The Study on Curcuminoids in Chromatography, Spectroscopy and Regioisomerism

Z Abdul Zahar, H F Mohsin and *AW Ibtisam
Department of Pharmacology & Chemistry, Faculty of Pharmacy, Universiti Teknologi MARA, 42300 Bandar Puncak Alam, Selangor Darul Ehsan, Malaysia

*ibtisam@uitm.edu.my

Abstract. The turmeric or Curcuma species was macerated with organic solvents. By executing both analytical and preparative thin-layer chromatographic analysis of the crude material, the chromatographic profile of the curcuminoids could be improved. The Nuclear Magnetic Resonance (NMR) spectra of the pure curcumin, demethoxycurcumin and bisdemethoxy-curcumin was acquired. Additional focus for discussion includes the keto–enol tautomerisation of the products. It is concluded that the understanding of this medicinal herb and its curcuminoidal content, is heightened with advanced technological aid.

1. Introduction
Medicinal plants include the turmeric, which is botanically known as Curcuma species (Zingiberaceae plant family). The natural dye pigment, known as curcuminoids (Figure 1), is extractable from the rhizome. The separation and determination of the physico-chemical characteristics of curcumin, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) was reported [1] and the selection of Curcuma species as the natural product to be investigated for university learners, was published [2]. In addition, a synthesis approach was introduced, to enhance the understanding of Nuclear Magnetic Resonance (NMR) spectroscopy. Following this report, sequential experiments were established [3] to teach the process of extraction, distillation and Thin Layer Chromatography (TLC) of curcumin to learners. In this paper, a comparable chemical analysis was organised for this herbal project.
Figure 1. The chemical structures of curcuminoids from Curcuma longa.

2. Methodology
The turmeric products were obtained from the retail stores. The powdered material was utilised for solvent extraction, both analytical and preparative Thin Layer Chromatography (TLC) and Nuclear Magnetic Resonance (500 MHz NMR) spectroscopy. By using the maceration procedure, the turmeric chloroform crude extract was obtained. Then, it was applied, with the help of a micropipette, on the precoated silica gel G60 F254 TLC plates (Merck). The plates were developed using mixtures of chloroform and methanol, as the solvent system.

3. Results and Discussion
The roles of curcumin as an anti-inflammatory product [4], to fight obesity [5] and in oral and dental care [6] were mentioned. From the literature search, the TLC conditions of curcuminoids were also reported. Curcumin displayed the retention factor (Rf) value of 0.45 on a silica gel 60 F254 plates (Merck), by using dichloromethane:methanol (99:1, v/v) as the eluent [7]. In addition, the Rf of the other main side components were recorded as 0.20 and 0.08. In the advanced technology of High Performance Thin Layer Chromatography (HPTLC), a complex mixture of three organic solvents was utilised instead [8]. By using toluene: ethyl acetate: methanol (18:1:1), the Rf value of curcumin was found at Rf 0.38, demethoxycurcumin (DMC) at Rf 0.3 and bisdemethoxycurcumin (BDMC) at Rf 0.24. These values were considered low; therefore, a more optimized condition was explored. The desired resolution of separation can be achieved by using a composition of chloroform:methanol (95:5) as the mobile phase [9]. The Rf value of curcuminoids were 0.75, 0.55, and 0.27 for curcumin, DMC, and BDMC, respectively. Better resolution of Rf values showed that chloroform and methanol were suitable for the separation of curcuminoids. Therefore, this composition was selected to enable the separation, as shown in Figure 2. Nevertheless, the technical skills in manually applying the compounds on the preparative scale, should be upgraded. A continuous line of the extract should be formed to indicate the compounds’ origin on the plate, instead of making disconnected spots. From Figure 2, three visible major bands (Band 1, 3 and 5) were noted in daylight. Another two bands (Band 2 and 4) could be detected via the ultraviolet ray. Following the success of the purification procedure, the purified Curcuma component could be characterized. The highest band of the extract was observed as the primary isolated compound. This band was labelled as the first compound. The proton NMR spectrum (1H-NMR, 500 MHz, CDCl3) of the first compound is displayed in Figure 3.

Figure 2. The analytical TLC of the Curcuma chloroform extract (left) and the measure-up separation of the crude extract was collected as several bands from the preparative silica gel plate (right).

In Figure 3, the NMR solvent’s peak (CDCl3, δH = 7.28 ppm, s) was recorded. Traces of organic solvents were also detected in δH = 1 - 4 ppm. The obvious peaks at δH = 3.95 ppm can be assignable to two methoxy groups (six equivalent hydrogens). Next, six different types of protons can be seen in
$\delta_H = 5.8 - 7.7$ ppm. The NMR spectra showed an AX spin system in the aromatic region at $\delta_H = 7.61$ and 6.50 ppm (respectively for 2H) with a spin coupling of 16.0 Hz. This system can easily be attributed to the protons of the trans olefinic bond. This AX spin system could be connected to the aromatic protons 2', 5' and 6' of the 1,3,4-trisubstituted aromatic ring systems. These protons display a very typical pattern and their individual assignment is obvious due to the small spin coupling between H-2' and H-6'(d, $J = 2.0$ Hz, 2H) at $\delta_H = 7.07$ ppm. The proton of H-5', 6.95 ppm, d, $J = 8.0$ Hz (2H), corresponded with another proton H-6', 7.14 ppm, dd, $J = 8.0, 2.0$ Hz (2H). Finally, the singlet at 5.82 ppm, which was integrating for only one proton, could be assigned to H-4. At this point, the symmetrical structure of curcumin, as drawn in the enolic form, could be explained (Figure 4).

**Figure 3.** The $^1$H-NMR spectra (500 MHz, CDCl$_3$) of the first compound, $\delta_H = 5.8-7.7$ ppm.

**Figure 4.** The enolic form of curcumin, which is a fully conjugated system.

Along with the spectral interpretation, the discussion would include why the enol form was stable, compared to the diketo form of curcumin. This debate could be supported by rationalising the two effects that could stabilize the enol form [7]. As drawn in Figure 4, the enol form is a fully conjugated system, which is accompanied by the release of energy of delocalization. Furthermore, an intramolecular hydrogen bonding could be established between the keto and the hydroxyl group, which then, could also release the energy from the bond. On the other hand, neither of those two effects would exist in the 1,3-diketo unit of curcumin (Figure 4). Therefore, another topic of regioisomerism (constitutional isomerism) could be incorporated. Such isomerism is the type that can be observed at the pair of keto–enol tautomers of curcumin.

Demethoxycurcumin (DMC, Figure 1) (band 3, Figure 2) is also a natural product of the Curcuma species, which is co-isolated with curcumin. It presents at approximately 20%, and this molecule occurs entirely as keto-enol tautomers [10]. The apparent peak at $\delta_H = 3.98$ ppm can be assignable to the methoxy group. However, the spectral integration only indicates the presence of three hydrogens. Therefore, the initial hypothesis of having DMC as the compound in band 3 was supported.

Different types of protons can be seen in $\delta_H = 5.3 - 7.8$ ppm (Figure 5). The $^1$H-NMR spectra showed two AX spin systems in the aromatic region at $\delta_H = 7.64/7.62$ ppm and $\delta_H = 6.53/6.49$ ppm (a total of 4H) with a spin coupling of 15.5/16.0 Hz. These systems can be assigned to the protons of the trans olefinic bond. The AX spin system could be attached to the aromatic protons of either 2', 5' and 6' of the 1,3,4-trisubstituted or to a mono substituted aromatic ring systems. The unsymmetrical structure of DMC could be projected (Figure 1). The aromatic protons display a very typical pattern and their individual assignment is clear. The mono substituted aromatic ring possesses protons as H-2'/6' (2H), $\delta_H = 7.50$ ppm, and H-3'/5' (2H), $\delta_H = 6.89$ ppm. They both appear as doublets, with $J = 8.5$ Hz.

A small spin coupling was seen between H-2'' and H-6'' (d, $J = 2.0$ Hz, 2H) at $\delta_H = 7.09$ ppm. The proton of H-5'', 6.98 ppm, d, $J = 8.0$ Hz (2H), was corresponding with another proton H-6', 7.15 ppm, $dd$, $J = 8.5, 2.0$ Hz (2H). A singlet at $\delta_H = 5.82$ ppm, which is for one proton, could be assigned to H-4.
What is intriguing is the fact that the singlet at $\delta_H = 5.89$ ppm, could also be assigned to H-4. It might mean the presence of another form of DMC tautomer. Lastly, a broad singlet, which is dedicated for a hydroxyl linkage, could be observed at $\delta_H = 5.42$ ppm.

Demethoxycurcumin (DMC) can possibly undergo tautomerization between 3-keto-5-enol (form A) and 5-keto-3-enol (form B) (Figure 6). These two isomers might not be equal; one form (form A) was the major one, and the other form (form B) was the minor one [11]. The two asymmetric keto-enol tautomers of curcumin and DMC rapidly interconvert in solution. There is no evident $\beta$-diketone tautomer in any of the 1D NMR spectra [10]. However, this is in contrast with a report by Pant [12]. On another note, the absence of methoxy protons at $\delta_H$ 3.91-3.95 ppm would indicate the isolation of bisdemethoxycurcumin (BDMC) (Figure 1).

Bisdemethoxycurcumin (BDMC) (band 5, Figure 2) is the third isolated natural product from this Curcuma sample. To reflect its symmetrical structure, the $^1$H-NMR spectrum of BDMC was studied. It contains one broad singlet at $\delta_H$ 1.64 ppm, possibly due to two exchangeable hydroxyl protons of the enol groups. Different types of protons can be seen in $\delta_H$ 5.5 – 7.8 ppm (Figure 7). The $^1$H-NMR spectra showed two AX spin systems in the aromatic region at $\delta_H$ 7.63 ppm and $\delta_H$ 6.52 (a total of 4H) with a spin coupling of 16.0 Hz (H1/7 and H2/6). These protons are attributed to the protons of
the trans olefinic character. The AX spin system is connected to the aromatic ring of a 1,4-disubstituted systems (H2’/6’ and H3’/5’). The spectral integration also aids in the interpretation process. Since the singlet for H-4 (δH = 5.80 ppm) could be intergrated as a single proton, therefore, the rest of the protons could be confidently interpreted as two protons for each olefinic carbons and four protons for each ortho-coupled aromatic carbons.

In terms of polarity, curcumin is the most non polar, followed by DMC and BDMC (Figure 1). This is due to the Rf values when the TLC bands were compared (Figure 2). In Figure 4, the enol form of curcumin has an extended chromophore with 20 π-electrons in conjugation. The chromophore contains phenyl rings and alkene units as weak chromophores and one keto group is included as a strong chromophore [7]. The pure diketo form of curcumin is also coloured, though the conjugation is interrupted. If one cuts it into two units, a substituted cinnamic acid remains (ferulic acid or hydroxycinnamic acid, Figure 8) which has a small conjugation as intensively coloured as the enol form.

![Figure 7. 1H-NMR spectra (500 MHz, CDCl3) of BDMC, the third compound, from the fifth band (δH = 4.5 – 7.8 ppm).](image)

The significance of curcuminoids, for the benefit of environment and conservation issues could be investigated. An antioxidant, which is also a potential biodiesel from curcumin, was reported [13]. Tetrahydrocurcumin (THC) (Figure 8) which is obtained from the hydrogenation of curcumin, is one of the main metabolites. When THC was compared to curcumin, it was found out that THC is a colorless component; therefore, this property would increase the marketability of THC as an additive for biodiesel. From this structural variety of curcuminoids, the students would be able to anticipate the ultraviolet (UV) absorption spectra of such organic compounds.

![Figure 8. The chemical structure of ferulic acid or hydroxycinnamic acid and tetrahydrocurcumin (THC), the metabolic product from curcumin.](image)
The source of the commercial Curcuma sample is not to be ignored. Curcuma grown in different locations might contain different amounts of various curcuminoids [14]. Therefore, to obtain a higher yield of certain curcuminoids, the area for plant collection should be considered. This guideline would provide a useful reminder for the standardization of curcuminoid extracts used in pharmaceutical products and cosmetics. In this study, the students have also selected two Curcuma samples for the comparative TLC (Figure 9). Both turmeric from different origins show different amounts of their pigments, due to the different spot measurements.

The thin layer chromatography profiles of turmeric products which were obtained via partial hydrothermal hydrolysis were presented [15] during the course of this investigation. It is anticipated that the other unknown two bands (Band 2 = Unknown 1 and Band 4 = Unknown 2) which could be detected via the ultraviolet ray, would be the deflorated and depigmented turmeric component. This is due to the Rf value which was within the range for curcumin (0.69 - 0.88), yet, more or less similar than DMC (Rf = 0.56 - 0.69) and BDMC (Rf = 0.38 – 0.56). The observed Rf value of curcuminoids in this study were 0.77, 0.53, and 0.24 for curcumin, DMC and BDMC. Meanwhile, the recorded Rf for both unidentified components were 0.67 and 0.35, respectively for Unknown 1 and Unknown 2. Though no heat was applied during the maceration, the contact between the silica gel and the extracts would induce the occurrence of partial hydrolyzed curcuminoids. It is indeed relatively challenging to identify the other phenolic constituents using TLC [15]. The reference compounds were not provided on the plate. Thus, this methodology offer permanent uncertainties, because two different compounds might show the same RF value. Therefore, a further characterization technique would be required to establish the identity of the unknowns at a higher confidence level. It is advisable to scrap all areas on the TLC plate, where the unknowns have migrated as well, followed by a solvent extraction and detailed chemical analysis, such as high field 600 MHz NMR spectroscopy. The scope for the next research should now be decided.

4. Conclusion
The extraction of curuminoids from a natural source, that is available as consumables, is deemed as an excellent choice to introduce how chemistry is related to daily life. In all NMR experiments, the spectra of curcumin, demethoxycurcumin and bisdemethoxycurcumin indicate only the presence of rapidly interconverting keto-enol tautomers.
References

[1] Péret-Almeida, L., Cherubino, A. P. F., Alves, R. J., Dufossé, L., Glória, M. B. A. 2005 Separation and determination of the physico-chemical characteristics of curcumin, demethoxycurcumin and bisdemethoxycurcumin, Food Res. Int., 38, pp. 1039–1044.

[2] Fagundes, T. da S. F., Dutra, K. D. B., Ribeiro, C. M. R., Epifanio, R. de A., Valverde, A. L. 2016 Using a Sequence of Experiments with Turmeric Pigments from Food to Teach Extraction, Distillation, and Thin-Layer Chromatography to Introductory Organic Chemistry Students, J. Chem. Educ., 93 (2), pp. 326-329.

[3] Wagner, C. E., Marshall, P. A., Cahill, T. M., Mohamed, Z. 2013 Visually Following the Hydrogenation of Curcumin to Tetrahydrocurcumin in a Natural Product Experiment That Enhances Student Understanding of NMR Spectroscopy, J. Chem. Educ., 90 (7), pp. 930-933.

[4] Araújo, C. A. C., Leon, L. L. 2001 Biological activities of Curcuma longa, L. Mem Inst Oswaldo Cruz, Rio de Janeiro, 96 (5), pp. 723-728.

[5] Ravindran, P. N., Nirmal Babu, K., Sivaraman, K., eds. 2007 Turmeric: the genus Curcuma, CRC Press.

[6] Sood S., Nagpal M. 2013 Role of curcumin in systemic and oral health: An overview, J. Nat. Sc. Biol. Med, 4 (1), pp. 3 - 7.

[7] Sicker, D., Berger, S., 2009, Classics in Spectroscopy: Isolation and Structure Elucidation of Natural Products”, Wiley pp. 207 – 220.

[8] Lawand, R. V., Gandhi, S. V. 2013 Comparison of Curcuma caesia Roxb. with other Commonly Used Curcuma Species by HPTLC, J. Pharmacogn. Phytochem., 2 (4), pp. 126-131.

[9] Revathy, S., Elumalai, S., Benny, M., Antony, B. 2011 Isolation, purification and identification of curcuminoids from turmeric (Curcuma longa L.) by column chromatography, J. Exp. Sci., 2 (7), pp. 21-25.

[10] Payton, F., Sandusky, P., Alworth, W. L. 2007 NMR Study of the Solution Structure of curcumin, J Nat Prod, 70 (2), pp. 143 – 146.

[11] Zeng, Y., Qiu, F., Liu, Y., Qu, G., Yao, X. 2007 Isolation and Identification of Phase 1 Metabolites of Demethoxycurcumin in Rats, Drug Metabolism and Disposition, 35, pp. 1564– 1573.

[12] Pant, N., Misra, H., Jain, D. C. 2013 Phytochemical investigation of ethyl acetate extract from Curcuma aromatica Salisb. Rhizomes, Arab. J. Chem., 6, pp. 279-283.

[13] Serqueira, D. S., Domellas, R. M., Silva, L. G., de Melo, P. G., Castellan, A., Ruggiero, R., Richter, E. M., Munoz, R. A. A. 2015 Tetrahydrocurcuminoids as potential antioxidants for biodiesels, Fuel, 160, pp. 490-494.

[14] Pothitirat, W., Gritsanapan, W. 2005 Quantitative Analysis of Curcumin, Demethoxycurcumin and Bisdemethoxycurcumin in the Crude Curcuminoid Extract from Curcuma longa in Thailand by TLC Densitometry, Mahidol University J. Pharm. Sci., 32 (1-2), pp. 23-30.

[15] Santana, Á. L., Meireles, M. A. A. 2016 Thin-Layer Chromatography Profiles of Non-Commercial Turmeric (Curcuma longa L.) Products Obtained via Partial Hydrothermal Hydrolysis, J. Public Health, 6 (1), pp. 15-25.

Acknowledgments
The authors acknowledge the Faculty of Pharmacy, Universiti Teknologi MARA and Atta-ur-Rahman Institute for Natural Product Discovery, Universiti Teknologi MARA Puncak Alam Campus.