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Extraction and Purification of Curcuminoids from Crude Curcumin by a Combination of Crystallization and Chromatography

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Abstract: In this work a method is developed for the extraction and purification of the three curcuminoids; Curcumin (CUR), Demethoxycurcumin (DMC) and Bisdemethoxycurcumin (BDMC) from commercially available crude curcumin. In a previous publication the extraction of pure curcumin by repeated crystallization has been described. The focus of this paper is on the following chromatographic treatment of the mother liquor from the crystallization to obtain pure DMC and BDMC, and to increase the yield of pure CUR. In the chromatographic process a mixture of chloroform and methanol is used as the mobile phase and silica gel is used as the stationary phase. Each fraction isolated in the chromatographic process was characterised by High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (LC-MS) techniques, and the pure CUR, DMC and BDMC solid phases were fully characterized by powder Xray diffraction (PXRD), differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA). Stability studies were performed on the purified curcuminoids where the degradation products were observed and analysed by HPLC/LC-MS. Overall, the combined purification method recovered from the crude: 88.5%, 79.7% and 68.8% of CUR, DMC and BDMC respectively, in highly pure form CUR (100%), DMC (98.6%) and BDMC (98.3%).

Keywords: Purification, Curcuminoids, Gram scale, Cooling Crystallization, Column Chromatography, HPLC analysis.

Introduction

Turmeric (*Curcuma longa L*) is a yellow Indian spice obtained from the *Zingiberaceae* family. The rhizomes of Turmeric have a range of health promoting factors which have been used for centuries in traditional oriental medicine in India and South East Asia.[1] Extracts obtained from Turmeric comprise a mixture of Curcumin (CUR), Demethoxycurcumin (DMC) and Bisdemethoxycurcumin (BDMC), altogether known as curcuminoids (Table.1). Among them, CUR, also known as *diferuloylmethane,* is the most important component as it has been shown to have a wide spectrum of biological activities[2,3,4], such as anti-inflammatory[5,6,7] anti-bacterial and anti-carcinogenic effects[8,9]. Even though CUR has a lot of health promoting factors, it has not yet been approved as a therapeutic agent, because of its poor stability and low solubility in water, and the poor bioavailability properties [10,11]. In addition, it is an unstable compound when it is exposed to light or

subjected to oxidative conditions, which cause the degradation of CUR into vanillin, vanillic acid and ferulic acid [12]. Thus, recently both academia and industry have focused their interest more onto DMC and BDMC compounds. Until now, little work has been done on the physico-chemical properties of these two compounds due to their non-availability in sufficient amounts as pure components (at least in gram scale).

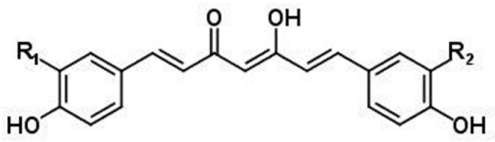
Commercially available crude CUR contains a significant amount of the other two curcuminoids, typically 17% of DMC and 3% of BDMC [13] . Purification is quite challenging, as the three curcuminoids are chemically quite similar, with the only differences being the presence or absence of a methoxy functional group on each of the aromatic rings. At a laboratory scale quite often chromatography methods are employed to isolate the individual curcuminoids, e.g. using silica as the stationary phase, and different mixtures of organic solvents as the mobile phase. So far, the best results have been obtained with chloroform: methanol mixtures, in milligram scale with a maximum purity of <95.5%. In our previous work,[13] CUR was successfully purified with 99% purity from the crude curcuminoid mixture by applying cooling crystallization in several steps. Using this method, highly pure CUR was separated but the other two curcuminoids (DMC and BDMC) were left in the mother liquor. However, since recent literature reports indicate that both DMC and BDMC have similar or even better biological properties than CUR [14] , there is also demand for the isolation of pure DMC and BDMC fractions.

In the literature, the main focus is on the separation techniques used to extract, purify and/or to identify[15,16,17,18, 19,20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31]the three curcuminoids, focusing more on the purification of pure CUR rather than on the purification of pure DMC and BDMC. In this work, the main focus is to develop a process that could be used commercially, i.e for process development or clinical trials, to obtain all three of the curcuminoids in pure form at high yield, adopting a combination of crystallization and liquid chromatography. In the multistep cooling crystallization [13] CUR is extracted in pure form (>99.1%) with 50 % yield. In the following column chromatography reported in the present paper the remaining mother liquors are treated to produce pure fractions of DMC and BDMC, and to increase the overall CUR yield.

The mother liquors remaining from the crystallization steps are enriched in DMC and BDMC concentration relative to the concentration of CUR, which facilitates their recovery. In accordance with favourable results presented previously[32, 33]on the purification of the curcuminoids by column chromatography, we used mixtures of chloroform and methanol as the mobile phase. CUR has a low solubility in chloroform and thus makes the chromatographic method time consuming and requires a large quantity of solvent. In the present work, the chromatographic conditions have been optimized (mobile phase to elute the pure components) to improve the yield and purity of each of the curcuminoid products. The work includes a detailed characterisation of different liquid fractions and of the purified curcuminoid solid phases using Mass Spectrometry, HPLC and Powder X-ray

diffraction. The degradation and stability of the curcuminoids is also investigated by using thermal analysis TGA and DSC.

Table 1. Chemical structures of CUR, DMC and BDMC displaying the differences in the R_1 and R_2 groups between the three curcuminoids.



Curcuminoid	CUR	DMC	BDMC
R_1	$-OCH_3$	-H	-H
R_2	$-OCH_3$	$-OCH_3$	-H

2. Experimental Section

Materials

Commercially available crude CUR was purchased from Merck (CUR >75% nominal purity - HPLC, area %; containing <20% DMC and <5% BDMC). All HPLC grade solvents such as chloroform (>99.9 %), methanol (99.8%), acetic acid (>99.9%) and analytical grade acetonitrile (>99.9%) were purchased from Sigma-Aldrich, and used without further purification. Analytical standards of CUR (nominal purity >98%) were purchased from Fluka. Vanillin and vanillic acid were purchased from VWR. Silica gel (70-150 mesh) was purchased from VWR. Purified water obtained by Millipore Milli-Q water purification system was used for the preparation of the buffer solutions for HPLC/LC-MS. The starting material for the chromatographic separation was obtained by collecting the mother liquors from the crystallization steps[13], and evaporating the mixture to dryness in a rotary evaporator. The resulting dry mixture contained 51.6% CUR, 36.5% DMC and 11.9% BDMC (*w/w*) as determined by HPLC/LC-MS analysis with pure curcuminoid reference standards (>98% *w/w).* This solid mixture is then completely dissolved in chloroform.

Column Chromatography

Besides various preliminary experiments, three column chromatography experiments were performed in total at two different scales, one at a smaller scale (column diameter: 20 mm) and two at a larger scale (column diameter: 73 mm). The results of these experiments are described in the Supplementary Information: Table S1 and Table S2, along with the types of columns and the procedures that were used. The best results obtained at a large scale are reported here in the main text. The adsorbent used as stationary phase was silica gel (70-150 mesh). Chloroform and methanol solvent mixtures were used as the mobile phase. Other solvents such as 1,2–dichloroethane were tested as the mobile phase in a preliminary experiment but did not give good separation of the curcuminoids. The column under operation is shown in Supplementary Information: Figure S1 (high-capacity column, 2000 mL, 73.0 mm × 610 mm, Aldrich). The bottom half of the column was packed

with dry silica gel to approximately 220 mm stationary bed column height. The methanol : chloroform (*w*/%) ratio used was always less than 10 percent as it is known that silica may dissolve in more than 10 percent methanol. The sample and chloroform mixture was mixed with silica gel (11 g of solid curcumin mixture mixed with 15 g of silica gel) and this silica slurry was placed on top of the silica gel. A piece of cotton wool was soaked with chloroform solvent and placed on top of the slurry to prevent disruption of the slurry layer. Using a constant mobile phase flow rate of 15 ml/min, a nonisocratic elution mode was used with a stepwise gradually increasing concentration of methanol in the chloroform-methanol mobile phase as given in Table 3. Each curcuminoid was obtained in pure form in the order CUR, DMC, BDMC by increasing the polarity of the mobile phase.

Stability Analysis

A stability study was performed on mixed curcuminoid samples as well as on the individual curcuminoid standards. The stability of the curcuminoids was assessed by analyzing individual curcuminoid samples dissolved in H_2O/ACN, exposed to light for 24 hours and subjected for HPLC analysis over a UV–Vis range of 280 – 425 nm. The known degradation products Vanillin, Vanillic acid and Ferulic acid each exhibited a significant absorbance at 280nm[34] (see Supplementary Information Figure S2(b)). Samples were analyzed for a percentage loss of individual curcuminoids along with the formation of degradation products using HPLC and LC-MS.

HPLC Analysis

The HPLC system used was an "Agilent Technologies 1260 Infinity Series" comprising of a solvent 1260 Quat delivery pump, manual injector, absorbance detector (UV lamp and Vis lamp) and Agilent ChemStation software. A C18 column (4.6 x 100 mm) was used with 2 % acetic acid in water/acetonitrile (60/40, v/v) as the mobile phase. The mobile phase was freshly prepared, filtered and degassed daily before use. The experimental methodology was; flow rate: 1.000 ml/min, simultaneous multichannel UV detection at 425 nm, 350 nm and 280 nm. This HPLC technique was employed to identify the purity of each column fraction and to detect and analyze degraded CUR samples. To perform the HPLC analysis 1 µl of a mixture made up of 50 µl of the fraction samples or degraded CUR samples diluted with 950 µl of mobile phase and subjected to chromatographic analysis at 33 °C.

Detection of the three curcuminoids was at 425 nm and detection of the keto form of the curcuminoids was at 350 nm. For all the three curcuminoids, sharp peaks (separated from the baseline) at 425 nm were obtained within 6 minutes and the order of elution was CUR, DMC and BDMC. The purity of the isolated fractions of individual curcuminoids is shown in Table 3.

The mobile phase used contained water (60 % acetic acid in H_2O/40 % ACN (60/40, v/v) solution) which facilitates the transformation of the enol tautomer to the keto form. Using a cold mobile phase along with fast needle injection showed either a decrease or disappearance of the keto

peak at 350 nm (Figure 1). It was then concluded that this peak at 350 nm was due to the keto form of CUR, which is supported by literature reports[35]. In HPLC experiments, the keto tautomer peak at 350 nm was observed when the experiments were performed for pure CUR as well as for degraded CUR samples. In the organic polar solvents like MeOH, the enol tautomer is the most predominant one. This is because the enol tautomer has a planar conformation, that is stabilized by strong intramolecular H-bonding and π-conjugation is maintained across the planar molecule, which results in a UV-Vis absorption maximum around 425 nm[35]. In contrast, less stable keto tautomer possess the nonplanar conformation where carbonyl groups are in anti-positions, forming two non - conjugated fragments resulting in an absorption peak around 350 nm. Addition of water stabilizes the keto form[35] as characterized by the presence of a UV adsorption shoulder at 350 nm; this observation was clearly explained in the literature based on the DFT calculations.[36]

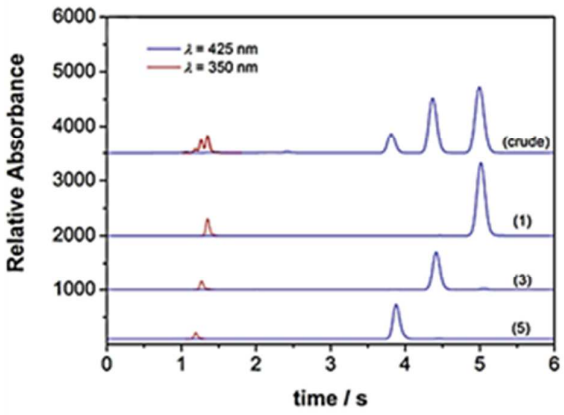


Figure 1. HPLC chromatograms of crude curcuminoid material (crude), individual curcuminoid fractions (1, 3, and 5) isolated by column chromatography.

Calibration standard curves were obtained using the HPLC by making up a stock of prepared/purified individual curcuminoids and known degradation products (Vanillin and Vanillic acid) in acetonitrile, (Figure 2). The linearity of the calibration curves, calibration factors and validation errors of the developed analytical method are presented in Table 2. All of the calibration curves of the three curcuminoids showed good linearity within the test range (R^2=0.999). The method showed good reproducibility provided that samples were analysed following storage in darkness using cold H_2O/ACN solvent for up to 24 hours (MREV < 3%).

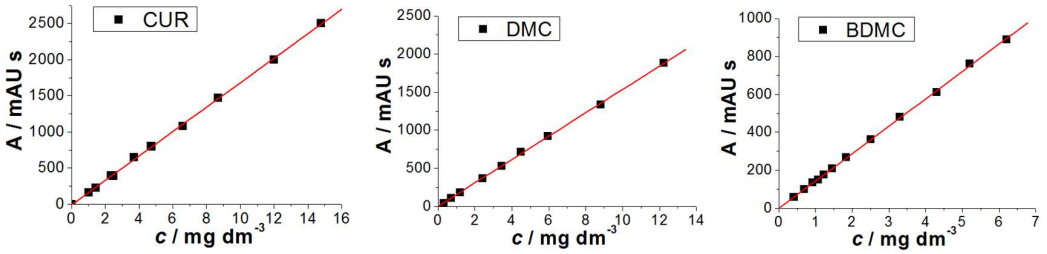


Figure 2. HPLC calibration curves for Curcumin, DMC and BDMC.

Table 2. Range and linearity of the calibration curves, calibration factors and validation errors of the HPLC analytical method for curcuminoids quantification.

	Range	Retention time	Calibration factor	R^2	MREV*
	mg/dm^3	min	µg mAU^{-1} s^{-1} dm^{-3}		%
CUR	0 – 15	5.01	6.1	0.999	2.9
DMC	0 – 12	4.38	6.5	0.999	2.1
BDMC	0 – 6	3.80	6.9	0.999	1.7

* MREV – mean relative error of validation

LC-MS Analysis

An Agilent Technologies 6120 Quadrupole LC-MS (model G6120B) was used for characterizing the purified curcuminoid components and to identify the degradation products. LC-MS analysis was performed for each isolated compound using an electrospray ionization source in positive ion mode. Capillary voltage was maintained at 4.6 kV, source temperature was set at 350 °C and nitrogen was used as the drying gas (12 dm^3/min). The new peaks which were identified by the HPLC method were considered as degradation products and were separated by LC-MS using an Agilent C18 column (2.7 µm, 100 x 4.6 mm; Poroshell 120 EC, Agilent) under isocratic elution flow of acetonitrile and water (70/30, v/v) in 0.1% formic acid with a flow rate of 0.2 mL/min at 33 °C.

Thermogravimetric Analysis (TGA)

A Q50 TGA from TA instruments was used for the TGA analysis. Experiments were performed using aluminium pans under a controlled N_2 environment (40 ml/min) over a temperature range of 0-300 °C at a heating rate of 20 °C/min. The weight of the samples were; CUR (7.0540 mg), DMC (1.0630 mg) and BDMC (8.1670 mg). The results were further analysed by using TA instruments Universal 2000 software (Universal V4. 5A).

Differential Scanning Calorimetry (DSC)

A Q2000 DSC from TA instruments was used to perform the thermal analysis of all three curcuminoid components. Experiments were carried out by using hermetic aluminium pans which contains a definite mass of each of the samples; CUR (2.100 mg), DMC (1.700 mg) and BDMC

(3.800 mg), and sealed with a Tzero press. The temperature range used for the analysis was 0 - 250 °C under N_2 atmosphere (40 ml/min) using a heat – cool run. The results were analyzed as heat flow (W/g) *vs* temperature (°C) using TA Instruments Universal 2000 software (Universal V4. 5A).

Powder X-ray Diffraction

All diffraction patterns were recorded on a PANalytical EMPYREAN diffractometer system using Bragg−Brentano geometry and an incident beam of Cu K-alpha radiation (λ = 1.5418 Å). Room temperature scans were performed on a spinning silicon sample holder**. (**Step size = 0.013 °2θ and step time = 32 (s)).

3. Results and Discussion

Chromatographic Separation

Using a constant mobile phase flow rate of 15 ml/min, a nonisocratic elution profile was used by gradually increasing the concentration of methanol in the chloroform-methanol mobile phase. Starting from pure chloroform solvent , pure CUR was eluted from the column. Then increasing the methanol content to 0.4 %, initially elutes a mixture of remaining CUR together with DMC, after which a pure DMC fraction is recoverd. In the third step the methanol concentration is increased to 0.7 %, which initially gives a mixture of remaining DMC together with BDMC, and then a fraction of pure BDMC is obtained. The overall history of the solvent used for each curcuminoids separation, mass collected from the column and purity of the each fraction is listed in Table 3. The overall mass of purified CUR, DMC and BDMC fractions obtained from 11.0 g of crude CUR was 8.7 g , i.e. 79 % overall yield. There was 6.7% loss of curcuminoids retained on the column and 13.6% loss in fractions where the separation of the curcuminoids is insufficient. The yield of each of the isolated curcuminoids in the column chromatography was 81% CUR, 79.7% DMC and 68.8% BDMC. Over the combined process of using a multistep crystallization followed by column chromatography of the remaining mother liquors the yield of pure CUR is 88.5%. Of course the mixed fractions collected between the pure fractions can be recycled to the next sample to be separated on the column. The purity of each fraction as determined by HPLC is very satisfactory with 100 % for CUR and above 98% for the other two curcuminoids.

Table 3. The elution profile from column chromatography: methanol fraction in chloroform (*w*), total volume of solvent used (*V*), mass collected and the purity of each fraction from a mixture sample containing 11 g of curcuminoids.

Fraction	*w*(MeOH)/%	*V*(eluent)/L	Curcuminoid	Purity (%)	Mass (g)
0	0	3.5	-	-	-
1	**0**	**8.5**	**CUR**	**100**	**4.6**
2	0.3-0.4	3.2	CUR/DMC mix	-	0.9
3	**0.4**	**4.0**	**DMC**	**98.6**	**3.2**
4	0.5-0.7	2.5	DMC/BDMC mix	-	0.7
5	**0.7**	**8.0**	**BDMC**	**98.3**	**0.9**

Crystal Structures

PXRD was performed on the purified curcuminoid solid samples. Sharp crystalline peaks were obtained for CUR and BDMC. DMC exhibited a sharp diffraction peak at a 2θ value greater than 38 °. The DMC pattern was more consistent with a disordered structure at interlayer d-spacing values more than 2.4 Å, i.e. peaks below 38 ° 2θ and an order in interlayer d-spacing values less than 2.4 Å, i.e. above 38° 2θ. The obtained PXRD pattern of separated CUR sample matched with the experimental PXRD of Form I (monoclinic) of CUR. The crystal structure of both DMC and BDMC are not yet reported in the literature. Thus, the PXRD pattern for both DMC (see Supplementary information: Figure S3, for clarity, the diffraction pattern has been collected from 5-80 ° 2θ value) and BDMC were reported as shown in the Figure 3. No match of DMC or BDMC was found with the other polymorphic forms of CUR[37] when the diffraction patterns were compared.

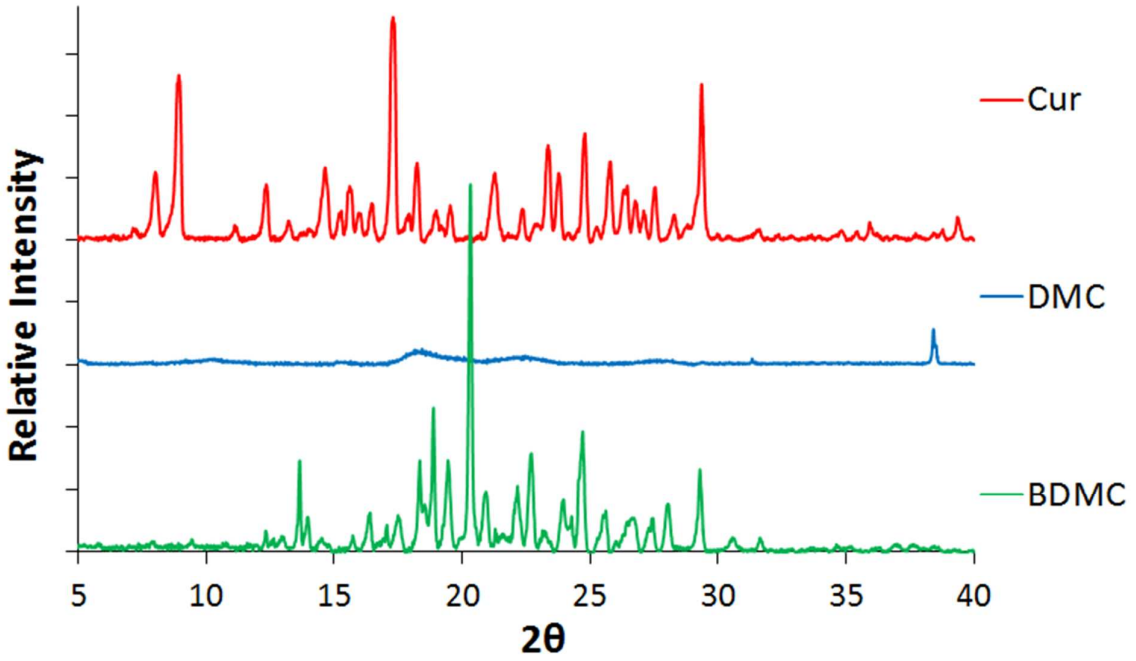


Figure 3. The XRD pattern of the three curcuminoids. (see Supplementary Information Figure S3 to view full diffractogram of DMC).

Chemical and Thermal Stability

The percentage loss of individual curcuminoids was calculated and plotted against time as shown in the Supplementary Information Figure S4. It was clearly understood that CUR rapidly degrades in an aqueous environment (H_2O/ACN, 60/40) and degrades even more when exposed to light[18] .50% of CUR had decomposed after 4 h; complete decomposition was observed after 24 h (solution loses its characteristic yellow colour and becomes transparent) when exposed to direct sunlight. On the other hand, (B)DMC shows somewhat better stability than CUR as 35% DMC and 15% of BDMC had degraded after 4 h, whereas 80% DMC and 20% BDMC had decomposed after 24 h.

Interestingly, contradictory results were obtained when the stability experiments were performed for a crude curcuminoid mixture; 50% of CUR remains after 24 h, and around 80% and 70% of DMC and BDMC, respectively.

During the stability experiments, a significant loss of the curcuminoids was observed but only trace amounts of the known degradation products vanillin (VAN) and vanillic acid (VAD) were detected by HPLC (about 3 % w/w with respect to initial curcuminoid). From this, it is understood that these are minor degradation products so that other unidentified products formed, as already reported in the literature [38, 39, 6, 40, 41]. MS analysis of degraded samples exhibited a number of new peaks, including a high-intensity peak at 248, identified as *trans*-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexenal [18, 38, 39] (see Supplementary information, Figure S5). The chemical structures of the degradation products are shown in Figure 4. The vanillin, vanillic acid and *trans*-6-(4′-hydroxy-3′-

methoxyphenyl)-2,4-dioxo-5-hexenal are obtained through photo-oxidation of the double bond which is present alpha to the carbonyl group of the curcuminoids, leads to the generation of the aldehydes and carboxylic acids. Other degradation products are formed by further oxidative degradation of the *trans*-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexenal component. No reports that the degradation products exist in a diketo – enol equilibrium have been found in literature so far.

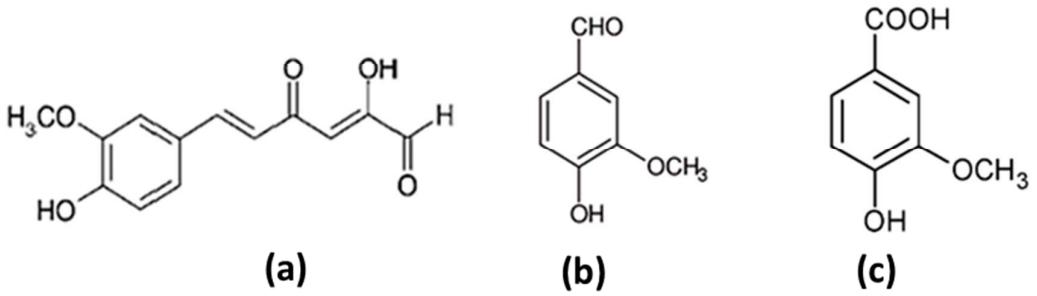


Figure 4. The chemical structure of (a) *trans*-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5- hexenal 21 [42] (b)Vanillin [43] and (c) Vanillic acid [44]

The TGA thermograms of the purified curcuminoid samples are shown in Figure 5 (a). CUR shows a weight loss at around 184 °C whereas DMC and BDMC components show a weight loss at 172 °C and 142 °C, respectively. In addition, the weight-loss of CUR and DMC exhibits a gradual decrease, while that of the BDMC occurs more rapidly at an earlier stage. Thus, based on the decomposition values, it was concluded that BDMC has a lower thermal stability than CUR and DMC. On the other hand, the DSC experiments (Figure 5 (b)) indicate that BDMC is thermodynamically more stable than Cur and DMC. The melting point for Curcumin (Table 4.) is in accordance with literature values [45] (Cur: 181.2 °C). The peak temperatures (Table 4.) are also in accordance with the literature (Cur: 184 °C, DMC: 172 °C, BDMC: 222 °C)[18]. The order of stability for the three curcuminoid components based on the decomposition temperature is BDMC < DMC < CUR, and based on melting temperature (onset) is DMC < CUR < BDMC.

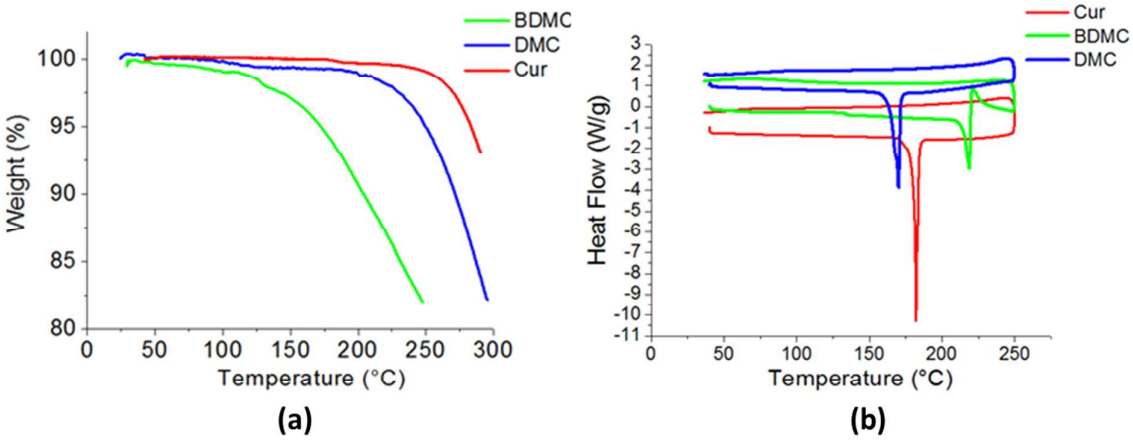


Figure 5. **(a)** TGA and **(b)** DSC of the purified curcuminoid samples.

Table 4. DSC thermal analysis data of the three curcuminoids components.

Curcuminoid:	*T*m onset (°C)	Peak Temperature (°C)
CUR	180.70	182.17
DMC	166.99	170.23
BDMC	218.84	219.84

Conclusion

Following a first step of repeated crystallizations to produce pure curcumin (CUR), the remaining mother liquors were successfully treated by silica gel column chromatography to yield pure fractions of Demethoxycurcumin (DMC) and Bisdemethoxycurcumin (BDMC). By using a mixture of chloroform and methanol as the mobile phase in nonisocratic operation, pure fractions of CUR (100%), DMC (98.6%) and BDMC (98.3%) were isolated by a stepwise increase of the methanol concentration. The yields in the chormatorgraphic process of the pure curcuminoids is 81%, 79.7% and 68.8% of CUR, DMC and BDMC respectively. Over the combined process of a multistep crystallization followed by column chromatography of the remaining mother liquors, the yield of pure CUR is 88.5%. It is found that CUR rapidly degrades under light and oxidative conditions, while BDMC and DMC are more stable. The presence of (B) DMC in crude curcuminoid mixtures enhances CUR stability.

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Supplementary Information (SI) available: High-Capacity Column Chromatography Setup, HPLC chromatograms of crude curcuminoid material (crude), individual curcuminoid fractions (1, 3, and 5) isolated by column chromatography, the degradation products of the three curcuminoids detected at 280 nm, full PXRD of DMC, degradation of curcuminoid samples dissolved in H_2O/ACN (60/40, v/v) and exposed to light; (a) individual pure curcuminoids and (b) crude curcuminoid mixture, MS spectra (ESI-negative mode) of purified curcuminoid samples: (a) CUR, (b) DMC, (c) BDMC and (d) degraded CUR (7h), Column chromatographic experiments.

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