Evaluation of thermo sensitivity of curcumin and quantification of ferulic acid and vanillin as degradation products by a validated HPTLC method

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Abstract: Charismatic therapeutic potential of curcumin in biological research have triggered an interest to explore the thermal degradation pattern of curcumin, formation of ferulic acid and vanillin as degraded metabolites at different temperatures in methanol and corn oil. The results revealed 47% w/w loss of curcumin along with formation of 17% w/w vanillin and 9% w/w ferulic acid at boiling temperature of methanol while oil samples show 38.9% w/w loss of curcumin but not confirming the formation of ferulic acid and vanillin. Findings of this study revealed that formation of vanillin in methanol starts around 50°C and its concentration goes on increasing up to 70°C and then further degraded at boiling temperature of methanol. Formation of ferulic acid begins around 60°C and initially increases with rise of temperature and then decreases approaching boiling point of methanol. Vanillin as well as ferulic acid was absent in corn oil samples though degradation of curcumin was observed by reduction in peak area of curcumin. The present study was done by applying a validated high-performance thin-layer chromatography method. The method involved glass-backed HPTLC plates precoated with silica gel 60_{F254} as the stationary phase and toluene: ethyl acetate: methanol (8:1:1, v/v/v) as mobile phase.

Keywords: Turmeric, Curcumin, HPTLC, Vanillin, Ferulic acid.

INTRODUCTION

Curcumin (fig.1a) is the active molecule present in *Curcuma longa* (Zingiberaceae) and known for its anti-tumour, anti-oxidant, anti-arthritic, anti-amyloid, anti-ischemic, anti-inflammatory activities. In addition, it may also be effective in treating malaria and prevention of cervical cancer (Gantait *et al*., 2011).

Curcumin is one of the most extensively studied natural compounds. Almost 3000 research articles have been published on curcumin and its effects (Preetha *et al*., 2008). Curcumin is a very sensitive compound and its bioavailability, solubility and stability is always been an issue of debate. Curcumin is poorly soluble in water at acidic or neutral pH, which makes curcumin hard to absorb. Limited stability of curcumin in aqueous environments is another cause of weak bioavailability (Tønnesen and Karlsen, 1985; Ansari *et al*., 2005). Curcumin exhibited more stability when formulated as liposomes as compared to free curcumin at 25°C (Yumeng *et al*., 2012). There are evidences about twelve fold increase in solubility of curcumin in water by applying heat (Kurien *et al*., 2007) and sometimes by making complexes with biopolymers like cyclodextrin (Tønnesen *et al*., 2002). Thermal degradation of curcumin revealed the maximium loss of curcumin content (up to 53%) was observed while pressure cooking for 10 minutes at 15p.s.i. and can be minimized to 30% by adding tamarind and providing acidic medium (Suresh *et al*., 2007). Decomposition products of curcumin may include trans-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexenal, ferulic acid (fig.1b), vanillin (fig.1c), feruloyl methane, vanillic acid, p-hydroxybenzaldehyde and p-hydroxybenzoic acid etc (Wang *et al.,* 1997; Khurana and Ho, 1988. Heating reduces the activity of enzyme polyphenol oxidase thereby enhancing the total phenolic contents in heat-treated samples than fresh rhizomes (Prathapan *et al.,* 2009).

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MATERIALS AND METHODS

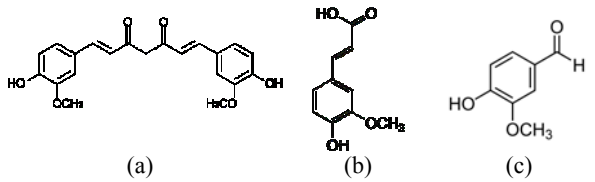
Materials, apparatus and reagents

Curcumin, ferulic acid and vanillin was obtained from Sigma Aldrich, Bayoumi Trading Co. Ltd. Al-Khobar, Saudi Arabia. HPLC grade toluene, ethyl acetate (EA) and methanol (MeOH) were purchased from BDH Laboratory supplies (Liverpool, UK). All other chemicals and reagents used were of analytical reagent (AR) grade. Glass-backed silica gel $60F_{254}$ precoated HPTLC plates (20×10cm) were purchased from Merck (Darmstadt, Germany). Methanol and oil samples of curcumin were applied to chromatographic plates band wise, by means of a CAMAG automatic TLC sampler-4 (CAMAG, Muttenz, Switzerland) and developed in ADC2 (automatic development chamber) (CAMAG, Muttenz, Switzerland). TLC Plates were then documented by CAMAG TLC Reprostar 3 and scanned by CATS 4 (CAMAG).

Preparation of standard stock solution

Stock solution of standard curcumin (1mg mL^{-1}) was prepared in methanol (95%). 1 ml of the stock solution

was diluted with 9mL methanol to make the concentration 100ng/µL. For calibration plot, curcumin standard solution (1-8µL) was applied to a HPTLC plate to furnish concentration in the range 100-800ng per band. Similar method has been applied for preparing stock solution of vanillin and ferulic acid and calibration curve was also made accordingly.



(a) (b) (c)

Fig. 1: Chemical structure of Curcumin (a); Ferulic Acid (b); Vanillin (c)

Preparation of samples

100ml of each of methanol (95%) and corn oil has been taken separately to dissolve 10mg curcumin to get the concentrations 100ng/µL for both the samples. All the samples were heated on a heating mental with controlled temperature. Sampling has been done for methanol samples (CM) at different temperatures like 50°C, 60 °C, 70°C and boiling point of methanol (CM1, CM2, CM3, and CM4 respectively), and oil samples (CO) at temperature 50°C, 100°C, 150°C and 200°C (CO1, CO2, CO3 and CO4 respectively). Methanol samples were applied as such while corn oil samples were first extracted with equal volume of methanol and then applied as spot on HPTLC plate.

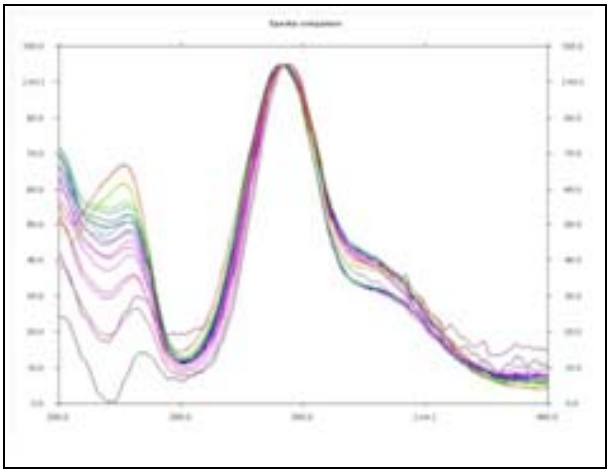


Fig. 2: Overlaying of absorption spectra of methanol samples of curcumin (collected at different temperature) with standard curcumin, ferulic acid and vanillin solution @293 nm.

HPTLC instrumentation and chromatographic conditions

Chromatographic analysis was done on 20×10cm precoated HPTLC plates. Samples were applied as bands 6mm wide and 7.7 mm apart by Linomat IV sample applicator. The application rate of sample on plate was 160nL S^{-1}. The plates were developed in previously saturated 20×10 cm twin-trough glass chamber [at room temp. (25±2°C) and rel. humidity (60±5%)], using solvents toluene: ethyl acetate: methanol (8:1:1, v/v/v) as mobile phase. The plates were dried at room temperature and then heated to identify compact bands. Quantitative analysis was performed at wavelength 293 nm in absorbance/reflectance mode with CATS 4 operated by WinCATS software (Version 1.2.0).

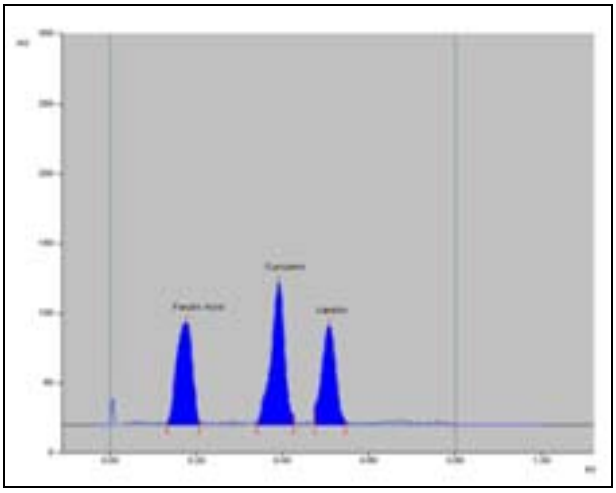


Fig. 3: Chromatogram of standards of curcumin, ferulic acid and vanillin (500 ng $spot^{-1}$) @ 293 nm

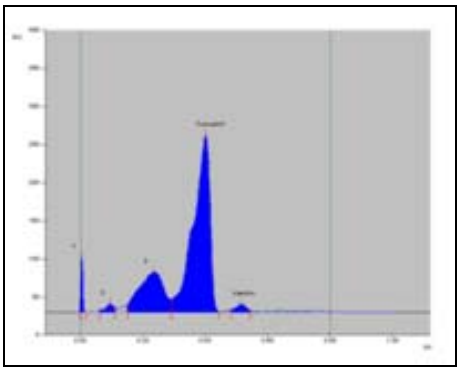


Fig. 4a: Chromatogram of CM1 (50°C) @ 293 nm

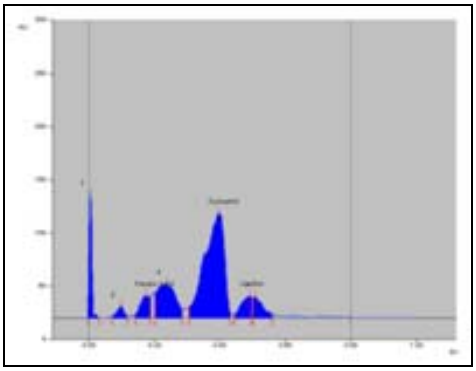


Fig. 4b: Chromatogram of CM2 (60°C) @ 293 nm

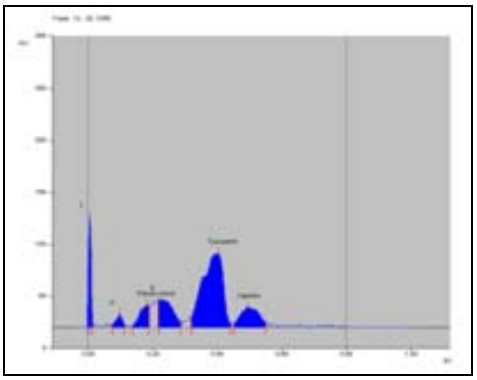


Fig. 4c: Chromatogram of CM3 (70□C) @ 293 nm

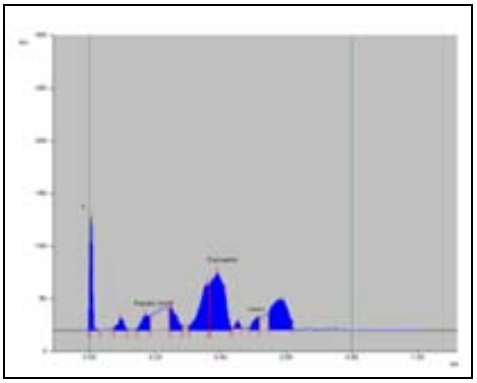


Fig. 4d: Chromatogram of CM4 (B.P) @ 293 nm

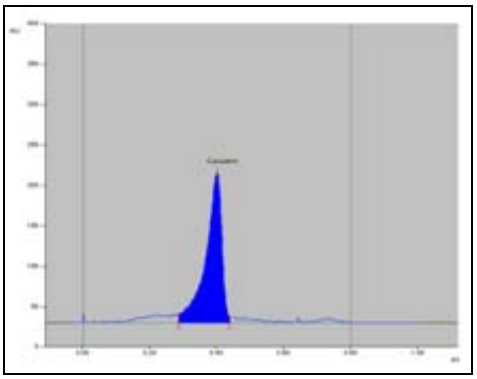


Fig. 5a: Chromatogram of CO1 (50□C) @ 293 nm

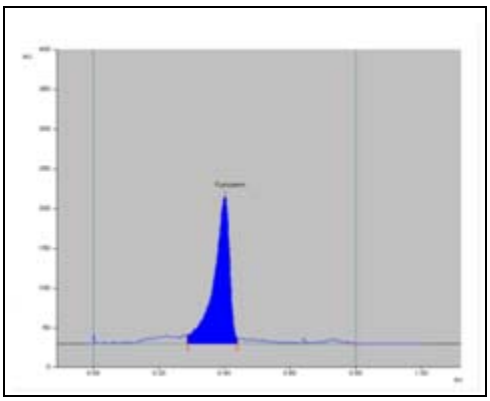


Fig. 5b: Chromatogram of CO2 (100□C) @ 293 nm

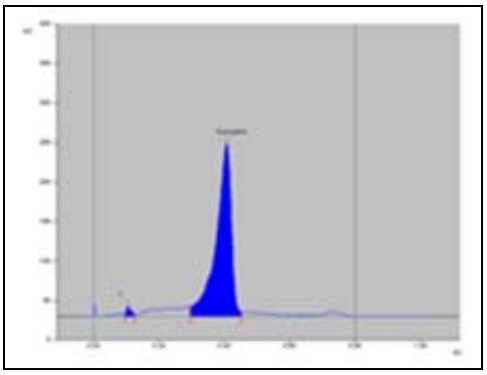


Fig. 5c: Chromatogram of CO3 (150□C) @ 293 nm

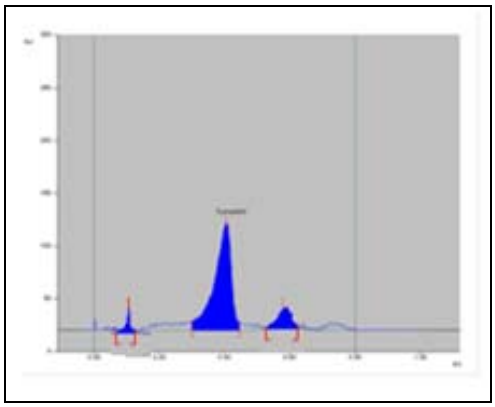


Fig. 5d: Chromatogram of CO4 (200□C) @ 293 nm

Preparation of calibration graphs

Calibration graph for standard curcumin was prepared by applying a series of spots of standard with eight different volumes so as to get different amount of curcumin per spot (100-800ng/spot). Calibration curve was prepared with respect to height and area vs amount per spot. Similar methods have been applied to prepare calibration graphs of ferulic acid and vanillin.

Method development

Chromatogram was developed for curcumin by selecting the mobile phase after trying several combinations of solvents. The best resolution was observed in the selected [toluene: ethyl acetate: methanol (8:1:1, v/v/v)] mobile phase. The same mobile phase has been employed for the separation of methanol and oil samples. The optimized saturation time was observed as 20min. The densitometric analysis was performed by optimizing the absorption maxima of wave length 293 nm in absorbance/reflectance mode.

Method validation

Validity of the adopted method was checked according to the parameters assigned by International Conference on Harmonization (ICH, 1996). These parameters include linearity range, precision, accuracy, robustness, limit of detection (LOD), limit of quantification (LOQ), and recovery. Precision (inter- and intra-day) and accuracy of

the assay were evaluated by performing replicate analyses (n=6) of samples at three quality-control (QC) levels, low, medium, and high of 150, 300 and 600ng band^{-1}, respectively. Repeating the intra-day assay on three different days gives inter-day precision and accuracy. Coefficient variation (% CV) of measured concentrations for each calibration level was used to express precision, whereas percentage recovery was used to express accuracy. Robustness of method was studied in triplicate at 300ng band^{-1} by making deliberate small changes to mobile phase composition, mobile phase volume, and duration of mobile phase saturation and activation of HPTLC plates. The results were examined in terms of relative standard deviation (% RSD) and standard error (SE) of peak areas. Mobile phases consist of toluene: ethyl acetate: methanol (8:1:1, v/v/v) in different proportions (7: 1.5: 1.5, *v*/*v*; 7: 2: 1, *v*/*v*; 7: 1: 2, *v*/*v*) were used for chromatography. Mobile phase volume and duration of saturation investigated were 20±2mL (18, 20 and 22mL) and 20±10min (10, 20 and 30 min), respectively The plates were activated at 110°C for 30 min before chromatography. Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) of the response and the slope (*S*) of the calibration curve at levels approaching LOD according to the formulae: (LOD=3.3 [SD/*S*] and LOQ=10 [SD/*S*]). The standard deviation of the response was determined based on the standard deviation of *y* intercepts of regression lines. Recovery was studied by applying the method to drug samples to which known

Table 1: R_f, linear regression data for the calibration curve and sensitivity parameter for Curcumin, Ferulic Acid and Vanillin

Parameter	Curcumin	Ferulic Acid	Vanillin
R_f	0.39±0.01	0.18±0.01	0.51±0.01
Linearity range (ng band^{-1})	100-800	100-800	100-800
Regression equation	Y=18.878X +2925.199	Y=13.175X+582.317	Y=34.132X+1181.587
Correlation coefficient	(r^2) 0.998	(r^2) 0.998	(r^2) 0.997
Slope±SD	18.878±0.031	13.175±0.054	34.132±0.042
Intercept±SD	2925.199±5.72	582.317±16.45	1181.587±10.25
Standard error of slope	*0.012*	*0.022*	*0.014*
Standard error of intercept	3.012	6.741	2.344
LOD	26 ng band^{-1}	31 ng band^{-1}	29 ng band^{-1}
LOQ	77 ng band^{-1}	92ng band^{-1}	88 ng band^{-1}

Table 2: Precision of Curcumin, Ferulic Acid and Vanillin

2A: Curcumin

	INTRADAY PRECISION			INTERDAY PRECISION		
Conc. (ng/spot)	Avg Conc. ± SD (n=6)	Standard Error	% RSD	Avg Conc. ± SD (n= 6)	Standard Error	% RSD
150	1384.50±22.04	9.94	1.74	1362.33±24.14	9.43	1.68
300	2588±41.24	17.31	1.63	2557.67±41.15	16.39	1.58
600	3720±18.24	23.23	1.54	3685.71±49.45	22.41	1.49

2B: Ferulic Acid

	INTRADAY PRECISION			INTERDAY PRECISION		
Conc. (ng/spot)	Avg Conc. ± SD (n= 6)	Standard Error	% RSD	Avg Conc. ± SD (n= 6)	Standard Error	% RSD
150	3515.44±14.23	17.14	1.19	3496.71±32.40	16.76	1.17
300	6185.45±47.76	28.34	1.12	5989.16±56.40	27.50	1.08
600	8281.65±78.24	36.02	1.05	8224.12±67.20	34.79	0.98

2C: Vanillin

	INTRADAY PRECISION			INTERDAY PRECISION		
Conc. (ng/spot)	Avg Conc. ± SD (n= 6)	Standard Error	% RSD	Avg Conc. ± SD (n= 6)	Standard Error	% RSD
150	1370.54±81.24	9.86	1.69	1341.22±81.24	9.01	1.59
300	2571.23±21.73	16.85	1.64	2529.57±21.98	16.42	1.49
600	3672.45±29.18	23.42	1.58	3629.87±35.67	21.69	1.32

amounts of marker corresponding to 50, 100 and 150% of the curcumin, vanillin and ferulic acid had been added. Each level was analyzed in triplicates. This was to check the recovery of curcumin, vanillin and ferulic acid at different levels in the samples.

Linearity range

A series of spots of different volumes (1µl-8µl) were applied so as to get 100-800ng quantity of curcumin per band respectively for determining the linearity range of standard curcumin. The plate was scanned at wavelength 293nm and a curve was prepared with respect to height and area vs amount per spot. The same concentration ranges were used for determining the linearity range of standard ferulic acid and vanillin.

HPTLC analysis of prepared samples

The developed method of HPTLC is applied for quantification of curcumin, ferulic acid and vanillin in samples of methanol (CM1, CM2, CM3 and CM4) and oil at different temperature (CO1, CO2, CO3 and CO4). From the HPTLC analysis of all the samples, quantity of curcumin, ferulic acid and vanillin is determined in each sample.

RESULTS

Method development

Chromatogram was developed for curcumin, ferulic acid and vanillin under chamber saturation conditions using solvents toluene: ethyl acetate: methanol (8:1:1, *v/v/v*) as solvent system. The same mobile phase was employed for the analysis of samples of curcumin in methanol and oil. The optimized saturation time was observed as 20 min. Densitometric analysis was performed at 293 nm in absorbance/reflectance mode. Compact, sharp, symmetrical, and high-resolution bands of curcumin, ferulic acid and vanillin were obtained at Rf 0.39±0.01,0.18±0.01 and 0.51±0.01 respectively. According to literature survey, there is no thermal degradation study with respect to decomposition products by HPTLC densitometric method was reported earlier. Therefore, attempts were made to develop and validate a cost-effective, simple stability indicating HPTLC method to quantify the ferulic acid and vanillin as decomposition products of curcumin. The developed method was found to be quite selective with good baseline resolution.

Method validation

Linearity of curcumin was validated by the linear regression equation and correlation coefficient. The eight-point calibration curve for curcumin was found to be linear in the range of 100-800ng. Regression equation and r^2values for the reference compound curcumin were observed as Y=18.878X +2925.199 and 0.998, respectively, which revealed a good linearity response for the developed method. The regression equation for ferulic acid was observed as Y=13.175X+582.317 with r^2=0.998 and for vanillin Y=34.132X+1181.587 with r^2=0.997 for concentration range similar to curcumin. LOD and LOQ for curcumin was found to be 26 and 77ng band^{-1}, for ferulic acid 31 and 92ng band^{-1}and for vanillin 29 and 88 ng band^{-1}, respectively (table 1). This indicates that the proposed method exhibits a good sensitivity for the quantification of the above compounds.

The mean value with ±SD of the slope for curcumin was 18.878±0.006,for ferulic acid 13.175±0.004 and for vanillin 34.132±0.002. No significant difference was observed in the slopes of standard plots (P>0.05) for curcumin, ferulic acid and vanillin. table 2 presents intra-day and inter-day precision and accuracy of the assay for curcumin at three QC levels (150, 300 and 600ng band-1). Both intra- and inter-day precisions were determined in terms of % CV. Intra- and inter-day precisions (n=6) for curcumin were found to be 1.54-1.74% and 1.49-1.68%, respectively. However, intra- and inter-day precisions (n=6) of ferulic acid was observed as 1.05-1.19 and 0.98-1.17 respectively while for vanillin intra and interday precision was found to be 1.58-1.69 and 1.32-1.59 respectively which demonstrated the good precision of proposed method.

The accuracy was calculated by recovery analysis, which afforded recovery of 98.88-99.61%, 98.41-99.6% and 98.07-99.35% for curcumin, ferulic acid and vanillin respectively after spiking the additional standard drug solution to the previously analyzed test solution. The values of % recovery and %RSD are shown in table 3. Low values of %RSD (1.04-1.54), (1.07-1.55) and (1.05-1.42) for curcumin, ferulic acid and vanillin respectively also indicated the good accuracy of proposed method.

The results of robustness are shown in table 4. Low values of %RSD (0.041-0.23), (0.39-0.21) and (0.40-0.24) for curcumin, ferulic acid and vanillin, respectively, obtained after introducing small deliberate changes into the densitometric HPTLC procedure proved the robustness of the proposed HPTLC method.

DISCUSSION

Curcumin molecule is poorly soluble in water and very sensitive to oxidation. Thermal decomposition of curcumin proves that heating enhances the solubility but provokes degradation. The experimental findings suggest the stability of curcumin is temperature dependent. These finding also supports the study performed by Jagannathan *et al*., 2012, on solubility and stability of curcumin. In another study curcumin was allowed to react at 70^{o}C with radical species generated from the pyrolysis of 2,2'-azobis (isobutyronitrile) and the degradation products were identified as ferulic acid, vanillin and a dimer possessing dihydrofuran moiety by HPLC (Toshiya *et al*., 1999). In

Table 3: Accuracy of Curcumin, Ferulic Acid and Vanillin

3A: Curcumin

Excess drug added to analyte (%)	Theoritical Content (ng)	Conc. Found (ng) ± *SD*	% Recovery	% RSD
0	300	296.67±1.13	98.88	1.54
50	450	446.34±*2.98*	99.18	1.38
100	600	595.67±*4.32*	99.27	1.20
150	750	747.12±*3.52*	99.61	1.04

3B: Ferulic Acid

Excess drug added to analyte (%)	Theoritical Content (ng)	Conc. Found (ng) ± *SD*	% Recovery	% RSD
0	300	295.23±3.53	98.41	1.55
50	450	446.03±1.23	99.12	1.39
100	600	594.97±2.76	99.16	1.28
150	750	747.01±2.16	99.60	1.07

3C: Vanillin

Excess drug added to analyte (%)	Theoritical Content (ng)	Conc. Found (ng) ± *SD*	% Recovery	% RSD
0	300	294.23±2.32	98.07	1.42
50	450	444.26±*1.76*	98.72	1.29
100	600	594.24±*7.62*	99.03	1.13
150	750	745.09±*1.34*	99.35	1.05

Table 4: Robustness of the method

Optimization condition	Curcumin	Ferulic Acid	Vanillin
	SD/%RSD	SD/%RSD	SD/%RSD
Mobile phase composition: Toluene: ethylacetate: methanol (7:1.5:1.5, v/v/v; 7:2:1, v/v/v; 7:1:2,v/v/v)	11.89/0.041	11.12/0.039	11.39/0.040
Mobile phase volume (18, 20 and 22 mL)	6.67/0.023	5.98/0.021	6.71/0.024
Duration of saturation (10, 20 and 30 min)	10.73/ 0.037	8.26/0.029	8.26/0.029

this study it seems that partial degradation of curcumin starts at around 50°C (Fig.4a) and the formation of vanillin begins at this temperature while formation of ferulic acid starts later at around 60°C (Fig.4b). The chromatogram revealed that concentration of vanillin in methanol goes on increasing up to 70°C (Fig. 4c) and then declined at boiling temperature of methanol (Fig. 4d). Concentration of ferulic acid initially increases with rise of temperature (Fig. 4a and 4c) and then decreases (Fig. 4d) at around boiling point of methanol. Vanillin as well as ferulic acid was absent in corn oil samples (Fig. 5a and 5b) though degradation of curcumin was observed by reduction in peak area of curcumin (Fig. 5d). The results revealed 47% loss of curcumin, formation of vanillin 17% and ferulic acid 9% at boiling temperature of methanol while oil samples show 38.9% loss of curcumin (fig. 5d) but not showing the formation of ferulic acid and vanillin. Decrease in concentration of vanillin at boiling temperature of methanol indicates the formation of some other degradation products. Oil samples on heating shows decomposition of curcumin by decrease in peak area of curcumin and formation of other products indicated by new peaks but not revealing the formation of ferulic acid and vanillin which indicate some possibilities like the quantity of these degradation products may be below detection limit, formation of complexes with co-constituents or enveloped by some molecular aggregates formed during heating. Further study is needed to explore these possibilities in the oil samples of curcumin. As we know the curcumin has antiulcer effect (dose 20-50mg/kg) as well as ulcerogenic effect (>50mg/kg) and these effects are only depend on the dose of curcumin (Ishita *et al*., 2004). Therefore it is necessary to know the actual dose of botanical ingredients to be delivered for a particular disease otherwise it may produce some deleterious effects.

CONCLUSION

Sometimes only one step of processing or manufacturing may impart unintentional damage to the herbal formulations. The outcomes of this experiment may be utilized in optimizing heat applications during in-process pharmaceutical formulations involving turmeric. We report here that the use of heat also has limitations and only mild to moderate heat can be recommended to maintain the integrity and efficacy of turmeric formulation particularly when heat is involved during the processing.

ACKNOWLEDGEMENT

The author is grateful to Research Centre, College of Pharmacy, King Saud University, Riyadh, Kingdom of Saudi Arabia for providing the facilities to carry out these studies.

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