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Optimization and Validation of HPLC-DAD Method for the Quantification of Curcuminoids in Turmeric (Curcuma Longa L.)

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Abstract

Curcumin and its derivatives named curcuminoids are naturally occurring polyphenolic compounds extensively marketed as a nutraceutical. The wide therapeutic potential of curcumin is highly explored by the authors. Therefore, pharma industries made many clinical inventions to bring it to the market as a drug. In this study, a simple, rapid, and sensitive High Performance Liquid chromatographic (HPLC) method for simultaneous determinations of curcumin, desmethoxycurcumin (DMC), and bis-desmethoxycurcumin (BDMC) was optimized and validated. Separation of curcumin, DMC, and BDMC was performed using the C18 column. Six factors, including the solvent type, ratio of mobile phase, flow rate of mobile phase, column temperature, injection volume, and wavelength, were investigated. The LOD and LOQ were observed to be 0.001 and 0.003 mg/L, R^2 value < 0,99, respectively. RSD (%) is 0.974 for inter-day, and 1,312 for intra-day. Taking into consideration the retention time, peak area, resolution, and tailing factor, the optimum conditions were preferred: methanol as solvent, THF/Citric Acid (40/60) as mobile phase, flow rate of mobile phase at 0.5 mL/min, temperature of column at 30^{o}C, injection volume at 20 µL and wavelength at 425 nm. The method is further used to determine ppm levels of curcumin derivatives, and recovery was found to be 90%. The proposed analytical method can be used for the quantification of curcumin in medicinal plants.

Keywords: Curcumin, Method validation, HPLC, Optimization in analysis

Introduction

Depending on its origin and the soil conditions, curcumin is the active ingredient of turmeric having 2%–9% curcuminoids. The "curcuminoid" mean a group of compounds such as curcumin, demethoxycurcumin (DMC) and bis-demethoxycurcumin (BDMC). Compared to curcumin, recent studies reported that DMC and BDMC also had similar or higher bioactivity [1]. For example, BDMC is the most effect anticancer activities compared to DMC and curcumin [2-3]. Furthermore, it is also reported that the mixture of three curcuminoids in a mixture of 77% pure curcumin, 17% DMC, and 3% BDMC is more effective than single curcuminoids separately [4]. This result can be attributed to the possible synergistic effect [1-4]. Curcumin can be dissolved in methanol, chloroform, ethanol, and acetone but more slightly in water. For separating curcumin from other components and its derivatives, column chromatography has been the most generally applied method using polar and non-polar organic solvents. In recent years, pulse ultrasonic and microwave-assisted extraction methods have been popular among commonly

used methods such as Soxhlet and ultrasonic extraction as well as microwave and similar methods [1, 5-6]. In column chromatography, the ability of curcumin and its derivatives are different for adsorbing on silica gel. These compounds using the solvents mixtures like methanol/chloroform or dichloromethane/ acetic acid can be separated, and three different fractions can be obtained. To further purify curcumin fraction on silica gel, the mixtures of chloroform/dichloromethane and ethanol/methanol as eluents are used [7].

The standard curcumin reagent contains approximately 80% curcumin, 15% DMC, 3% BDMC components [3]. In addition to its biological activity, curcumin can chelate metals by forming new complexes thanks to the β-diketo group in its structure [8]. To determine curcumin and its derivatives in turmeric, several methods are used. These methods include UV-Vis, HPLC, LC/ MS, and the spectrofluorimetric procedure [6-13]. Among them, the most used technique is HPLC due to its high separation capability compared to the other methods [2, 10, 12-14].

In this study, the HPLC-DAD method was used by optimizing the conditions for the separation of curcumin, DMC, and BDMC from turmeric (*Curcuma longa L.*). To achieve those goals, the method was examined to find the best conditions with different parameters including the mobile phase composition, rate of flowing, column temperature, wavelength, and injection volume. With the method applied in this article, better LOD and LOQ values were obtained than the results obtained from previous similar studies [16-18].

Materials and Methods

Reagents

The solvents include methanol (Merck), acetonitrile (Merck), formic acid (Merck), ethyl acetate (Sigma-Aldrich), isopropanol (Sigma-Aldrich), acetic acid (Merck), orthophosphoric acid (Merck), ethanol (Sigma-Aldrich), 1-butanol (Sigma-Aldrich), hydrochloric acid (Merck), tetrahydrofuran (Merck), citric acid (Fluka), acetone (Sigma-Aldrich) was used.

Instruments

The analysis was carried out with an Agilent 1260 HPLC-DAD and ACE 5 C18 (125 x 4.0 mm, 5 µm) column. The C18 column and detector signal after each injection were stabilized by waiting the necessary time.

Sample collection and extraction

In this study, turmeric (*Curcuma longa* L.) was obtained from the herbalist. After being properly cleaned, it was dried in the open air so as not to be exposed to sunlight. The samples brought to constant weighing were ground in an electric grinder until they turned into powder. It was kept under moisture-proof until extraction.

Extraction was performed with 8 different solvents, the most common in the literature. So, various solvents having different polarities were used for extraction. These solvents were ethanol, 1-butanol, ethyl acetate, methanol, 1% HCl: methanol, 80% isopropanol, acetonitrile, and distilled water. The yield was determined after enrichment of each extract, and the percentage of each curcuminoid component in the extract was determined by using HPLC-DAD method.

In the extraction procedure, 15 mL of solvent was added to one g of plant sample. The mixture was sonicated in an ultrasonic bath for 25 min and centrifuged to separate the supernatant from the solid phase. 10 mL of solvent was added to the precipitate and extracted again in an ultrasonic bath for 25

min. It was centrifuged, and the supernatant was combined with the previous extract. The extracts were passed through the injection filter (0.45 µm) and stored at -4 ^{o}C until analyzed.

Optimization

The following parameters were tried in HPLC measurements to determine the optimum conditions using 25 mg curcumin/L dissolved in methanol. Mobile phase, wavelength, injection volume, column temperature and flow rate were determined for the optimum conditions. The most suitable parameters were examined by considering peak symmetry, peak area, and retention time. Nine different solvents/solvent mixings were tried to determine the optimum mobile phase. To determine the most suitable wavelength, the wavelengths of 245, 254, 280, 340, 415, in ranges of 420-433, and 539 nm, which are the most common in the literature for curcumin, were examined. While no peak was observed at 245, 254, 280, and 539 nm, a negative peak at 340 nm was seen. To determine the optimum injection volume, volumes in ranges of 5-30 µL were injected. The temperatures in the range of 22-50ºC were applied to determine the optimum column temperature. In determining the optimum flow rate, volumes in ranges of 0.2-1.0 mL/min were examined.

Calibration and Validation of the Method

Calibration curves were constructed in the ranges of 0.04-1 µg/mL; 1-10 µg/mL, and 10-200 µg/mL for standard curcumin, to cover the ranges expected to occur in the samples. Each working standard solution was analyzed five times in optimum conditions. Calibration plots were drawn according to the concentrations corresponding to the peak areas obtained as a result of the measurements.

In optimized conditions, analytical performance criteria of HPLC were determined with parameters such as limit of detection (LOD), the limit of quantification (LOQ), precision, sensitivity, and accuracy.

Selectivity test

The selectivity was studied by comparing the chromatograms obtained for standard curcumin solutions, including three curcuminoids. In the selection of selectivity, well separation in the peaks observed in the chromatograms and no co-eluting peaks at retention times of Curcumin, DMC, and BDMC were considered.

Linearity and sensitivity

The linearity was considered by applying calibration data using least squares regression with six concentrations of curcumin between 0.04-1.0, and five different concentrations between 2-10 and 25-200 µg/mL. The calibration curves were obtained by plotting the peak area (y-axis) against the concentration of curcumin (x-axis). The linearity was evaluated using correlation coefficient (R^2-value) and intercept value. R^2 values are evaluated according to whether they exceed 0.99 or not. Reproducibility was evaluated by calculating the % RSD. Relative Standard Deviation (RSD) of intra- and inter-day precisions were satisfactorily found.

The LOD and LOQ values were calculated using the slope of the calibration curve and the standard deviation of the smallest value in the calibration curve. These values were calculated with; LOD = 3.3 x S/b and LOQ = 10 x S/b; where “S” is the standard deviation and “b” is the slope of the calibration curve.

Certainty and reliability

The precision that means certainty was assessed for the HPLC method using the repeatability test by injecting more than five replicates of the samples. To assess the reliability of the method, the accuracy was found by spiking 8, 20, and 50 mg/L of curcumin standards and estimating the recovery values.

Results and Discussion

In this study, three curcumin compounds from *Curcuma longa* were measured using HPLC. To enough extraction of the curcuminoids from the studied samples, the factors involved in the analytical scheme were optimized.

Related with the solvent selection, the identity of each peak using different solvents/or their mixing were confirmed by determination of retention times and by spiking with standards. From Fig. 1, curcumin was found to be the major compound in most of the tested extracts, followed by DMC and BDMC. It was found that curcumin in methanol extracts was higher than all of the other extracts. Hence, methanol extract was selected because it can be a good source in the isolation of curcumin and its derivatives (curcuminoids).

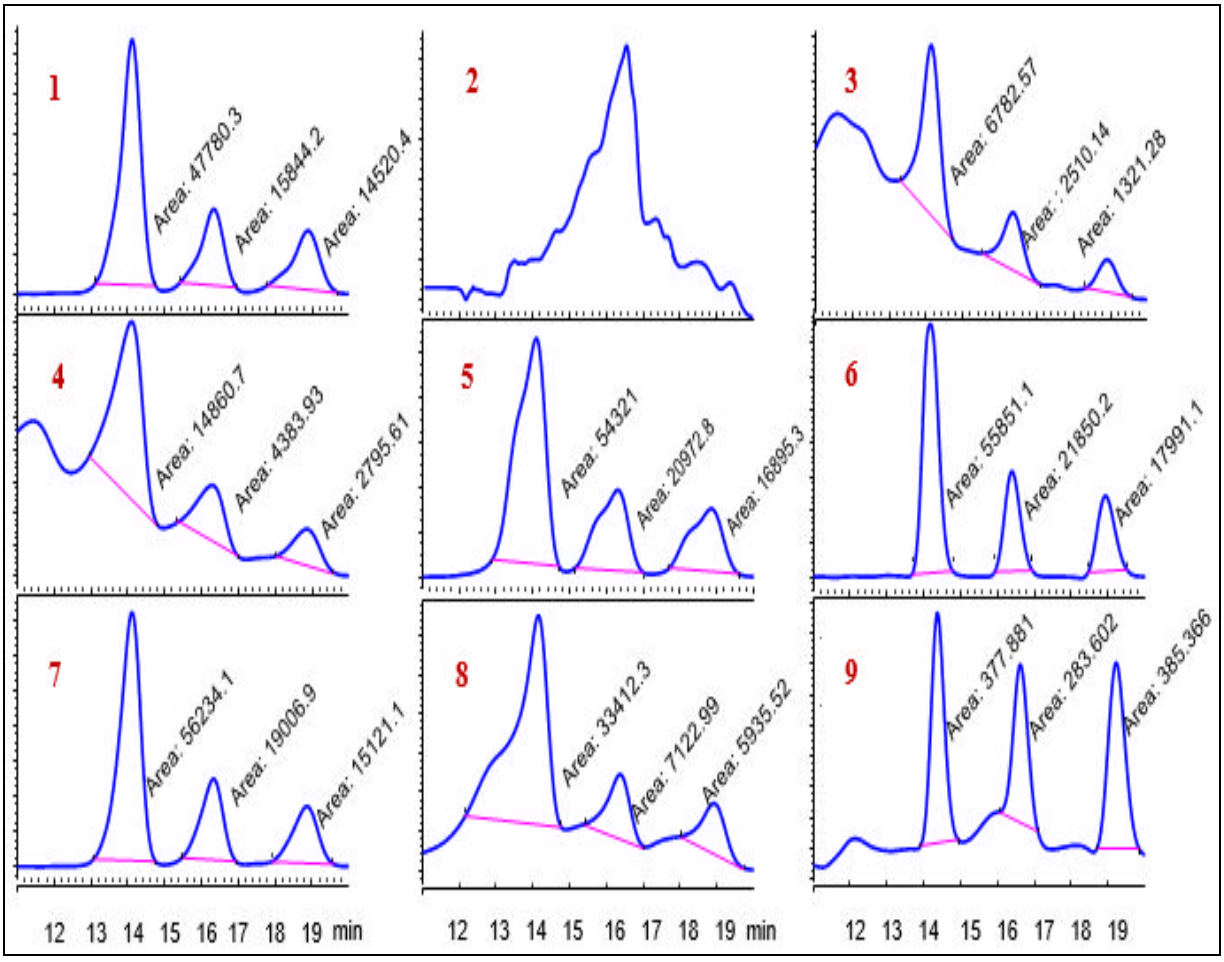

Figure 1. **Effect of solvent type on curcuminoids extraction in Turmeric. 1-80% acetonitrile; 2- acetone; 3-ethyl acetate; 4-1-butanol; 5-%80 ethanol; 6-%1HCl:methanol; 7-methanol; 8-80%isopropanol; 9-distilled water**

To determine the optimum mobile phase, the chromatograms of the tested four mobile phases or their mixing ratios were given in Fig. 2 due to obtaining the peak only for four of them. Considering the peak symmetry and peak area, it was determined that the best mobile phase in HPLC was the mixing of THF: %1citric acid (40:60).

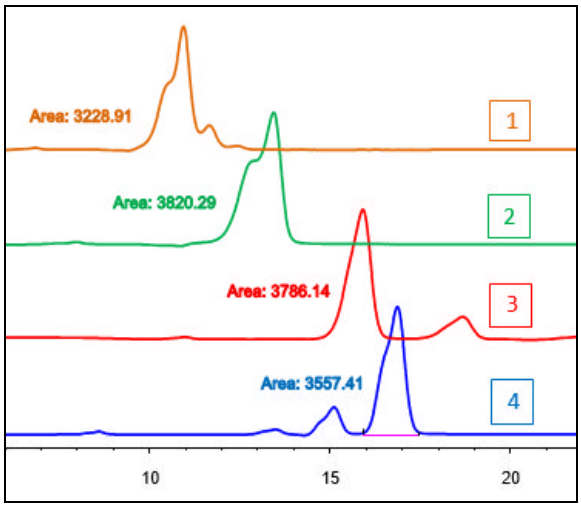

Figure 2. **Effect of Mobile phase on curcuminoids peak -1: Methanol: tetrahydrofuran(THF) : H_3PO_4 (55:10:35); 2: %1ethyl acetate: methanol (40:60); 3:THF: %1citric acid (40:60); 4: %2 Acetic acid (AA): Acetonitrile (ACN) (60:40)**

Related to the optimum wavelength selection, Fig. 3 shows the chromatograms obtained at different wavelengths. Considering the peak areas and symmetry, 425 nm was selected as the optimum wavelength.

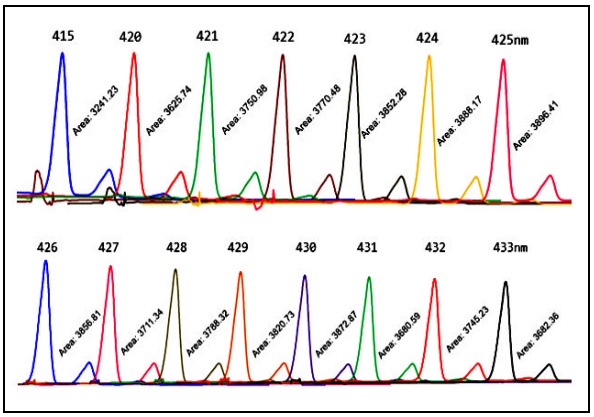

Figure 3. **Effect of wavelength on curcuminoids peak using THF/1%:citric acid (40/60)**

To determine the optimum injection volume, it was observed that volumes larger than 20 µL had broken down in peak symmetry. Since the peak area increased depending on the injection volume, it was determined that the most suitable injection volume was 20 µL (Fig.4).

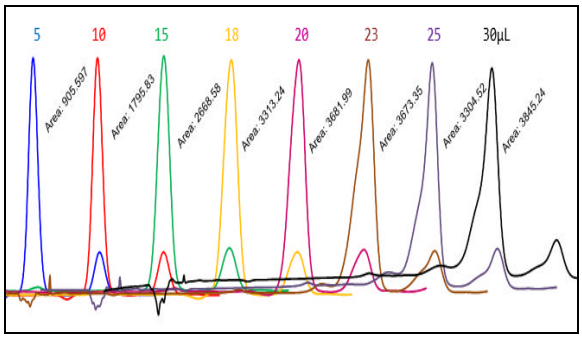

Figure 4. **Effect of injection volume on curcuminoids peak**

From Fig. 5, the highest peak area and good peak symmetry were observed for 30°C, and this temperature was decided to be the optimum column temperature.

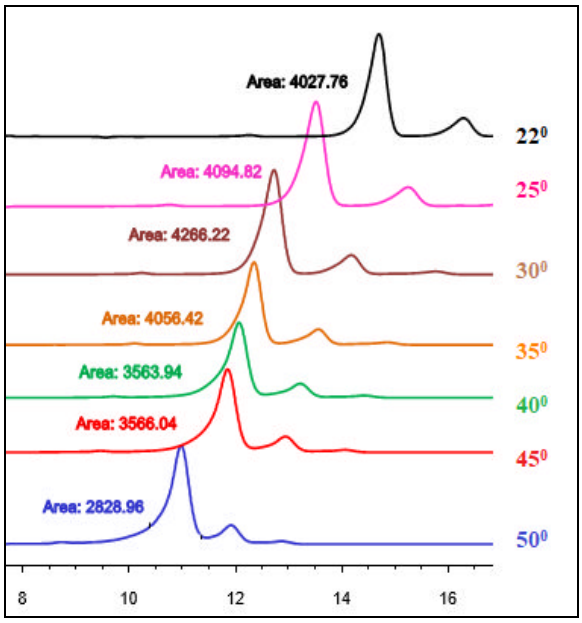

Figure 5. **Effect of column temperature on the curcuminoids peak**

To determine optimum flow rate, it was observed that the peak area decreased

after the flow rate of 0.5 mL/min. For this reason, 0.5 mL/min has been accepted as the most suitable flow rate (Fig. 6).

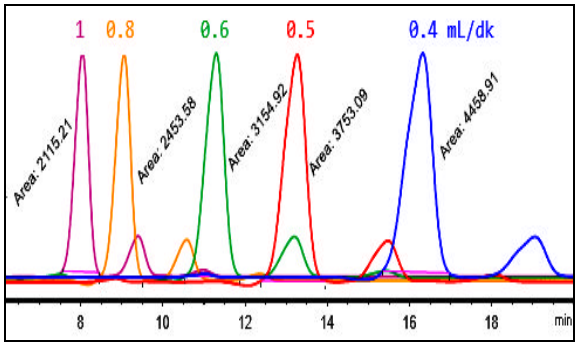

Figure 6. **Effect of flow rate on the curcuminoids peaks**

Briefly, the optimum conditions in HPLC-DAD analysis are given in Table 1.

Table 1. **The optimum conditions obtained for HPLC-DAD measurements.**

Mobile Phase	Tetrahydrofuran:%1 citric acid (4:6)
Wavelength	425 nm
Injection volume	20 µL
Column temperature	30ºC
Flow rate	0.5 mL/min

A calibration graph was constructed between concentrations of standard curcumin solutions and the peak area. Results show a linear relationship in the measuring ranges, but peak symmetry disrupts for 500 ppm, and this concentration was excluded. Calibration graphs are shown in Fig. 7.

Method suitability tests were performed using a freshly prepared 25 mg/L standard curcumin stock solution. All curcuminoid peaks were well divided, tailless, and symmetric. The order of curcuminoids exiting the column and appearing in the chromatogram is curcumin, DMC, and BDMC, and the retention time was for curcumin 14.137±0.020 min.

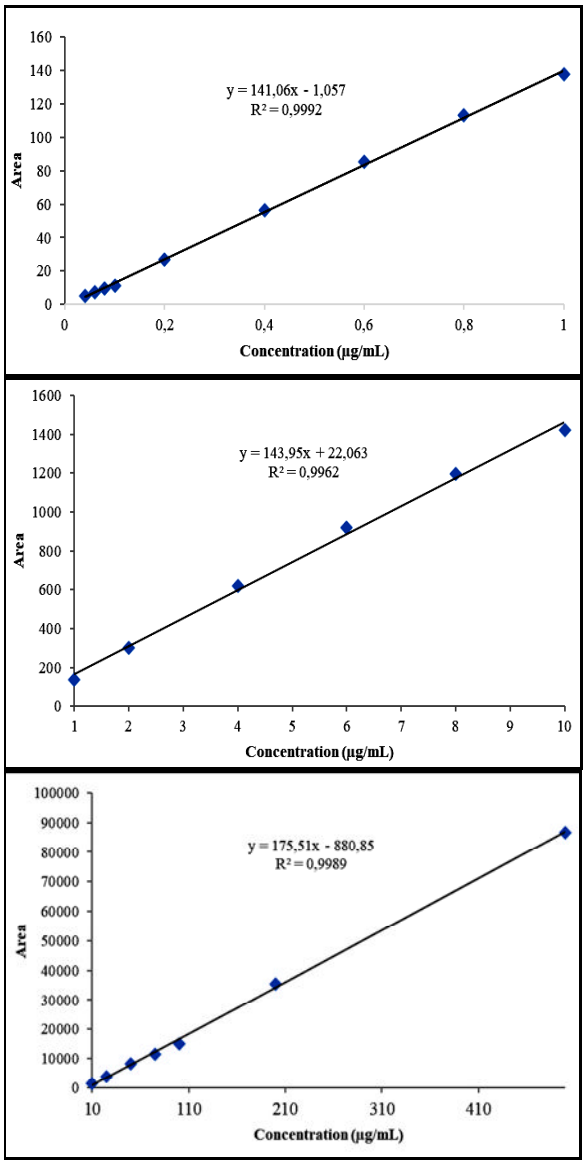

Figure 7. **Calibration plot of curcumin for a) 0.04-1 µg/mL b) 1-10 µg/mL c) 10-200 µg/mL range**

Correlation coefficient (R^2), %RSD, LOD, and LOQ were listed in Table 2. The accuracy of measurements was determined in three concentration ranges, and results were assessed by percentage recovery. After adding standard curcumin solutions to the turmeric samples, the recovery was found to be an average of 90% for 8, 20, and 50 µg/mL additions. Linearity is an acceptable level because its correlation coefficients were all >

0.999 on the inter-day and intra-day calibration curves were created. The LOD and LOQ were observed to be 0.001 and 0.003 mg/L, respectively. % RSD was found for intra-day 0.974 and inter-day 1.012 for curcumin.

Table 2. Validation parameters of HPLC-DAD method.

Linear range (mg/L)	Retention time (min)	LOD (mg/L)	LOQ (mg/L)	R^2	% RSD (intra-day)	% RSD (inter-day)
0.04-200	14.137±0.020	0.001	0.003	0.999	0.974	1.012

Turmeric plant extracts were analyzed by HPLC-DAD instrument with a C18 column under optimum conditions. Peak splitting was observed as the curcumin concentration extracted in turmeric exceeded the maximum detection limit of the device. For this reason, turmeric extracts were diluted and analyzed. Curcumin chromatograms obtained from turmeric (Curcuma longa L.) plant extract are given in Fig. 8. It is seen that there are three derivatives of curcumin (from left to right, respectively curcumin, DCM, and BDCM) in the chromatogram.

The optimum conditions for both extraction and instrument were determined using the purposed method. Using the linear calibration plots, quantitative values were obtained. The observed results revealed that the mobile-phase composition and the column temperature significantly affect the total extraction yield and the concentrations of curcuminoids. Thus, any researcher can apply the mobile-phase composition of THF:1% citric acid (40/60) and the column temperature of 30°C for the best extraction and separation from turmeric, even if it is done the first time. The obtained results show that the retention time, total yield, and resolution decreased when column temperature increased. Again, the highest curcumin concentration was found in the 100% methanol extract.

In this study, three major compounds of curcumin derivatives were extracted by optimization of the analytical scheme. Three major curcuminoids were simultaneously determined using the reliable and selective HPLC diode-array method. For this purpose, the operating conditions were experimentally optimized. The obtained results can investigate the constituents of natural products and the resources of pharmaceutical, nutrition, and cosmetic products. In this study, the obtained concentrations of curcuminoids in dried turmeric plants are in the range of 30-50 mg/g (3-5%) for curcumin, 6.3-11.2 mg/g (0.63-1.12%) for DMC, and 1.9-6.7 mg/g (0.19-0.67%) for BDMC. The results obtained agree with most of the studies published in the literature, although very different concentrations were reported [12-15].

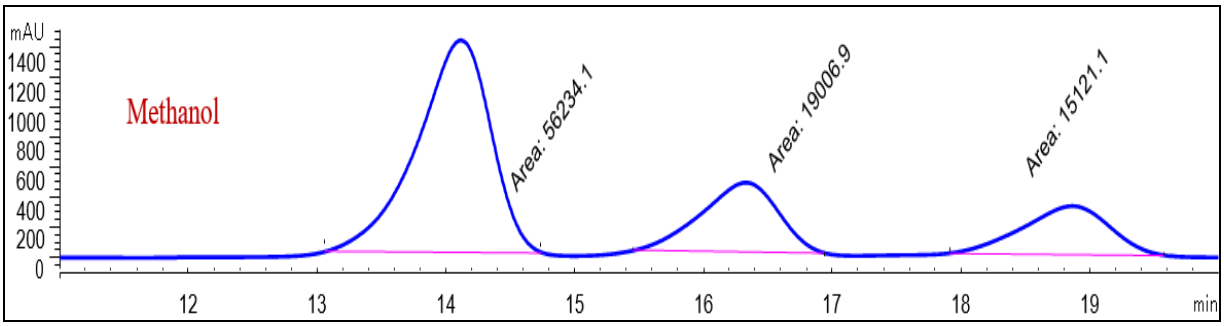

Figure 8. **Chromatogram of Curcuma longa (turmeric plant)**

Conclusion

With our developed method, three curcuminoid compounds can be detected simultaneously with HPLC-DAD, which is reproducible, simple, specific, accurate, reliable, and selective, and excellent peak values can be observed for curcumin and its derivatives. The optimization and validation procedure are described for curcuminoids in turmeric, and the results revealed that the mobile-phase composition, column temperature, injection volume, and flow rate are significantly effective. The method was successfully applied to determine curcuminoids in turmeric. From the results obtained, it can be concluded that the application of the optimized method is reliable, precise, and applicable to the much more plant species.

Conflict of Interest

The authors declare that they have no conflict of interest.

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