/*v*) as the mobile phase and resulted with Rf 0.84 for vanillin. For better peak detection and Rf, the polarity can be reduced. Other mobile phases were compared which resulted in tailing of peaks (Sharma et al. 2007) using methanol/water/isopropanol/acetic acid (30:65:2:3; *v*/*v*) and inappropriate separation of both the compounds with toluene/ethyl acetate/formaldehyde (6:3:1; *v*/*v*) (Srivastava et al. 2008), no identification of vanillin peak using hexane/ethyl acetate (5:2; *v*/*v*) (Hennig et al. 2011) and 1-butanol/acetic acid/water (66:17:17; *v*/*v*) (Males et al. 2001). Chloroform/methanol/formic acid (85:15:1; *v*/*v*) with spraying reagent 2, 4-dinitrophenylhydrazine was used for vanillin detection but resulted in poor peak visualization and separation of vanillin (Mabinya et al. 2006). Sharp peaks were obtained with a presaturation of mobile phase for 20 min. FA and vanillin was quantified using UV detector and scanning at 320 and 312 nm. The peaks corresponding to FA and vanillin in samples had the same retention time when compared to their respective standards.

Linearity

Linearity was achieved with concentrations ranging from 50 to 250 ng/band for FA and 50 to 200 ng/band for vanillin (Figures 1 and 2). The regression equation and correlation coefficient of FA was found to be $y = 37.94 \times X + 621.04$, $R^2 = 0.9975$ with SD = 1.95% and for vanillin $y = 36.016 \times X + 1128.3$, $R^2 = 0.9991$ with SD = 1.60%.

Precision

To get accurate chromatographic results, the precision of the chromatographic method must be analyzed and confirmed whether it is fit for purpose which is adequate to the analytical requirements and it is evaluated in terms of intra- and inter-day precision. The standard deviation and percent relative standard deviation will evaluate the variation limit of the analysis. The value lower than 2% indicate the method is more precise to variation which assumes that the chromatograph does not malfunction after the system precision testing has been performed (Indrayanto 2011). Peak areas measurement of 100, 150, and 200 ng concentration of FA and vanillin showed %RSD less than 2 as shown in Table 1. Both intra- and inter-day results suggested an excellent method of precision which ensures the objective of the method development phase to be reproducible.

Recovery

Recovery is an important parameter as it offers information about the recovery of the analyte from the sample preparation and the effect of matrix. If the recovery is close to 100% then it implies that the proposed analytical method is free from constant and proportional systematic error (Srivastava 2011). Recovery of FA at three different levels obtained was 98.47%, 99.1%, and 98.75% with 98.77% average recovery and of vanillin was 96.51%, 99.19%, and 99.65% with 98.45% average

Table 3 Specificity of FA and vanillin

Retention factor (Rf)	Peak start	Peak apex	Peak end
FA[a]	0.43	0.48	0.51
Vanillin[a]	0.49	0.56	0.62
FA[b]	0.41	0.47	0.50
Vanillin[b]	0.51	0.56	0.60

[a]Standard ferulic acid and vanillin; [b]crude extracts.

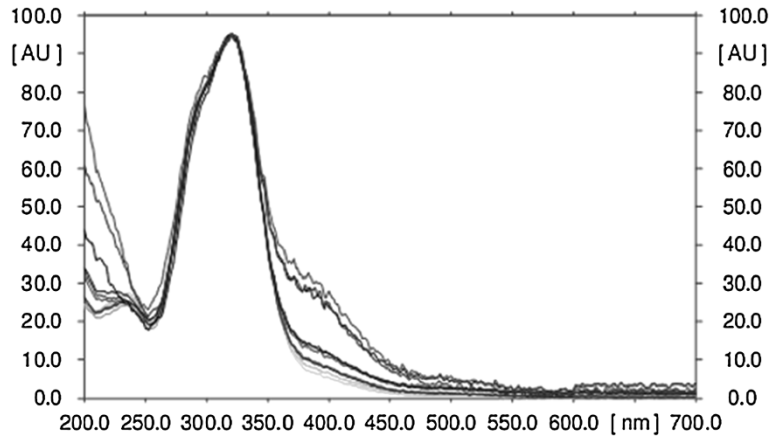

Figure 6 Overlay of UV absorption spectra of standard FA with crude extract.

recovery. The results are shown in Table 2. The percentage recovery was close to 100% which indicated no interference of any other compound and representing the accuracy of the method. This indicates the suitability of the method for the routine analysis.

Specificity

Specificity is a method which provides a response for only a single analyte. This study is performed to check how accurately and specifically the analyte of interest is estimated in the presence of other components with system interference during detection and quantification of analyte (Indrayanto and Yuwono 2010; Kakde et al. 2008). Retention factor values of standard FA and vanillin were compared with sample extract. There was no interference of other peaks as shown in Figures 3, 4, and 5. The standard and sample was compared with respect to peak start, peak apex, and peak end of the bands which showed the specificity between extracts and standard compound and purity of the peaks (Table 3). Overlay of the standard and sample compound using spectral scan was compared to confirm the specificity (Figures 6 and 7). Spectral scan of both the compounds showed maximum absorption at 320 nm for ferulic acid and 312 nm for vanillin. Thus, results obtained from comparison of peak and spectral scan showed the method is specific for detection of FA and vanillin.

Robustness

Robustness can be described as the ability to reproduce the analytical method under different circumstances and provides an indication of its reliability during normal phase. It was introduced to avoid problems in

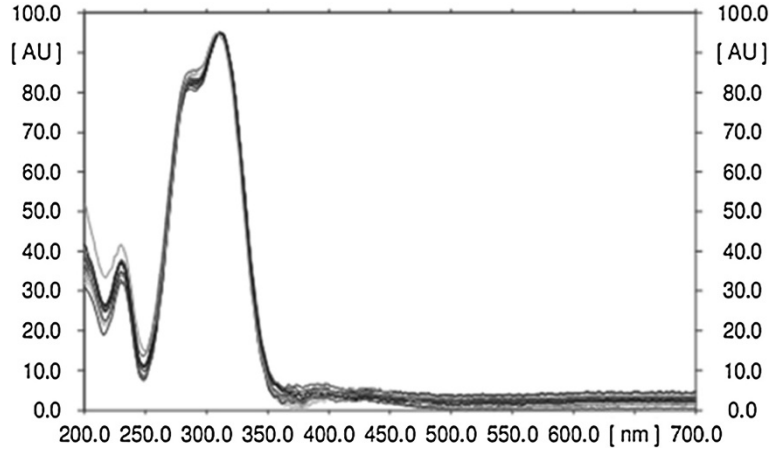

Figure 7 Overlay of UV absorption spectra of standard vanillin with crude extract.

Table 4 Robustness of FA & Vanillin

Parameters	FA		Vanillin	
	SD[a]	%RSD[b]	SD[a]	%RSD[b]
Mobile phase (toluene/1,4-dioxan/acetic acid) (9{±1}:2.5{±1}:0.4{± 0.2}) (*v*/*v*)	2.95 ± 0.13	0.039	2.526 ± 0.05	0.039
Mobile phase vols. 10, 12 and 14 ml	3.00 ± 0.09	0.027	3.626 ± 0.12	0.056
Saturation time 10, 15 and 20 min	2.20 ± 0.06	0.027	2.699 ± 0.07	0.058
Temperature 25, 30 and 37°C	2.25 ± 0.12	0.050	3.351 ± 0.10	0.052

[a]SD, standard deviation of peak area ($n = 3$); [b]RSD, percent relative standard deviation ($n = 3$).

inter-laboratory studies and to discover the potentially repairable factors (Van der Hyden et al. 2001). According to the ICH guidelines, the evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. For robustness evaluation by chromatographic method, the acceptance criteria are that, the Rf values of all standards should lie within the acceptance criteria of the precision method by performing variations in considered parameters. Calculations of standard or relative error are common ways to look at the data and departures from deviations in the data will directly affect robustness. Variations made in mobile phase composition, mobile phase volume, saturation time, and temperature showed less %RSD and SD for FA and vanillin by spotting 150 ng/spot ($n = 3$) (Table 4). No significant change in Rf or response to FA and vanillin was observed which indicated robustness of the method. Robustness study has provided valuable information about the quality and reliability of the method and no further development or optimization is necessary.

Limit of detection and limit of quantification

LOD is based on the analyte response sensitivity (response per amount or concentration per time, using either peak height or area), and LOQ is the lowest amount of analyte that can be quantitatively determined in sample with defined precision and accuracy under standard conditions. LOQ is usually a multiple of LOD (Kakde et al. 2008). Standard deviation and slope used in the equations are used to determine LOD and LOQ which is equivalent to instrument sensitivity for the specific analyte, reinforcing that the LOD/LOQ details, are expressed in units of analyte concentration (Apostol et al. 2012). It is important to determine sensitivity using analyte amounts near to their detection limits. LOD and LOQ with signal-to-noise ratio of FA were found to be 13.63 and 45.42 ng/band, respectively. LOD and LOQ of vanillin were found to be 10.59 and 35.30 ng/band, respectively. Sensitivity of the method is evaluated with regard to LOD and LOQ (Kakde et al. 2008). These results indicate the sensitivity of the method which can be used for quantification of the compound.

System suitability

System suitability test (SST) is an integral part of many analytical procedures. The test is based on the conception that, the equipment, electronics, analytical operations, and samples to be analyzed represent an integral system that can be evaluated. System suitability test parameters to be established for a particular procedure depend upon the type of procedure being validated. It is the ability of the analytical method to detect analyte quantitatively in the presence of other components which are expected to be present in the sample and they should be chromatographed along with the analyte to check the system suitability and retention factor of the required analyte (Dolan 2004). On the basis of repeatability relative standard deviations of peak response, SST was analyzed. Retention factor of FA and vanillin were 0.48 and 0.56, respectively. Standard deviation of FA was 2.95 ± 0.13 with 0.039% RSD and standard deviation of vanillin was 3.62 ± 0.12 with 0.056% RSD. The low %RSD indicates the reproducibility and the system suitability of the method.

Conclusion

The simultaneous detection of FA and vanillin by the HPTLC method can be performed as there is noteworthy difference in their retention factor values. The proposed method was developed and validated by ICH guidelines which is simple, rapid, accurate, precise, sensitive, and eco-friendly. The mobile phase selected was toluene/1, 4-dioxan/acetic acid 9:2.5:0.4 (*v*/*v*) which resulted in proper peak separation as compared to different mobile phase which were not able to separate the compounds properly. Other chromatographic methods like HPLC, GC, and spectrophotometric mentioned may not be applied for routine check because of the various shortcomings as compared to HPTLC. The total optimized method is therefore useful in both qualitative and quantitative analysis for routine assays in pharmaceutical and food industry within acceptable limits.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions
SH and SD has performed all the experimental and analytical work and drafted the manuscript. The guidelines for all the mentioned part was provided by UA. All authors read and approved the final manuscript.

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