ORIGINAL ARTICLE

A validated stability-indicating HPLC method for simultaneous determination of Silymarin and Curcumin in various dosage forms

Mohamed A. Korany [a], Rim S. Haggag [a], Marwa A.A. Ragab [a,*], Osama A. Elmallah [b]

[a] Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Alexandria, El-Messalah, Alexandria 21521, Egypt
[b] Pharco-B International Company for Pharmaceuticals, Borg El-Arab, Alexandria, Egypt

Abstract   A new, reliable, sensitive and stability-indicating gradient HPLC method was introduced for the simultaneous determination of two anti-hepatotoxic polyphenolic drugs (Silymarin and Curcumin). The method was adapted to analyze both drugs in their dosage forms (tablets and capsules) with no interference from common excipients. The photo diode array detector was used as a tool for peak identification and purity confirmation especially that both drugs have several reported peaks. In order to assess the stability-indicating power of the assay procedure, SIL and CUR were subjected to different forced degradation studies: acidic, alkaline and neutral hydrolysis, photo-degradation, oxidative degradation and dry heat. The developed method could efficiently separate the parent drug peak from the degradation products peaks. The method was validated according to the ICH guidelines with respect to linearity, detection and quantitation limits, accuracy, precision, specificity, and robustness. Finally, the results of the proposed method for determination of SIL were statistically compared to the official BP method and no significant difference was found between them.

© 2013 Production and hosting by Elsevier B.V. on behalf of King Saud University.

1. Introduction

Silymarin (SIL) is an anti-hepatotoxic polyphenolic substance isolated from the milk thistle plant, *Silybum marianum*, family (Asteraceae) (United States Pharmacopeia, 2011). SIL (Sweetman, 2009), which is a mixture of flavonolignans including the isomers silibinin, silicristin, and silidianin -of which silibinin is the major component- is claimed to be a free radical scavenger and to have hepatoprotectant properties. It has been used in

various liver disorders, as well as to prevent hepatotoxicity associated with poisoning (Sweetman, 2009).

Curcumin (CUR) which is a polyphenolic compound present in the rhizomes of the turmeric (Curcuma longa Linn.) family (Zingiberaceae) (Sweetman, 2009), has a wide biological and pharmacological profile. It is reported to have antioxidant and hepatoprotective activity (Park et al., 2000). This compound exhibits numerous biological activities including anti-inflammatory, antiprotozoal, antibacterial, anti-HIV and anti-cancer activities against several malignancies. Also, hepato, neuroprotective, hypoglycemic, and antirheumatic effects of curcumin were reported (Anand et al., 2007). The safety of CUR at very high doses has been proved in various animal and human studies (Anand et al., 2007). These studies led to the approval of CUR as a 'Generally Regarded as Safe' ingredient by the Food and Drug Administration (FDA) of the United States of America, by the Natural Health Products Directorate of Canada and the Expert Joint Committee of the Food and Agriculture Organization/World Health Organization (FAO/WHO) on food additives (JECFA) (Basnet and Skalko-Basnet, 2011).

The USP 34 (United states Pharmacopeia, 2011) monograph of SIL (Powdered Milk Thistle Extract) describes a gradient HPLC-UV method for its assay in pure form, in capsules and in tablets, with a limit for resolution between Silybin A and B not less than 1. However, the BP 2012 (British Pharmacopoeia, 2012) monograph of SIL (Milk-thistle Fruit) describes also a gradient HPLC method for its analysis in pure form, with a limit for resolution between Silibinin A and B not less than 1.8.

Various methods are available for the determination of SIL, including stability-indicating HPTLC method for quantitative estimation of Silybin in bulk drug and pharmaceutical dosage form (Parveen et al., 2010). HPLC and capillary electrophoresis have been used for the determination of SIL from dried fruits (Quaglia et al., 1999).

UPLC-UV method was reported for the simultaneous determination of active compounds in SIL (Liu et al., 2009). Several reversed phase HPLC-UV methods have been published for the analysis of Silibinin in rat plasma and bile (Wu et al., 2007), in the seed extract of some Milk Thistle (Radjabian et al., 2008), in pharmaceutical preparations (Hadad et al., 2009), and in human plasma (Kosina and Bartek 2000). UPLC-MS methods were also used to analyze and separate the active constituents of the silymarin extract (Wang et al., 2010).

A differential pulse voltammetric method has been used for the determination of SIL and vitamin E acetate mixture in pharmaceuticals (Hassan et al., 2008).

Determination of SIL using spectrophotometry in bulk drug and pharmaceutical formulations (Moin et al., 2010) has also been found.

A survey of the literature showed that different analytical techniques have been used for the analysis of CUR. CE methods have been used for the detection and determination of CUR (Lechtenberg et al., 2004), also different TLC and HPTLC methods have been reported for the simultaneous quantification and determination of Curcuminoids in Curcuma longa (Phattanawasin et al., 2009). In addition, a stability-indicating HPTLC method for the determination of CUR in bulk drug and pharmaceutical formulations (Ansari et al., 2005) was published.

Several fluorimetric methods have been used for the determination of CUR (Wang et al., 2008). NMR methods have also been reported for the rapid quantitation of CUR (Gören et al., 2009).

Different HPLC methods have been described for the analysis of CUR, such as HPLC methods with fluorescence detector which have been used for its quantification in biological samples (Schiborr et al., 2010). HPLC-MS methods have been used for the assay of CUR in plasma (Yang et al., 2011).

HPLC-UV methods were also used, such as: HPLC methods for the quantitative determination of CUR in biological fluids (Han et al., 2011). Simultaneous determination of Curcuminoids in different types of extracts (Wichitnithad et al., 2009) and in food products was done.(Nagappan et al., 2009).

A stability-indicating LC method dealing with the quantitative determination of CUR in laboratory samples has been found (Dandekar and Patravale, 2009).

A literature survey revealed that no analytical method has been reported for the simultaneous analysis of SIL and CUR and for their simultaneous analysis in the presence of their forced degradation products.

In the present work, a new validated stability-indicating HPLC method has been developed for the simultaneous analysis of SIL and CUR. Furthermore, the different conditions for optimization of the HPLC parameters for the simultaneous identification and determination of both drugs in their dosage forms (tablets and capsules) were studied using HPLC- DAD. The diode array detector which is an elegant part of the HPLC instrument enhances the performance of the instrument and is very efficient in confirming the peak purity of the several reported peaks of SIL and CUR.

To validate the stability-indicating power of the developed analytical method, SIL and CUR were subjected to forced degradation studies including the effect of hydrolysis (acidic, alkaline and neutral), oxidation, photolysis and dry heat. The proposed method could effectively separate the drug peaks from those of their degradation products.

2. Experimental

2.1. Materials and reagents

Silymarin and Curcumin were kindly supplied by EMITCO Pharmaceuticals (Alexandria, Egypt). Methanol and acetonitrile used were of HPLC grade and were obtained from LAB-SCAN (UK). Other reagents were of analytical grade including: potassium dihydrogen phosphate and orthophosphoric acid obtained from LOBA-Chemie, NaOH, HCl and H_2O_2 obtained from EL-NASR company-ADWIC for chemical industries (Egypt).

2.2. Pharmaceutical formulations

Legalex® Tablets labeled to contain 70 mg Silymarin per tablet were manufactured by the Alexandria Company for Pharmaceutical and Chemical Industries (Alexandria, Egypt). Hepapro® Capsules labeled to contain 100 mg Silymarin and 50 mg Curcumin per capsule were manufactured by EMITCO Pharmaceuticals (Alexandria, Egypt).

2.3. Apparatus

A Waters HPLC system consisting of: Alliance e HPLC with a 2695 separation module consisting of a solvent management module, a sample management module and a column oven.

Empower 2 chromatography data software for the recording and integration of the chromatograms. The detector was a PDA (Photodiode Array) absorbance detector (2998 Waters detector).

The column used: Waters X-bridge® (C18) column, packing material: Hybrid particle, dimension: (250X 4.6 mm i.d.) and Particle size: 5 μm.

2.4. Stock standard solutions preparation

2.4.1. Preparation of stock Silymarin and Curcumin standard solutions

Stock solutions were prepared by dissolving SIL or CUR in methanol to obtain solutions having concentrations of 1 and 0.5 mg mL^{-1}, respectively. The solutions were stable in a refrigerator at 5 °C for a week.

2.4.2. Preparation of stock Silymarin and Curcumin solutions for forced degradation

Stock solutions were prepared by dissolving SIL or CUR in methanol to obtain solutions having a concentration of 2.5 and 1.25 mg mL^{-1}, respectively. The solution was stable in a refrigerator at 5 °C for a week.

2.5. Standard solutions preparation

2.5.1. Preparation of working Silymarin and Curcumin standard solutions

Working SIL and CUR standard solutions were prepared by accurately transferring different volumes (3–7 mL) of each stock solution into 20-mL volumetric flasks, completing to volume with a diluting solvent composed of 0.05 M KH$_2$PO$_4$ solution pH 2.3: methanol (65:35 V/V) to obtain different working standard solutions of SIL and CUR in the range of (0.15–0.35) and (0.075–0.175) mg mL^{-1}, respectively. These solutions were directly injected into the HPLC system.

2.5.2. Preparation of working Silymarin and Curcumin standard mixture solution

Working standard mixture solution of SIL and CUR was prepared by accurately transferring a volume of 5 mL from each corresponding stock solution, into a 20-mL volumetric flask. The volume was completed with diluting solvent to obtain a final concentration of 0.25 and 0.125 mg mL^{-1} of SIL and CUR, respectively and the solution was mixed well, and then injected directly into the HPLC system.

2.6. Chromatographic conditions

Column: Waters X-bridge® (C18) column.
  Packing material: Hybrid particle.
  Dimension: (250 × 4.6 mm i.d) and Particle size: 5 μm.
  Mobile phase: Gradient elution of mobile phase components A, B and C, table (1).
  Mobile phase A: 0.05 M KH$_2$PO$_4$ adjusted at pH 2.3.
  Mobile phase B: Methanol.
  Mobile phase C: Acetonitrile.
  Flow rate: 1 mL/min.
  Temperature: Column oven was adjusted at 30 °C.

Detection wavelength: Using photodiode array detector set at 288 nm.
Injection volume: 20 μL.

2.7. Analysis of Silymarin and Curcumin different dosage forms

2.7.1. Preparation of test Legalex® Tablets solution

Test solution was prepared by weighing 20 tablets of Legalex® (labeled to contain 70 mg of SIL per one tablet) to determine their average weight, then an amount of powdered tablets equivalent to 100 mg of SIL was transferred into a 100-mL volumetric flask, 40 mL of methanol was added, then the solution was sonicated for 15 min and the volume was completed with methanol, to obtain a stock test solution having a concentration of 1 mg mL^{-1}. The stock test solution was filtered; 5 mL was transferred into a 20-mL volumetric flask and completed to volume with diluting solvent, mixed well to obtain a working test solution of final concentration 0.25 mg mL^{-1}. This solution was injected directly into the HPLC system.

2.7.2. Preparation of test Hepapro® Capsules solution

Test solution was prepared by emptying the content of 20 capsules of Hepapro® (labeled to contain 100 mg of SIL and 50 mg of CUR per capsule) to determine their average weight. Then an amount equivalent to 100 mg of SIL and 50 mg of CUR was transferred into a 100-mL volumetric flask, and then preceded as under the Section 2.7.1, in order to obtain a working test solution of final concentration 0.25 and 0.125 mg mL^{-1} of SIL and CUR, respectively.

2.8. Forced degradation study
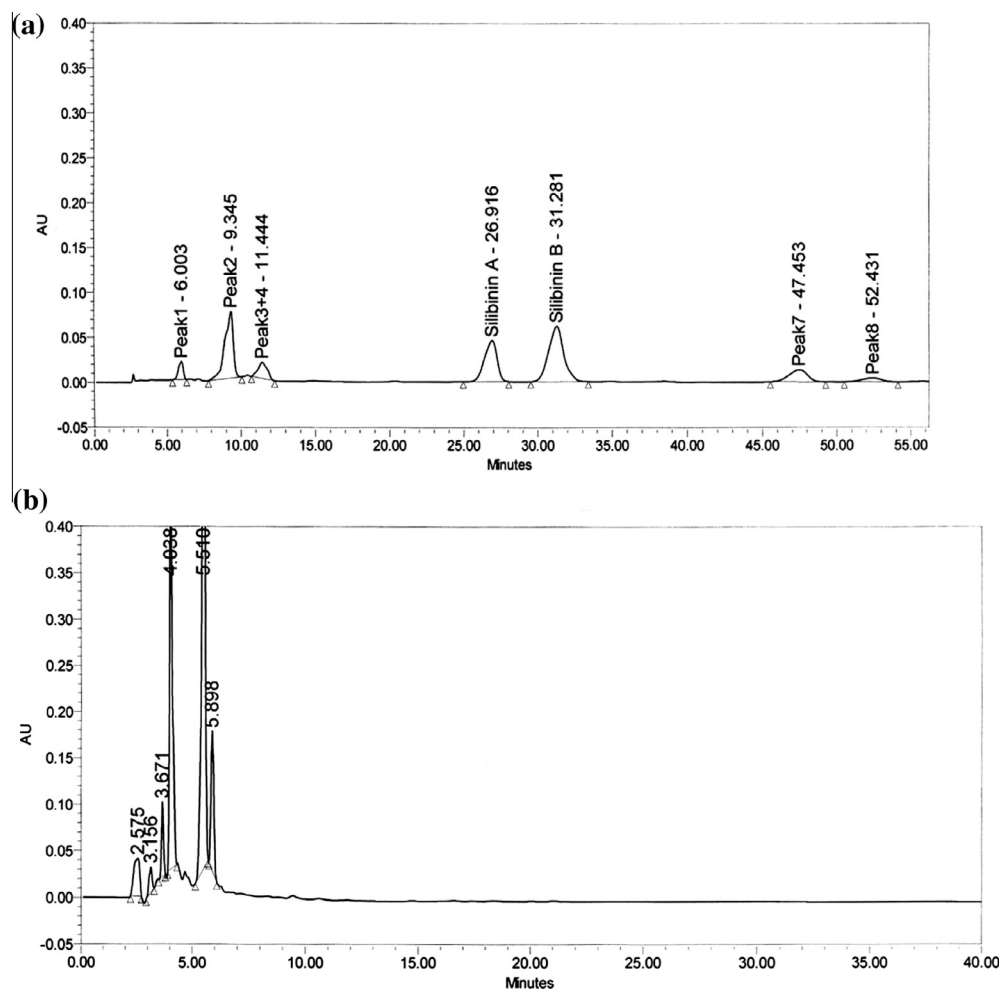
2.8.1. Acidic hydrolysis

A volume of 10 mL of the stock SIL or CUR solution for forced degradation was mixed with 10 mL of 2 N HCl into a 250-mL conical flask, heated in a water bath adjusted at 70 °C for four hours and cooled. Then the solution was neutralized with 2 N NaOH, transferred quantitatively into a 100-mL volumetric flask, completed to volume with diluting solvent and mixed well to give a final concentration of 0.25 mg mL^{-1} of SIL or 0.125 mg mL^{-1} of CUR. Finally these solutions were filtered and injected into the HPLC system using chromatographic conditions previously stated.

2.8.2. Alkaline hydrolysis

A volume of 10 mL of the stock SIL or CUR solution for forced degradation was mixed with 10 mL of 2 N NaOH into a 250-mL conical flask, heated in a water bath adjusted at 70 °C for 4 h and cooled. Then the solution was neutralized with 2 N HCl, and the procedure was continued as described under Section 2.8.1.

2.8.3. Neutral hydrolysis

A volume of 10 mL of the stock SIL or CUR solution for forced degradation was mixed with 10 mL of distilled water into a 250-mL conical flask, heated in a water bath adjusted at 70 °C for four hours and cooled, transferred quantitatively into a 100-mL volumetric flask, then the procedure was continued as described under Section 2.8.1.

Figure 1 HPLC chromatograms of working standard Silymarin solution (0.25 mg mL^{-1}) using methanol percentage of 35% (a) and 65% (b) in the mobile phase.

2.8.4. Photo-degradation

A volume of 10 mL of the stock SIL or CUR solution for forced degradation was mixed with 10 mL of distilled water into a 250-mL conical flask, exposed to sunlight for 8 h, transferred quantitatively into a 100-mL volumetric flask, then the procedure was continued as described under Section 2.8.1.

2.8.5. Oxidative degradation

A volume of 10 mL of the stock SIL or CUR solution for forced degradation was mixed with 10 mL of 30% H$_2$O$_2$ solution into a 250-mL conical flask, heated in a water bath adjusted at 70 °C for four hours, cooled, transferred quantitatively into a 100-mL volumetric flask, then the procedure was continued as described under Section 2.8.1.

2.8.6. Dry heat

500 mg of SIL or CUR working standard was weighed into a petri dish, inserted in an oven previously adjusted to 105 °C, left for 4 h then cooled. An amount of the powder was weighed and dissolved in methanol to obtain a solution having a concentration of 2.5 mg mL^{-1} of SIL or 1.25 mg mL^{-1} of CUR. A volume of 10 mL from each previous solution was transferred into a 100-mL volumetric flask and the volume was completed with a diluting solvent to obtain solutions having fi-

nal concentrations of 0.25 mg mL^{-1} and 0.125 mg mL^{-1} for SIL and CUR, respectively. Finally these solutions were filtered and injected into HPLC system using chromatographic conditions previously stated.

3. Results and discussion

3.1. Optimization of chromatographic conditions

3.1.1. Effect of the column type

The effect of different column types (packing material) on the simultaneous separation and determination of SIL and CUR peaks was studied using four different C18 columns (Symmetry, Symmetry sheild, Zorbax XBD and X-bridge column). This was done using a mobile phase composed of MeOH: phosphate buffer pH 2.3 in a ratio of 4:6 (v/v) and the most suitable separation between SIL peaks with the highest resolution between Silybin A and B peaks was achieved using the X-bridge column.

3.1.2. Effect of mobile phase mode

The effect of mobile phase mode (isocratic and gradient) on the simultaneous separation and determination of SIL and CUR peaks was studied. Isocratic elution was firstly tried

Table 1 Gradient elution of mobile phase for the chromatographic separation of Silymarin, Curcumin and their forced degradation products.

Time	0.05 M KH$_2$PO$_4$ pH 2.3% (V/V)	Methanol% (V/V)	Acetonitrile% (V/V)
0–3	65	35	–
3–12	65 → 55	35 → 45	–
12–15	55 → 50	45 → 50	–
15–24	50	50	–
24–24.1	50 → 55	50 → 0	0 → 45
24.1–35	55	–	45
35–35.1	55 → 65	0 → 35	45 → 0
35.1–38	65	35	–
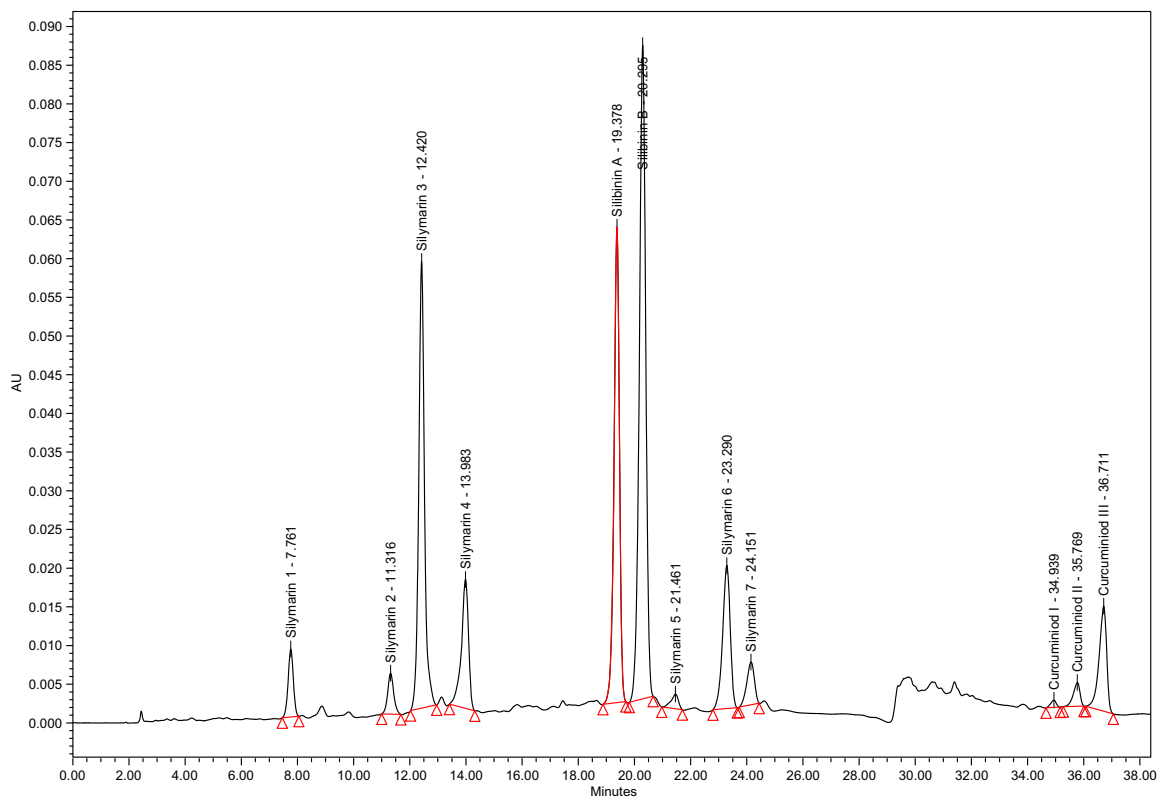
Figure 2 HPLC chromatogram of standard mixture solution of Silymarin 0.25 mg mL^{-1} and Curcumin 0.125 mg mL^{-1} obtained by the proposed HPLC method.

**Table 2** Chromatographic characteristics of Silymarin, and Curcumin peaks by the proposed HPLC method.

	t_r[a]	N[b]	K'[c]	[d]	R_s[e]	T_f[f]
Silybin A	19.38	49375	6.75			0.89
Silybin B	20.30	38290	7.11	1.05	2.45	0.92
Bisdemethoxycurcumin	34.94	179064	11.29	–	–	0.83
Demethoxycurcumin	35.77	102910	13.30	1.03	2.07	0.80
Curcumin	36.71	89022	13.68	1.03	2.07	0.80

[a] Retention time, in minutes.
[b] Number of theoretical plates.
[c] Capacity factor.
[d] Selectivity.
[e] Resolution, between each two successive peaks.
[f] Tailing factor.

using different MeOH ratios with the phosphate buffer pH 2.3 (Fig. 1). Low MeOH ratio resulted in an increase in the retention time of the peaks more than 55 min and this was unsuitable for HPLC, while increasing the MeOH ratio resulted in overlapping of SIL peaks. As a result, a gradient elution was selected (Table 1).

3.1.3. Effect of the temperature of the column

The effect of temperature of the column on the separation of SIL peaks was studied. Upon using a temperature of 25 °C, the retention time of SIL peaks was considered high and inapplicable for HPLC separation while at a temperature of 40 °C, the resolution between Silybin A and B was
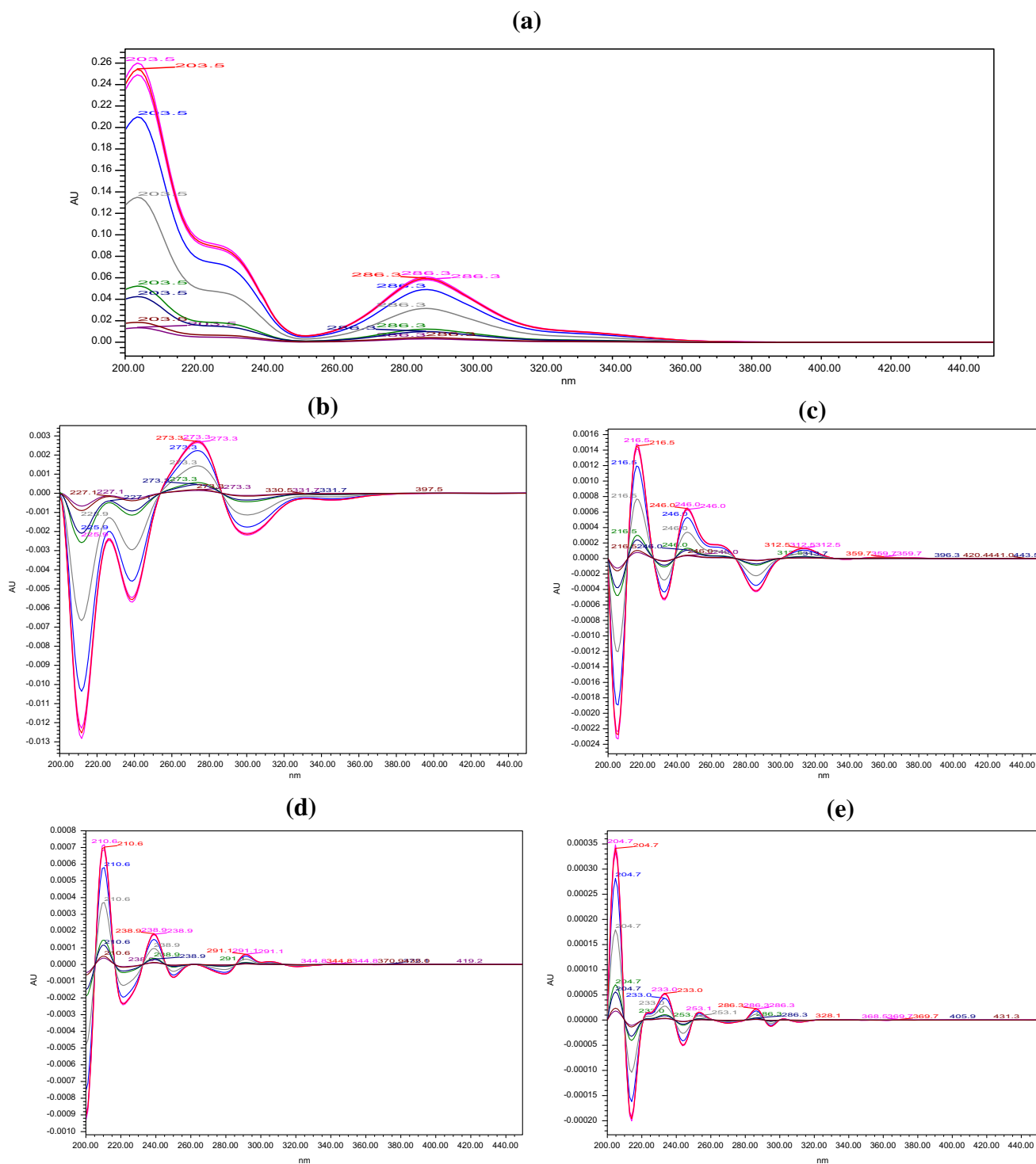
Figure 3 Spectra of Silybin A (a), as a representative example of confirming the purity of SIL peaks, at different time intervals, 1st derivative spectra (b), 2nd derivative spectra (c), 3rd derivative spectra (d) and 4th derivative spectra (e).

not suitable and did not conform to both USP 34 (United states Pharmacopeia, 2011) and BP (British Pharmacopoeia, 2012) 2012 monographs. However, at a temperature of 30 °C, SIL peaks eluted at shorter retention times compared to those at 25 °C and the resolution between Silybin A and B was conformed to both USP 34 and B.P 2012 monographs. So, 30 °C was chosen for the chromatographic measurement.

(a)

(b) **(c)**

(d) **(e)**
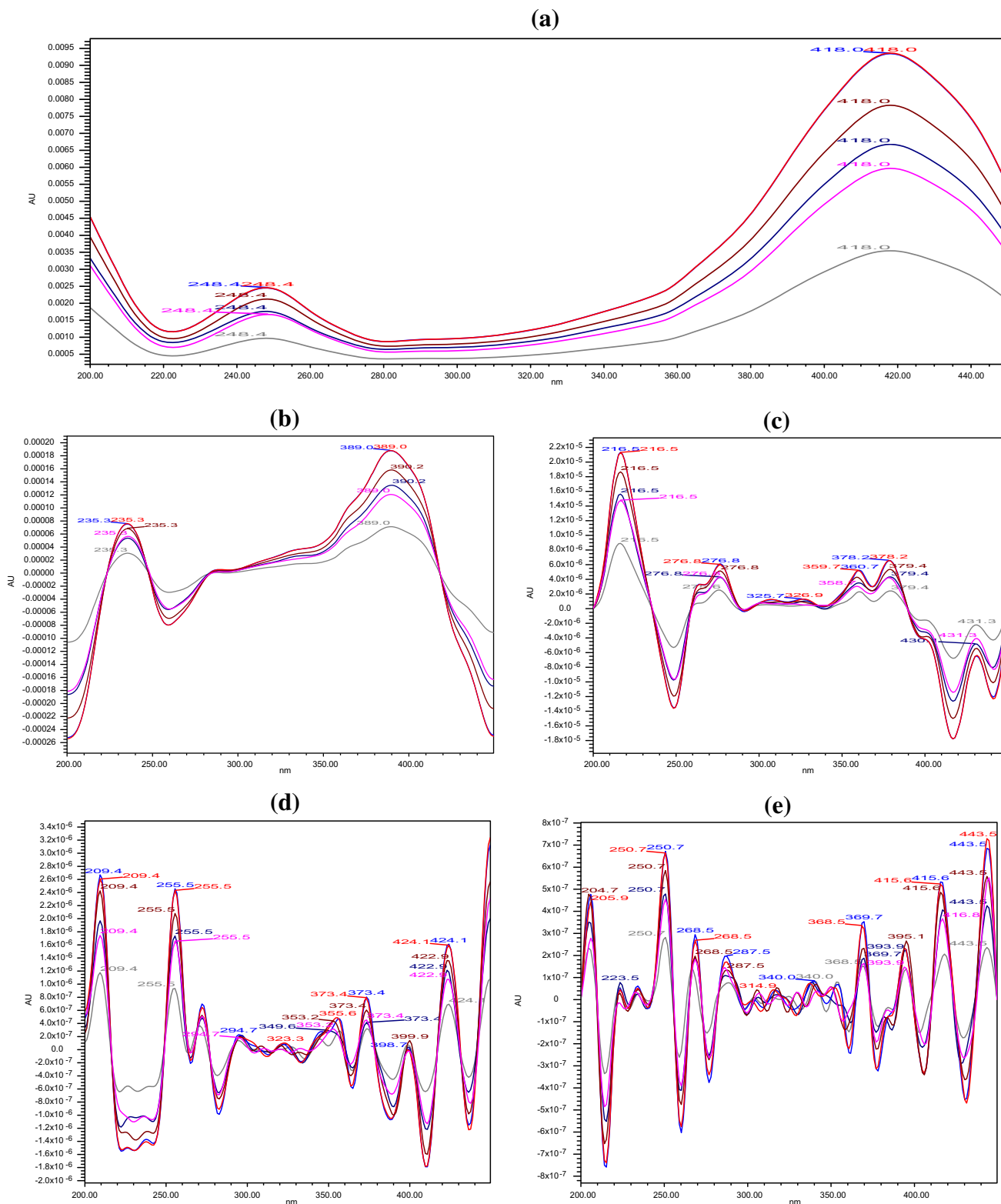
Figure 4 Spectra of bisdemethoxycurcumin (a), as a representative example of confirming the purity of CUR peaks, at different time intervals, 1st derivative spectra (b), 2nd derivative spectra (c), 3rd derivative spectra (d) and 4th derivative spectra (e).

3.1.4. Selection of phosphate buffer pH

The pH of phosphate buffer was selected according to buffer capacity of phosphate which resulted in more resistance to pH changes and this, in turn, led to less variation in retention times and to a more robust method.

3.2. HPLC method development

Gradient elution was used for the simultaneous determination of SIL and CUR (Fig. 2) with good chromatographic characteristics, Table 2. The gradient elution was beneficial in the separation of high number of peaks belonging to both drugs (SIL and CUR) in a relatively short run time which greatly saves time and money and helps in increasing the number of theoretical plates (N).

The HPLC method achieved a good resolution between Silybin A and B (good resolution and selectivity values) which is conformed to both USP and BP monograph requirements, and a good resolution between the three CUR peaks (Curcumin, demethoxycurcumin and bisdemethoxycurcumin) within a reasonable run time (suitable capacity factors). In addition, high column efficiency was indicated from the large number of theoretical plates of both SIL and CUR peaks. The degree of peak symmetry was also evaluated using the tailing factor which did not exceed the critical value (1.2) indicating an acceptable degree of peak asymmetry. (Table 2 and Fig. 2).
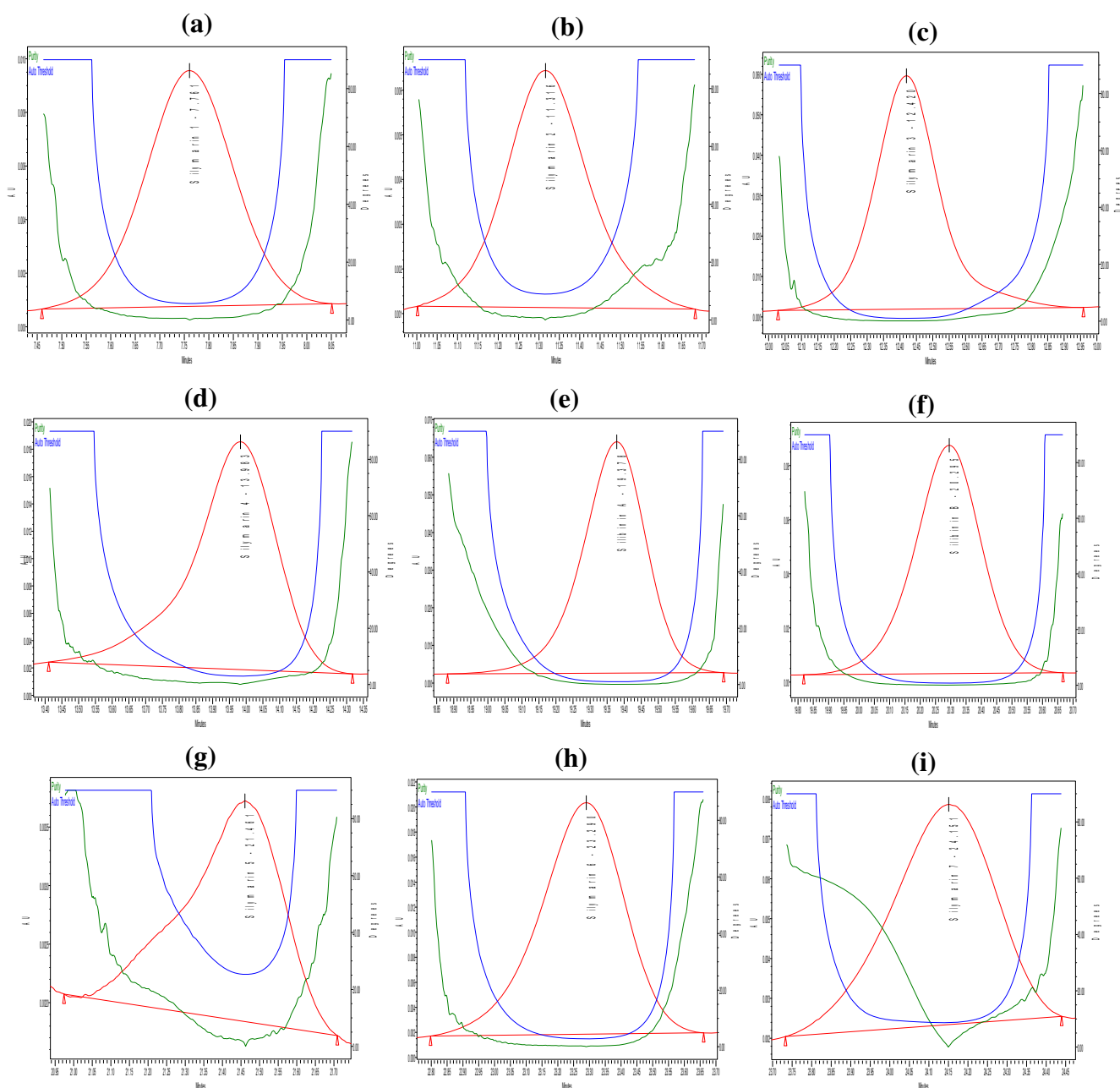
Figure 5   Peak purity plots of Silymarin peaks, SIL 1-4 (a–d), silybin A and B (e and f) and SIL 7-9 (g–i), respectively.

3.3. Peak purity assessment

Fig. 2 shows nine SIL peaks and three CUR peaks well separated from each other and within suitable retention times. For SIL, seven SIL peaks were separated and named SIL1 to SIL 7 with the two peaks of Silybin A and B (Silibinin A and B) identified as the main two peaks in SIL(United states Pharmacopeia, 2011; British Pharmacopoeia, 2012; Liu et al., 2009; Radjabian et al. 2008 and Hadad et al., 2009). The three CUR peaks were separated and identified as bisdemethoxycurcumin, demethoxycurcumin and Curcumin, respectively (Lechtenberg et al., 2004; Phattanawasin et al., 2009). In the present work the chromatographic peak purity was assessed as follows:

3.3.1. Use of peak profile

The spectra (A vs λ), D^1, D^2, D^3 and D^4 of Silybin A, Silybin B, bisdemethoxycurcumin, demethoxycurcumin and Curcumin, were recorded at different time intervals across their chromatographic peaks obtained from the DAD detector indicating the purity of the peaks (Figs. 3 and 4)

3.3.2. Use of peak purity plot

The peak purity plot in which the purity angle was found to be less than the purity threshold, gave additional confirmation of the purity of the nine SIL peaks and the three CUR peaks (Figs. 5 and 6).
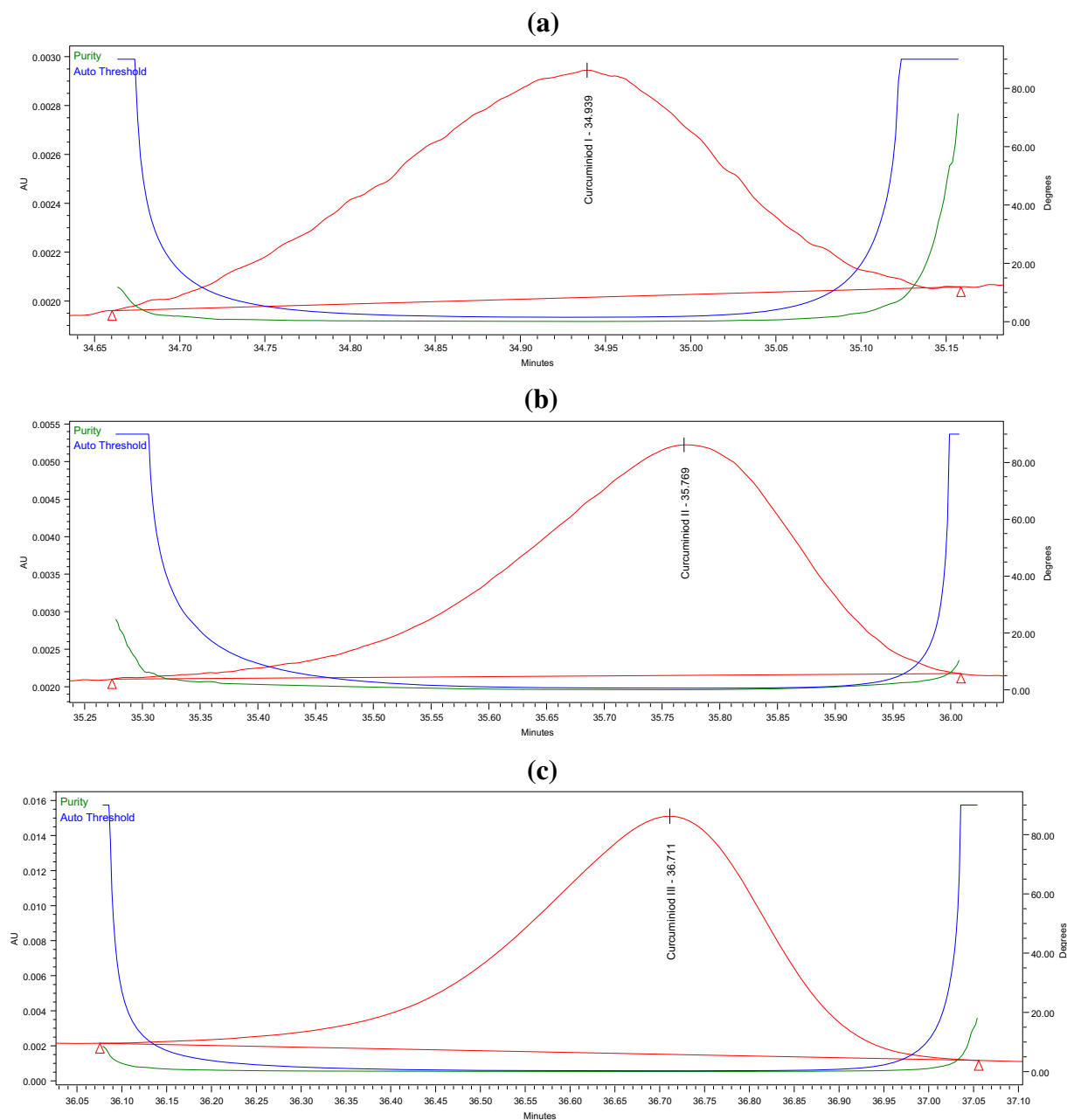
Figure 6 Peak purity plots of Curucmin peaks, bisdemethoxycurcumin (a), demethoxycurcumin (b) and Curcumin (c), respectively.

Table 3 Evaluation of the accuracy for the determination of Silymarin (SIL) and Curcumin (CUR) by the proposed HPLC method according to ICH guidelines.

Nominal value (mg mL^{-1})	Mean recovery%[a] ± SD	Grand mean[b]	Pooled SD[c]	S.E[d]
SIL				
0.20	100.20 ± 0.408	100.05%	0.946	0.315
0.25	100.02 ± 1.27			
0.30	99.92 ± 0.950			
CUR				
0.10	100.51 ± 0.618	100.12%	0.883	0.294
0.125	100.26 ± 1.21			
0.15	99.58 ± 0.696			

[a] Mean of %recovery of the three different solutions at each concentration level for each drug.
[b] Mean of recovery of the three different concentration levels.
[c] Pooled standard deviation of all recoveries for the three different concentration levels.
[d] Standard error of the mean.

Table 4 Evaluation of the intra-day precision for the determination of Silymarin and Curcumin by the proposed HPLC method according to ICH guidelines.

Assay number	Mean recovery%[a]	
	Silymarin	Curcumin
1	101.81	101.33
2	101.70	101.17
3	102.01	101.96
4	101.75	102.34
5	101.62	101.66
6	101.34	101.65
Mean%[b]	**101.71**	**101.69**
SD[c]	**0.222**	**0.424**
RSD%[d]	**0.218**	**0.417**

[a] Recovery of each individual assay, for a concentration of 0.25 mg mL^{-1} Silymarin and 0.125 mg mL^{-1} Curcumin.
[b] The mean of all recoveries of all determinations ($n = 6$).
[c] Standard deviation of the recoveries of all determinations.
[d] Percentage relative standard deviation of all determinations.

Table 5 Evaluation of the inter-day precision for the determination of Silymarin (SIL) and Curcumin (CUR) by the proposed HPLC method according to ICH guidelines.

	Mean recovery%[a]	
Day	SIL	CUR
1	100.33	101.45
2	100.70	101.14
3	99.99	100.24
Grand mean%[b]	**100.34**	**100.94**
Pooled SD[c]	**0.869**	**0.457**
RSD%[d]	**0.866**	**0.453**

[a] Mean recovery, for a concentration of 0.25 mg mL^{-1} Silymarin and 0.125 mg mL^{-1} Curcumin for each day.
[b] The grand mean of recoveries of all determinations in the three different days.
[c] Pooled standard deviation of all recoveries for the three different days.
[d] Percentage relative standard deviation of all determinations.

3.4. Method validation

The method was validated according to ICH guidelines (International Conference on Harmonization, 2005). The following validation characteristics were addressed.

3.4.1. Linearity
Peak area (sum of all nine peaks of SIL and sum of three peaks of CUR) against concentration curves were obtained for both SIL and CUR in the ranges of 0.15–0.35 and 0.075–0.175 mg mL^{-1}, respectively. An excellent linearity ($r = 0.9999$ for SIL and 0.9997 for CUR) was obtained with regression equations of $y = 27196878x - 115236$ and $y = 10134416x + 12168$, for SIL and CUR, respectively. Good regression lines (Armitage and Berry, 1994) showing high (r) values with low ($S_{y/x}$) values of 28,486 and 11,996 for SIL and CUR, respectively were obtained.

3.4.2. Detection and quantitation limits
Limits of detection (LOD) and quantitation (LOQ) were calculated by using the residual standard deviation of the regression line ($S_{y/x}$) of the calibration curve and its slope (b). LOD is equal to ($y_B + 3 \ S_B$) where y_B is the value of the calculated intercept and S_B is the $S_{y/x}$, while LOQ is equal to ($y_B + 10 \ S_B$) (Miller and Miller, 2005). LOD and LOQ calculated by this statistical method were 0.0031, 0.0035 and 0.0104, 0.0118 mg mL^{-1} for SIL and CUR, respectively.

3.4.3. Accuracy
The accuracy of the proposed HPLC method was evaluated by analyzing three laboratory prepared solutions of a standard mixture of SIL and CUR (added to placebo) at three different concentration levels within the working linearity range of both drugs. Accuracy was expressed as the recovery% (International Conference on Harmonization, 2005) and standard error for SIL and CUR. Table 3 illustrates the method accuracy for SIL and CUR with satisfactory recovery%. Small values for both standard error and pooled standard deviation were obtained.

3.4.4. Precision
The precision of an analytical method gives information on the random errors. It expresses the closeness of agreement between a series of measurements obtained from multiple sampling of

Table 6 Evaluation of the robustness for the determination of Silymarin and Curcumin by the proposed HPLC method according to ICH guidelines.

| Parameters		Recovery%[a]		% Change [b]		Mean recovery% ± SD[c]		RSD%[d]	
		SIL	CUR	SIL	CUR	SIL	CUR	SIL	CUR
pH of phosphate buffer (2.2, 2.3 and 2.4)	2.2	100.84	102.05	−0.069%	0.860%	100.57 ± 0.535	101.52 ± 0.463	0.532	0.456
	2.3[*]	100.91	101.18	–	–				
	2.4	99.95	101.34	−0.951%	0.158%				
Temperature (29, 30 and 31 °C)	29	100.12	100.25	0.250%	−0.516%	99.75 ± 0.448	100.68 ± 0.397	0.449	0.394
	30[*]	99.87	100.77	–	–				
	31	99.25	101.03	−0.621%	0.258%				

[*] Analytical pH and temperature of the proposed method.

[a] The recovery of the Silymarin and Curcumin samples at each pH value and temperature for a concentration of 0.25 mg mL^{-1} Silymarin and 0.125 mg mL^{-1} Curcumin.

[b] % change in the recovery from the analytical point.

[c] The mean of recoveries at different pH values and temperatures ± their standard deviation

[d] Percentage relative standard deviation at the three pH values and temperatures.
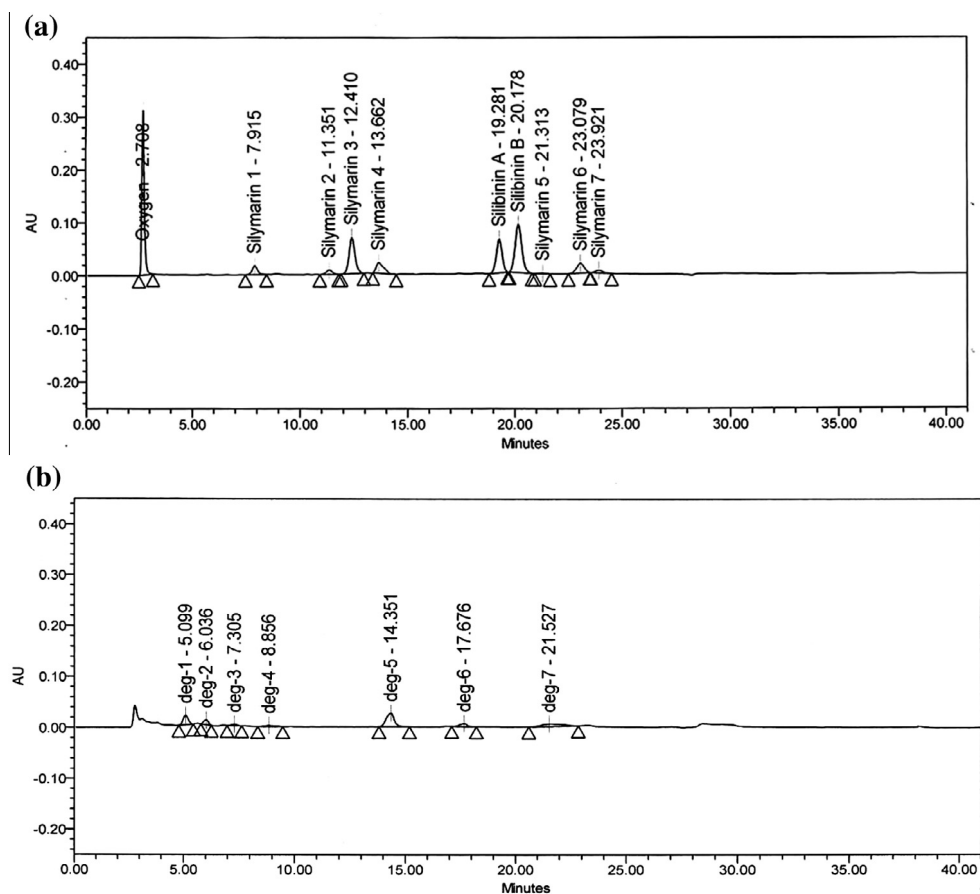
Figure 7 HPLC chromatograms of a working standard solution of Silymarin 0.25 mg mL^{-1} after oxidative degradation with 30% H$_2$O$_2$ (a) and alkaline hydrolysis with 2 N NaOH (b).

the same homogenous sample under prescribed conditions (International Conference on Harmonization, 2005). It is divided into repeatability (intra-day precision), intermediate precision (inter-day precision).

3.4.4.1. Repeatability (intra-day). The intra-day variation for the determination of SIL and CUR was carried out by analyzing dosage form sample solutions (Hepapro® Capsules) pre-

pared at 100% of test concentration stated in the method (0.25, 0.125 mg mL^{-1} of SIL and CUR, respectively) using the analytical procedure, within the same laboratory, using the same analyst, with the same equipment, on the same day. Repeatability was assessed using six determinations each injected twice, by calculating the RSD% values obtained by repeating the assay six times on the same day (Table 4).

3.4.4.2. Intermediate precision (Inter-day). The inter-day precision for the determination of SIL and CUR was carried out as described under repeatability but on three consecutive days, each of two determinations ($n = 6$). Precision was expressed as the percentage relative standard deviation (% RSD) for SIL and CUR (Table 5). The data indicated a good agreement among the individual test results. The criterion for intra-day and inter-day precisions demands a RSD less than 2%.

3.4.5. Robustness

Robustness of the proposed method was evaluated by analyzing SIL and CUR dosage form (Hepapro® Capsules) at the same concentration level used under precision at intentional slight variation of the selected parameters indicated in Table 6. The RSD% of the recovery obtained by analyzing the same sample after introducing small deliberate changes in the method parameters was calculated and was found to be less than 2% indicating the robustness of the method (Table 6).

3.4.6. Specificity

The simultaneous determination of SIL and CUR in their pharmaceutical preparations revealed that the normally existing excipients and additives did not interfere in the applied HPLC method. So, pharmaceutical preparations containing SIL (Legalex® Tablets) and pharmaceutical preparations containing both SIL and CUR (Hepapro® Capsules) were successfully analyzed by the proposed HPLC method.
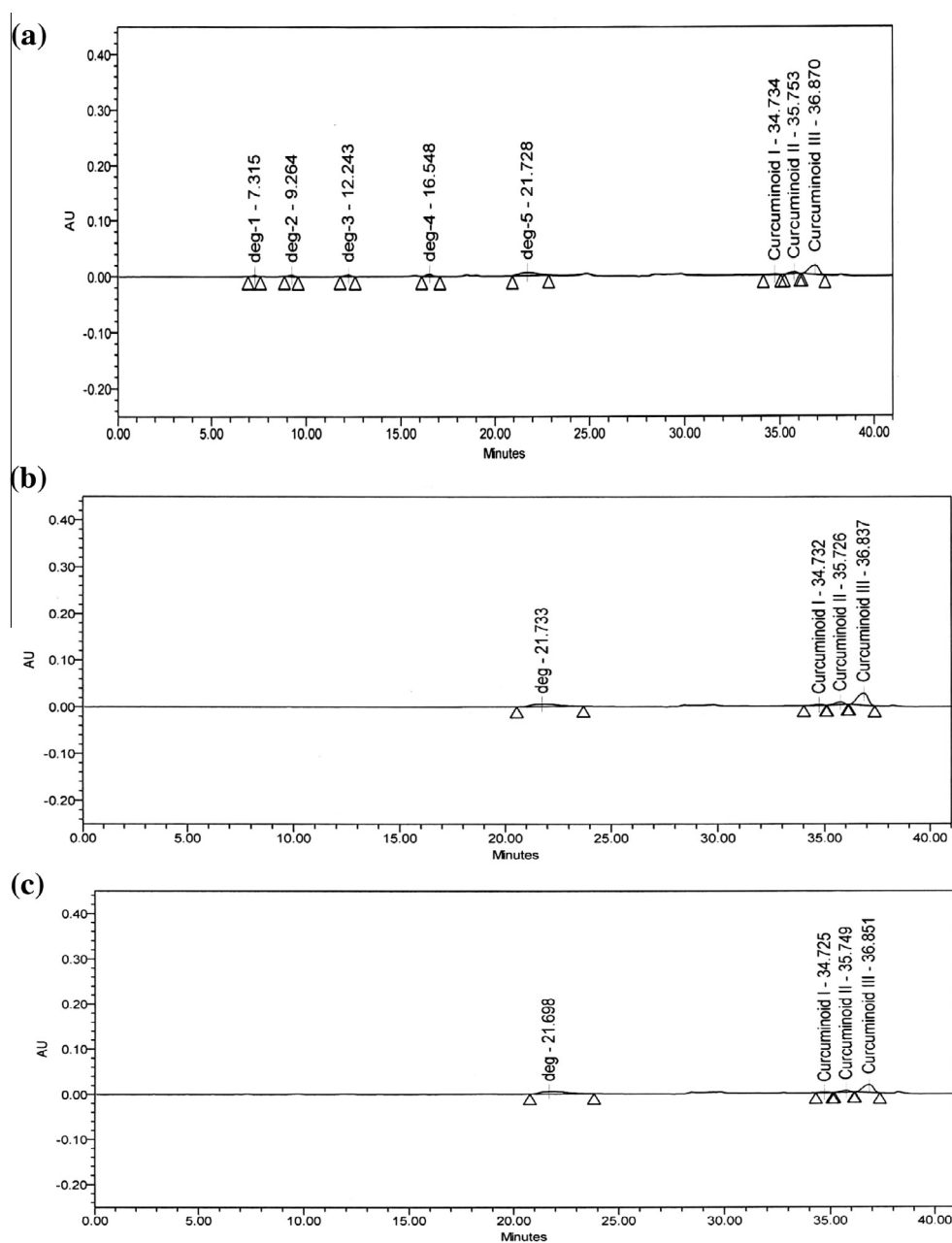
Figure 8 HPLC chromatograms of a working standard solution of Curcumin 0.125 mg mL^{-1} after acid hydrolysis with 2 N HCl (a) neutral hydrolysis with H$_2$O (b) and photo-degradation with sunlight (c).
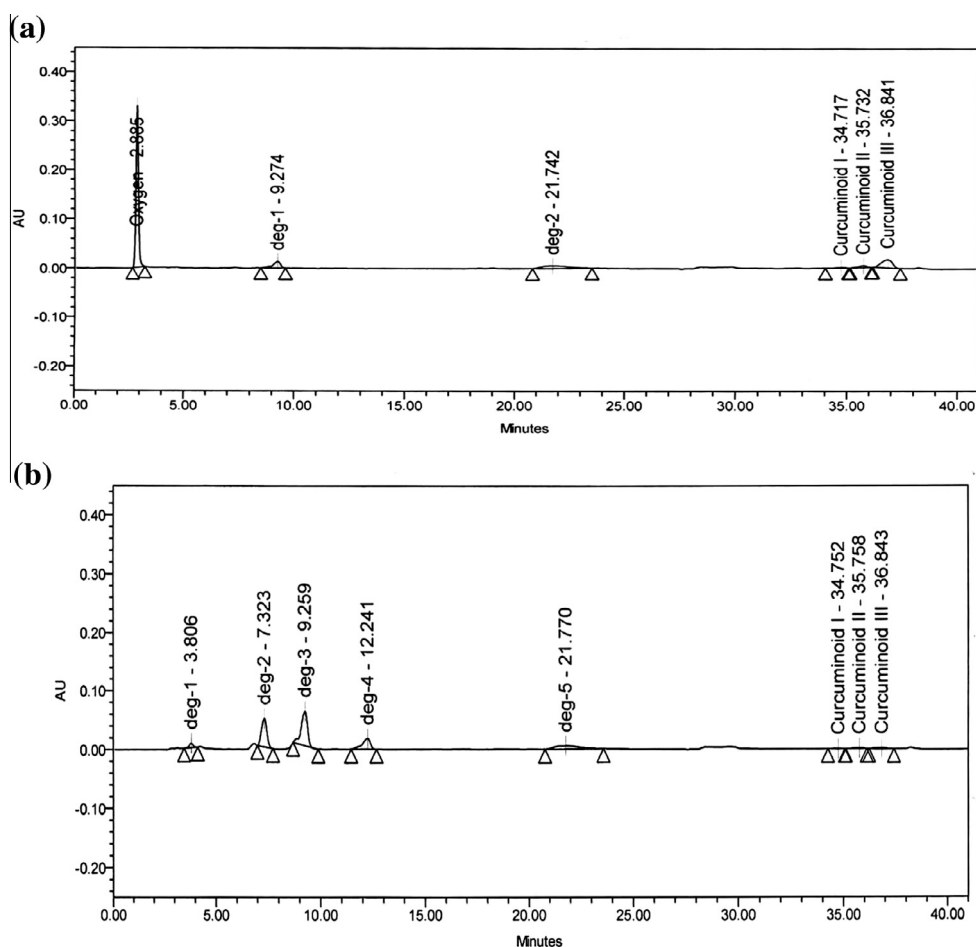
Figure 9 HPLC chromatograms of a working standard solution of Curcumin 0.125 mg mL^{-1} after oxidative degradation with 30% H$_2$O$_2$ (a) and alkaline hydrolysis with 2 N NaOH (b).

Table 7 Analysis of Silymarin and Curcumin in different dosage forms by the external standard method.

Dosage form Legalex® tablets	Recovery%[a]	Mean recovery% ± RSD%[b]
	Silymarin	100.19 ± 0.374
	100.45	
	99.92	
Hepapro® Capsules	*Silymarin*	100.49 ± 0.380
	100.76	
	100.22	
	Curcumin	100.78 ± 0.603
	100.35	
	101.21	

[a] The recovery% of the duplicate determinations of a concentration of 0.25 mg mL^{-1} of Silymarin in Legalex® Tablets and Hepapro® Capsules dosage forms and of a concentration of 0.125 mg mL^{-1} of Curcumin in Hepapro® Capsules.
[b] The mean recovery% ± RSD% of duplicate determinations.

3.4.7. Forced degradation study

The effect of the presence of different forced degradation products on the purity of SIL and CUR peaks was studied and the method was found to be stability-indicating.

Table 8 Analysis of Silymarin tablets dosage form by the proposed method and the reference method (BP method).

Number of assay	Reference method	Proposed method
	Recovery%[a]	Recovery%[a]
1	100.72	101.11
2	101.88	102.11
3	101.55	100.95
4	101.64	100.75
5	99.98	100.29
Mean recovery%[b]	101.15	101.04
SD[c]	0.788	0.672
Variance	0.621	0.452
RSD%[d]	0.780	0.665
F-test[*]	1.374	
t-test[*]	0.238	

[a] The recovery% of a concentration of 0.25 mg mL^{-1} of the Silymarin in Legalex Tablets dosage form.
[b] The mean of all recoveries of all determinations ($n = 5$).
[c] Standard deviation of the recoveries of all determinations.
[d] Percentage relative standard deviation of all determinations.
[*] Theoretical values for F and t tests at $p = 0.05$ are 6.388 at (4, 4) degree of freedom and 2.31 at (8) degree of freedom, respectively.

3.4.7.1. Silymarin. The peaks of SIL were not affected by acidic and neutral hydrolysis, photo-degradation, oxidative degradation or dry heat. Their peak areas did not change. For the oxidative degradation, a peak attributed to oxygen eluted at 2.708 min, far from the retention time of both SIL peaks and CUR peaks (Fig. 7a).

For the alkaline hydrolysis, the peaks of SIL were totally destroyed with the appearance of seven other foreign degradation peaks which eluted far from the retention times of both SIL peaks and CUR peaks (Fig. 7b). In all cases of the forced degradation, the purity angles of SIL peaks were found to be smaller than purity threshold which indicated the purity of these peaks.

3.4.7.2. Curcumin. For the acidic and neutral hydrolysis, photo-degradation and oxidative degradation, the peaks of CUR were partially degraded as the peak areas of CUR were decreased by 43%, 26%, 37% and 41%, respectively with the appearance of different degradation product peaks eluted far from the retention time of both SIL and CUR peaks (Figs 8a–c). For the oxidative degradation, in addition to the degradation product peaks, the oxygen peak eluted far from the retention time of both SIL and CUR peaks (Fig. 9a).

For the alkaline hydrolysis, the peaks of CUR were nearly completely destroyed as the peak areas of CUR were decreased by 92% with the appearance of five degradation products peaks eluted far from the retention times of both SIL and CUR peaks (Fig. 9b).

For the dry heat, the peaks of CUR were not affected and their peak areas did not change. In all cases of forced degradation, the purity angles of CUR peaks were smaller than the purity threshold which indicates the purity of these peaks.

3.5. Analysis of pharmaceutical formulation

Using the proposed HPLC method, determination of SIL in its dosage form (tablets) and of both SIL and CUR in their dosage form (capsules) was carried out using the external standard method. SIL was analyzed in different dosage forms including: Legalex® Tablets and Hepapro® Capsules while CUR was analyzed in Hepapro® Capsules. The assay was evaluated in duplicate. Satisfactory results were obtained for each dosage form in a good agreement with the label claims (Table 7).

The results of the assay of SIL in Legalex® Tablets were statistically compared to those of the pharmacopoeial method, BP method (British Pharmacopoeia, 2012), using the student's *t*-test and variance ratio *F*-test (Table 8). The experimental values of *t*-test and *F*-test did not exceed the theoretical ones, thus, indicating the absence of any significant difference between the compared methods.

4. Conclusion

A gradient HPLC method has been developed for the simultaneous determination of Silymarin and Curcumin in their dosage forms. The advantages of the proposed method over the previously reported ones is the use of a DAD detector which is widely available in the ordinary laboratories, with no need for the more sophisticated mass or fluorescence detectors. Another noted advantage is its stability-indicating power and its ability to selectively analyze the studied drugs in the presence of their forced degradation products.

References

Anand, P., Kunnumakkara, A., Newman, R., Aggarwal, B., 2007. Bioavailability of Curcumin: problems and promises. Mol. Pharm. 4, 807.

Ansari, M.J., Ahmad, S., Kohli, K., Ali, J., Khar, R.K., 2005. Stability-indicating HPTLC determination of curcumin in bulk drug and pharmaceutical formulations. J. Pharma. Biomed. Anal. 39, 132.

Armitage, P., Berry, G., 1994. Statistical methods in medical research, third ed. Blackwell Scientific Publications, Oxford, England.

Basnet, P., Skalko-Basnet, N., 2011. Review Curcumin: an anti-inflammatory molecule from a curry spice on the path to cancer treatment. Molecules 16, 4567.

British Pharmacopoeia, 2012. The Stationary Office, London.

Dandekar, P.P., Patravale, V.B., 2009. Development and validation of a stability-indicating LC method for Curcumin. Chromatographia 69, 871.

Gören, A.C., ÇikrikÇi, S., Çergel, M., Bilsel, G., 2009. Rapid quantitation of curcumin in turmeric via NMR and LC–tandem mass spectrometry. J. Food Chem. 113, 1239.

Hadad, G.M., Emara, S., Abdel-Salam, R.A., 2009. Validated optimized method for simultaneous analysis of active silymarin components and dimethyl-4,40-dimethoxy-5,6,50,60-dimethylene dioxybiphenyl-2,20-dicarboxylate in a pharmaceutical preparation by use of a monolithic silica C18 column. Chromatographia 70, 217.

Han, Y.R., Zhu, J.J., Wang, Y.R., Wang, X.S., Liao, Y.H., 2011. A simple RP-HPLC method for the simultaneous determination of curcumin and its prodrug, curcumin didecanoate, in rat plasma and the application to pharmacokinetic study. Biomed. Chromatogr. 25, 1144.

Hassan, E.M., Khamis, E.F., El-Kimary, E.I., Barary, M.A., 2008. Development of a differential pulse voltammetric method for the determination of Silymarin/Vitamin E acetate mixture in pharmaceuticals. Talanta 74, 773.

International Conference of Harmonization, 2005. Q2 (R1), Validation of Analytical Procedures: Text and Methodology, International Conference on Harmonization, Geneva, (http://www.ich.org/LOB/media/MEDIA417.pdf).

Kosina, P., Bartek, J., 2000. Determination of Silybin in blood plasma using high performance liquid chromatography with solid phase extraction. Chem-Listy 94, 1115.

Lechtenberg, M., Quandt, B., Nahrstedt, A., 2004. Quantitative determination of Curcuminoids in curcuma rhizomes and rapid differentiation of *Curcuma domestica* Val. and *Curcuma xanthorrhiza* Roxb. by capillary electrophoresis. Phytochem. Anal. 15, 152.

Liu, H., Du, Z., Yuan, Q., 2009. A novel rapid method for simultaneous determination of eight active compounds in silymarin using a reversed-phase UPLC-UV detector. J. Chromatogr. B 877, 4159.

Miller, J.N., Miller, J.C., 2005. Statistics and Chemometrics for Analytical Chemistry, fifth ed. Prentice Hall, Harlow, England.

Moin, A.M., Patel, C.N., Dave, J.B., Badmanaban, R., Patel, J.A., 2010. Validated method for Silymarin by spectrophotometry in bulk drug and pharmaceutical formulations. J. Chem. Pharm. Res. 2, 396.

Nagappan, K.V., Meyyanthan, S.N., Raja, R.B., Kannan, E., 2009. A liquid chromatography method for the simultaneous determination of Curcumin and piperine in food products using diode array detection. Asian J. Res. Chem. 2, 115.

Park, E.J., Jeon, C.H., Ko, G., Kim, J., Sohn, D.H., 2000. Protective effect of curcumin in rat liver injury induced by carbon tetrachloride. J. Pharm. Pharmacol. 52, 437.

Parveen, R., Ahmed, S., Baboota, S., Ali, J., Alka, A., 2010. Stability-indicating HPTLC method for quantitative estimation of silybin in bulk drug and pharmaceutical dosage form. Biomed. Chromatogr. 24, 639.

Phattanawasin, P., Sotanaphun, U., Sriphong, L., 2009. Validated TLC-image analysis method for simultaneous quantification of Curcuminoids in Curcuma longa. Chromatographia 69, 397.

Quaglia, M., Bossu, E., Donati, E., Mazzanti, G., Brandt, A., 1999. Determination of Silymarin in the extract from the dried Silybum marianum fruits by high-performance liquid chromatography and capillary electrophoresis. J. Pharma. Biomed. Anal. 19, 435.

Radjabian, T., Rezazadeh, S., Huseini, H.F., 2008. Analysis of Silymarin components in the seed extracts of some milk thistle ecotypes from Iran by HPLC. Iranian J. Sci. Technol. 32, 141.

Schiborr, C., Eckert, G.P., Rimbach, G., Frank, J., 2010. A validated method for the quantification of curcumin in plasma and brain tissue by fast narrow-bore high-performance liquid chromatography with fluorescence detection. Anal. Bioanal. Chem. 397, 1917.

Sweetman, S.C., 2009. Martindale: The Complete Drug Reference, thirty-sixth ed. Pharmaceutical Press.

United States Pharmacopeia, 2011. Thirty-fourth ed. National formulary 29th ed. Asian ed. By authority of the United States Pharmacopeial Convention, Prepared by the Council of Experts and its Expert Committees. The United States Pharmacopeial Convention, 12601 Twinbrook Parkway, Rockville, MD 20852.

Wang, F., Hung, W., Wang, Y., 2008. Fluorescence enhancement effect for the determination of curcumin with yttrium(III) – curcumin – sodium dodecyl benzene sulfonate system. J. Lumin. 128, 110.

Wang, K., Zhang, H., Shen, L., Du, Q., Li, J., 2010. Rapid separation and characterization of active flavonolignans of Silybum marianum by ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry. J. Pharma. Biomed. Anal. 53, 1053.

Wichitnithad, W., Jongaroonngamsang, N., Pummangura, S., Rojsitthisak, P., 2009. A simple isocratic HPLC method for the simultaneous determination of Curcuminoids in commercial turmeric extracts. Phytochem. Anal. 20, 314.

Wu, J.W., Lin, L.C., Hung, S.C., Chi, C.W., Tsai, T.H., 2007. Analysis of silibinin in rat plasma and bile for hepatobiliary excretion and oral bioavailability application. J. Pharma. Biomed. Anal 45, 635.

Yang, X., Ma, J., Ye, Y., Zhang, Y., Lin, G., Zheng, Y., Li, X., Wang, X., 2011. LC–APCI–MS quantitative analysis of Curcumin in rabbit plasma. Chromatographia 73, 605.