Research Article

Phytochemical Analysis

Received: 20 February 2008, Revised: 18 June 2008, Accepted: 21 June 2008 Published online 5 August 2008 in Wiley Interscience

(www.interscience.wiley.com) DOI 10.1002/pca.1086

Application of Scion Image Software to the Simultaneous Determination of Curcuminoids in Turmeric (*Curcuma longa*)

Uthai Sotanaphun,[a]* Panadda Phattanawasin[b] and Lawan Sriphong[b]

ABSTRACT:

Introduction – Curcumin, desmethoxycurcumin and bisdesmethoxycurcumin are bioactive constituents of turmeric (*Curcuma longa*). Owing to their different potency, quality control of turmeric based on the content of each curcuminoid is more reliable than that based on total curcuminoids. However, to perform such an assay, high-cost instrument is needed.

Objective – To develop a simple and low-cost method for the simultaneous quantification of three curcuminoids in turmeric using TLC and the public-domain software Scion Image.

Methodology – The image of a TLC chromatogram of turmeric extract was recorded using a digital scanner. The density of the TLC spot of each curcuminoid was analysed by the Scion Image software. The density value was transformed to concentration by comparison with the calibration curve of standard curcuminoids developed on the same TLC plate.

Results – The polynomial regression data for all curcuminoids showed good linear relationship with $R^2 > 0.99$ in the concentration range of 0.375 – 6 μg/spot. The limits of detection and quantitation were 43 – 73 and 143 – 242 ng/spot, respectively. The method gave adequate precision, accuracy and recovery. The contents of each curcuminoid determined using this method were not significantly different from those determined using the TLC densitometric method.

Conclusion – TLC image analysis using Scion Image is shown to be a reliable method for the simultaneous analysis of the content of each curcuminoid in turmeric. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: Scion Image; TLC image analysis; method validation; turmeric; curcuminoids; *Curcuma longa*

Introduction

Turmeric (*Curcuma longa* Linn., family Zingiberaceae) is a popular herb worldwide. The rhizome of the herb has been proven to be clinically effective for the treatment of dyspepsia (Thamlikitkul *et al.*, 1989) and peptic ulcer (Kositchaiwat *et al.*, 1993; Prucksunand *et al.*, 2001). A pilot study indicated that it could reduce the symptoms of irritable bowel syndrome (Bundy *et al.*, 2004). The possibility of using the herb for the prevention or retardation of atherosclerosis has been suggested (Ramirez Boscá *et al.*, 2000a, b). Turmeric also exhibits anti-inflammatory activity (Yegnanarayan *et al.*, 1976) and inhibitory activity against some dermatological micro-organisms (Lutomski *et al.*, 1974; Banerjee and Nigam, 1978).

Currently, official monographs assess the quality of turmeric on the basis of its total content of essential oils and curcuminoids (*Standard ASEAN Herbal Medicine*, 1993; *Thai Herbal Pharmacopoeia*, 1995; Blumenthal *et al.*, 2000). Curcumin is the most studied curcuminoid with respect to several biological activities and its remarkable anti-inflammatory, antioxidant and bile flow stimulation activities have been reported. However, some experiments have demonstrated that two other curcuminoids present in turmeric, namely, desmethoxycurcumin and bisdesmethoxycurcumin, also possess biological activities, but their potency was different from that of curcumin (Chatterjee *et al.*, 1999; Deters *et al.*, 1999; Portes *et al.*, 2007). Therefore, to ensure the repeatability of clinical efficacy, the quality control of turmeric based on the contents of the three curcuminoid should be taken into account.

HPLC (Taylor and McDowell, 1992; Jayaprakasha *et al.*, 2002), CE (Sun *et al.*, 2002; Lechtenberg *et al.*, 2004) and TLC densitometric (Ansari *et al.*, 2005; Pathania *et al.*, 2006) methods have been described for the analysis of curcuminoids in turmeric. However, in consideration of its simplicity and low cost, the present study proposes a convenient TLC image analysis method using computer technology. A digital image of a TLC chromatogram was obtained with a flat scanner, and densities of the TLC spots were analysed using the Scion Image program. Scion Image is public computer software modified from the NIH Image software of the National Institutes of Health, USA (Scion Corporation, 2000–2001). Its main application is in the field of biological image analysis. Only a few applications concerning TLC image analysis have been reported (Lancaster *et al.*, 2005; Mabinya *et al.*, 2006). This study is the first report on a validated TLC image analysis using Scion Image program for the quality control of herbal raw material.

* Correspondence to: U. Sotanaphun, Faculty of Pharmacy, Silpakorn University, Nakhon-pathom 73000, Thailand. E-mail: uthai@email.pharm.su.ac.th

[a] Department of Pharmacognosy, Faculty of Pharmacy, Silpakorn University, Nakhon-pathom 73000, Thailand

[b] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Silpakorn University, Nakhon-pathom 73000, Thailand

Contract/grant sponsor: Silpakorn University Research and Development Institute, Thailand.

Copyright © 2008 John Wiley & Sons, Ltd.

Jan – Feb

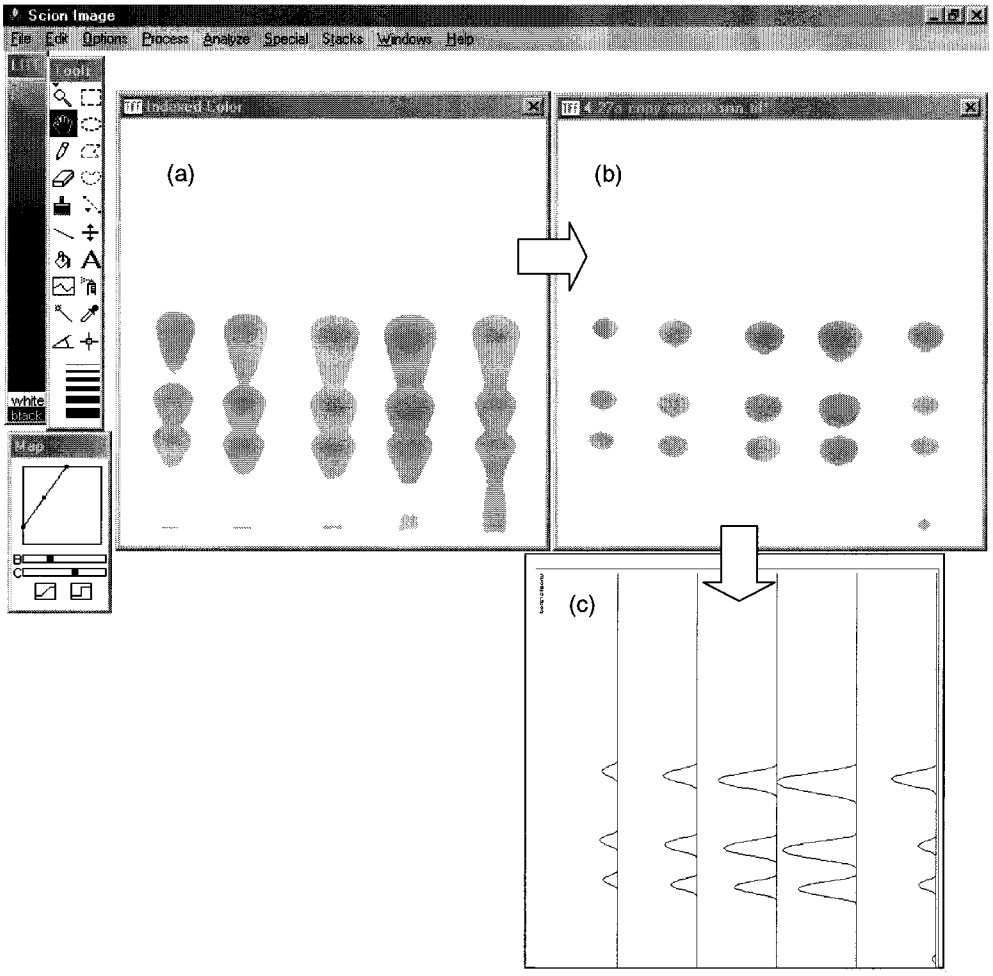

Figure 1. Processing of TLC image analysis by Scion Image software: (a) converting the image to greyscale; (b) smoothing the image to achieve the separated spots; and (c) chromatogram profiles obtained from the smoothing image. (From left to right lanes: standard curcuminoids 0.75, 1.5, 3 and 6 μg/spot and a turmeric sample.)

Experimental

Materials. Curcumin, desmethoxycurcumin and bisdesmethoxycurcumin were purified from turmeric by column chromatography. The identification of these standards was based on the analysis of ^{1}H-NMR spectra and mass spectra in comparison with those previously reported (Péret-Almedia *et al.*, 2005). The purity of each isolated curcuminoid (<99%) was assessed by a TLC densitometric method in the absorbance mode at 254 nm.

Turmeric samples were collected from local markets in Thailand during 2002, and identified by Associate Professor Uthai Sotanaphun. Voucher specimens (US-02-Cl01 to US-02-Cl06) are deposited at the Herbarium of the Department of Pharmacognosy, Silpakorn University, Thailand.

Sample preparation. A sample (25–100 mg) of dried turmeric (425 μm powder) was suspended in 50 mL of methanol and sonicated for 1 h for exhaustive extraction (Supinya, 1993). The extract was dried and re-dissolved in 2.0 mL of methanol.

Chromatographic condition. A sample solution (2 μL) was spotted onto a pre-coated silica gel 60F_{254} plate (0.25 mm thickness; aluminium-backed; Merck, Darmstadt, Germany). The applied spots were pre-concentrated into a band by developing in 100% methanol for a distance of 6 mm. After air-drying, the TLC plate was twice developed to a distance of 80 mm with hexane: chloroform:methanol (10:10:1, v/v/v) in a TLC chamber previously saturated for 30 min.

Imaging and processing. An image of the TLC chromatogr was taken using a Hewlett Packard (Palo Alto, CA, USA) model SCAN Jet 3500C and saved as in .tiff format at a resolution of 600 dpi. The image file was opened with Scion Image for Windows version Alpha 4.0.3.2 (Scion Corporation, Maryland, USA) (Fig. 1). The natural colour was converted to greyscale, and the smooth function (available in the process toolbar) was applied 20–25 times until all of the overlapped spots were clearly separated. A profile plot along the chromatogram was generated using the macro Gelplot2. Finally, the wand tool was used to select the peak corresponding to each curcuminoid in order to allow the measurement of the area under the curve (AUC).

Calibration curve. Standard solutions of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin (each at 0.1875–3 μg/μL) were prepared in methanol. Aliquots (2 μL) of each solution were spotted onto a TLC plate to obtain the concentrations of 0.375, 0.75, 1.5, 3 and 6 μg/spot of each curcuminoid. The chromatogram was developed and the image was scanned and analysed

Copyright © 2008 John Wiley & Sons, Ltd.

as described above. Least square regression analysis was employed in the construction of the calibration curves.

Method validation. Selectivity of the method was determined in relation to the interference from other compounds in turmeric. The concentrations in the lower part of the linear range of the calibration curve were used to construct the linear equations. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on the standard deviation (SD) of the *y*-intercept and the slope (*a*) as 3 SD/*a* and 10 SD/*a*, respectively.

The repeatability (intra-day precision), the intermediate precision (inter-day precision) and the accuracy were determined by analysing six replicates of three different concentrations (0.75, 1.5 and 3 μg/spot) of each curcuminoid. The intermediate precision was determined on two consecutive days. The precision was expressed as the percent relative deviation (%RSD), whereas the accuracy was expressed as a percentage (observed concentration × 100/loading concentration).

A turmeric sample was spiked with curcumin, desmethoxycurcumin and bisdesmethoxycurcumin to give additional concentrations of 0.75, 1.5 and 3 μg/spot. The contents of each curcuminoid were analysed by the proposed method and the recovery of the added curcuminoids was calculated.

TLC densitometric method. Dried powdered turmeric (100 mg) was macerated in methanol (10 mL) at room temperature for 24 h. An aliquot (2 μL) of the extract was applied to a pre-coated silica gel $60F_{254}$ plate (0.25 mm thickness; Merck). The mobile phase consisted of benzene:chloroform:methanol (15:80:5, v/v/v)[a]. The TLC chamber was previously saturated for 1 h and the developing distance was 80 mm. Densitometric scanning was performed using a Camag (Muttenz, Switzerland) TLC scanner II in the absorbance mode at 254 nm in conjunction with Camag CAT3.1 software.

Results and Disscussion

The TLC mobile phase initially employed was benzene: chloroform:methanol (49:49:2, v/v/v) based on the method published in an official pharmacopoeia (*Thai Herbal Pharmacopoeia*, 1995). However, the distance moved by the curcuminoids was so small that it was not suitable for quantitative analysis. The ratio of the mobile phase was then adjusted to 27:70:5 (v/v/v), giving suitable R_f values of all curcuminoids. However, the resolution was not satisfactory because of the tailing and, moreover, benzene is not recommended for use in routine analytical system by virtue of its toxicity. In order to overcome these problem, the mobile phase hexane: chloroform:methanol (10:10:1, v/v/v) was developed. This gave a good resolution and suitable R_f values when the TLC plate was developed twice. The R_f values of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin were 0.42, 0.25 and 0.18, respectively. The concentration and volume of the applied sample were also adjusted to give clear spots of the separated curcuminoids when detected by the naked eye under daylight.

The imaging program Scion Image was selected for this study because it contains many useful application functions. Moreover, it is a public domain software that can be downloaded from www.scioncorp.com (Scion Corporation, 2000–2001). A profile plot of the TLC chromatogram (Fig. 1) was generated using the macro Gelplot2 in the software package. The smooth function was also applied before transforming the TLC spot into a peak. Because of plate-to-plate variability, repetition of the smoothing function was carried out 20–25 times until clearly separated peaks for each curcuminoid were obtained.

Data for the calibration curves of standard curcuminoids are shown in Table 1. The polynomial regressions showed good linearity relationships ($R^2 > 0.99$) over the range from 0.375–6 μg/spot for all curcuminoids.

The developed method was validated in compliance with the International Conference on Harmonization (ICH) guideline [ICH-Q2 (R1), 2005]. Figure 1 clearly shows that, at the R_f values of all curcuminoids, no interfering peaks of other chemical compositions in turmeric are observed. Therefore the selectivity of the method was confirmed. In order to determine the LOD and LOQ values, the linear regressions from the low concentration range (0.375–1.5 μg/spot) of each curcuminoid were calculated (Ansari *et al.*, 2005), giving $y = 1668.67x - 256.67$ ($R^2 = 0.9939$),

Table 1. Polynomial regression in the form of $y = ax^2 + bx + c$ (where y = a number of pixels, x = μg/spot, and $n = 3$) for three curcuminoids in turmeric

Parameter	Curcumin	Desmethoxycurcumin	Bisdesmethoxycurcumin
TLC imaging analysis			
A	−111.16 ± 5.24	−95.70 ± 4.14	−69.74 ± 4.28
B	1753.80 ± 29.56	1361.00 ± 34.06	1004.16 ± 25.79
C	−240.14 ± 23.67	49.14 ± 14.47	100.74 ± 15.17
R^2	0.9990 ± 0.0003	0.9985 ± 0.0006	0.9978 ± 0.0012
Range (μg/spot)	0.375–6	0.375–6	0.375–6
LOD (ng/spot)	43	69	73
LOQ (ng/spot)	143	230	242
TLC densitometric method			
A	−257.20 ± 32.01	−178.75 ± 31.78	−46.25 ± 11.86
B	1609.80 ± 34.22	1637.70 ± 201.91	1512.63 ± 263.71
C	27.87 ± 29.97	−67.01 ± 12.22	−109.35 ± 43.03
R^2	0.9999 ± 0.0001	0.9997 ± 0.0001	0.9989 ± 0.0002
Range (μg/spot)	0.0625–2	0.0625–2	0.0625–2
LOD (ng/spot)	55	25	107
LOQ (ng/spot)	183	83	355

Copyright © 2008 John Wiley & Sons, Ltd.

Table 2. Precision and accuracy data for the determination of three curcuminoids in turmeric

Compound	Loading (μg/spot)	Amount detected (μg/spot)	%RSD	Accuracy (%)
Repeatability (n = 6)				
Curcumin	0.75	0.77 ± 0.02	2.90	103.31
	1.50	1.60 ± 0.03	1.64	106.66
	3.00	2.91 ± 0.02	0.82	96.99
Desmethoxy-curcumin	0.75	0.78 ± 0.02	2.99	104.25
	1.50	1.60 ± 0.03	2.05	106.94
	3.00	2.90 ± 0.03	1.07	96.81
Bisdesmethoxy-curcumin	0.75	0.79 ± 0.03	3.83	104.98
	1.50	1.61 ± 0.03	2.00	107.50
	3.00	2.90 ± 0.03	0.89	96.55
Intermediate precision (2 days, n = 12)				
Curcumin	0.75	0.77 ± 0.03	3.67	103.21
	1.50	1.58 ± 0.03	2.20	105.06
	3.00	2.93 ± 0.03	0.93	97.63
Desmethoxy-curcumin	0.75	0.79 ± 0.03	3.80	104.81
	1.50	1.58 ± 0.04	2.76	105.48
	3.00	2.92 ± 0.03	1.07	97.29
Bisdesmethoxy-curcumin	0.75	0.79 ± 0.04	4.78	105.18
	1.50	1.59 ± 0.05	3.10	105.75
	3.00	2.91 ± 0.03	1.11	97.15

Table 3. Recovery studies ($n = 3$) for the determination of three curcuminoids in turmeric

Compound	Amount added (μg/spot)	Recovery (%)	Average recovery (%)
Curcumin	0.75	94.95 ± 3.29	101.87 ± 9.68
	1.50	112.93 ± 1.92	
	3.00	97.73 ± 3.43	
Desmethoxy-curcumin	0.75	99.33 ± 2.01	104.79 ± 6.03
	1.50	111.26 ± 0.96	
	3.00	103.79 ± 1.76	
Bisdesmethoxy-curcumin	0.75	86.23 ± 5.20	101.49 ± 13.23
	1.50	109.80 ± 2.78	
	3.00	108.44 ± 1.80	

$Y = 1290.63x + 32.67$ ($R^2 = 0.9922$) and $y = 970.74x + 75.83$ ($R^2 = 0.9900$) for curcumin, desmethoxycurcumin and bisdesmethoxycurcumin, respectively. The LOD and LOQ values were the concentrations that gave AUC values that were 3 × SD and 10 × SD above the *y*-intercept, respectively. The results (Table 1) were comparable with the LOD and LOQ values obtained from the TLC densitometric method. Precision of the method was studied using three concentrations of curcuminoids at 0.75, 1.5 and 3 μg/spot. The results revealed that the repeatability and intermediate precision of the method was satisfactory (Table 2). The accuracy of the method is demonstrated in Table 2 and was in the range of 97–106%. For the examination of recoveries, known amounts of curcuminoids were added to a turmeric sample. Mean recoveries for curcumin, desmethoxycurcumin and bisdesmethoxycurcumin were 101.87, 104.79 and 101.49%, respectively (Table 3). These data indicated a good accuracy of the method.

Curcuminoids in six turmeric samples were analysed by the developed method. The results were comparable with those obtained using the conventional TLC densitometric method (Table 4). The analytical results of both methods were not significantly different ($p > 0.05$) as determined using Student's paired *t*-test. All of this information suggests that this low-cost and simple TLC image analysis method could be used as a routine method for the quality control of each curcuminoid in turmeric. The previous application of the Scion Image with respect to herbal analysis has involved only the screening of the content of ferulic acid in plant material, but the method was not validated (Mabinya *et al.*, 2006). Therefore the application of Scion Image for the quantitative TLC analysis of herbal raw material has been demonstrated herein for the first time.

Acknowledgements

The authors thank Silpakorn University Research and Development Institute, Thailand, for the financial support of part of this work.

 Copyright © 2008 John Wiley & Sons, Ltd.

Table 4. Contents of three curcuminoids (%w/w) in turmeric sample determined by TLC image analysis and TLC densitometry ($n = 3$)

Sample	Curcuminoid	TLC image analysis with Scion Image	TLC densitometry
1	Curcumin	3.00 ± 0.18	2.99 ± 0.12
	Desmethoxycurcumin	1.20 ± 0.07	1.20 ± 0.07
	Bisdesmethoxycurcumin	1.14 ± 0.12	1.20 ± 0.04
2	Curcumin	4.95 ± 0.09	4.89 ± 0.07
	Desmethoxycurcumin	1.79 ± 0.03	1.73 ± 0.07
	Bisdesmethoxycurcumin	2.06 ± 0.04	2.08 ± 0.06
3	Curcumin	3.42 ± 0.19	3.30 ± 0.17
	Desmethoxycurcumin	1.55 ± 0.06	1.50 ± 0.06
	Bisdesmethoxycurcumin	1.44 ± 0.07	1.46 ± 0.03
4	Curcumin	5.91 ± 0.17	5.98 ± 0.10
	Desmethoxycurcumin	2.45 ± 0.03	2.26 ± 0.07
	Bisdesmethoxycurcumin	2.86 ± 0.03	2.75 ± 0.13
5	Curcumin	4.34 ± 0.13	4.35 ± 0.09
	Desmethoxycurcumin	1.95 ± 0.07	1.90 ± 0.04
	Bisdesmethoxycurcumin	2.53 ± 0.09	2.44 ± 0.04
6	Curcumin	7.50 ± 0.09	7.51 ± 0.10
	Desmethoxycurcumin	3.03 ± 0.03	3.05 ± 0.12
	Bisdesmethoxycurcumin	4.15 ± 0.05	4.10 ± 0.16

References

Ansari MJ, Ahmad S, Kohli K, Ali J and Khar RK. 2005. Stability-indicating HPTLC determination of curcumin in bulk drug and pharmaceutical formulations. *J Pharm Biomed Anal* **39**: 132–138.

Banerjee A and Nigam SS. 1978. Antimicrobial efficacy of the essential oil of *Curcuma longa*. *Indian J Med Res* **68**: 864–866.

Blumenthal M, Goldberg A and Brinckman J. 2000. *Herbal Medicine. Expanded Commission E monographs*. American Botanical Council: USA; 379–384.

Bundy R, Walker AF, Middleton RW and Booth J. 2004. Turmeric extract may improve irritable bowel syndrome symptomology in otherwise healthy adults: a pilot study. *J Altern Complement Med* **10**: 1015–1018.

Chatterjee S, Padwal Desai SR and Thomas P. 1999. Effect of γ-irradiation on the antioxidant activity of turmeric (*Curcuma longa L.*) extracts. *Food Res Int* **32**: 487–490.

Deters M, Siegers C, Muhl P and Häensel W. 1999. Choleretic effect of curcuminoids on a acute cyclosporine-induced cholestasis in the rat. *Planta Med* **65**: 610–613.

ICH-Q2 (R1) 2005. *Validation of Analytical Procedures: Text and Methodology*. International Conference on Harmonization: Geneva.

Jayaprakasha GK, Rao LJM and Sakariah KK. 2002. Improved HPLC method for the determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin. *J Agric Food Chem* **50**: 3668–3672.

Kositchaiwat C, Kositchaiwat S and Havanondha J. 1993. *Curcuma longa* Linn. in the treatment of gastric ulcer comparison to liquid antacid: a controlled clinical trial. *J Med Assoc Thai* **76**: 601–605.

Lancaster M, Goodall DM, Bergstrom ET, McCrossen S and Myers P. 2005. Quantitative measurements on wetted thin layer chromatography plates using a charge coupled device camera. *J Chromatogr A* **1090**: 165–171.

Lechtenberg M, Quandt B and 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**: 152–158.

Lutomski J, Keasia B and Debska W. 1974. Effect of an alcohol extract and of active ingredients from *Curcuma longa* on bacteria and fungi. *Planta Med* **26**: 9–19.

Mabinya LV, Mafunga T and Brand JM. 2006. Determination of ferulic acid and related compounds by thin layer chromatography. *Afr J Biotechnol* **5**: 1271–1273.

Pathania V, Gupta AP and Singh B. 2006. Improved HPTLC method for determination of curcuminoids from *Curcuma longa*. *J Liq Chromatogr Relat Tech* **29**: 877–887.

Péret-Almedia L, Cherubino APF, Alves RJ, Dufossé L and Glória MBA. 2005. Separation and determination of the physico-chemical characteristics of curcumin, demethoxycurcumin and bisdemethoxycurcumin. *Food Res Int* **38**: 1039–1044.

Portes E, Gardrat C and Castellan A. 2007. A comparative study on the antioxidant properties of tetrahydrocurcuminoids and curcuminoids. *Tetrahedron* **63**: 9092–9099.

Prucksunand C, Indrasukhsri B, Leethochawalit M and Hungspreugs K. 2001. Phase II clinical trial on effect of the long turmeric (*Curcuma longa* Linn.) on healing of peptic ulcer. *Southeast Asian J Trop Med Public Health* **32**: 208–215.

Ramirez Boscá A, Soler A, Carrión-Gutiérrez MA, Diaz-Alperi J, Bernd A, Quintanilla C, Quintanilla Almagro E and Miquel J. 2000a. An hydroalcoholic extract of *Curcuma longa* lowers the apo B/apo A ratio. Implications for atherogenesis prevention. *Mech Ageing Dev* **119**: 41–47.

Ramirez Boscá A, Soler A, Carrión-Gutiérrez MA, Mira DP, Zapata JP, Diaz-Alperi J, Bernd A, Quintanilla Almagro E and Miquel J. 2000b. An hydroalcoholic extract of *Curcuma longa* lowers the abnormally high values of human-plasma fibrinogen. *Mech Ageing Dev* **114**: 207–210.

Scion Corporation, 2000–2001. *Scion Image for Windows version Alpha 4.0.3.2*. Maryland, USA.

Standard ASEAN Herbal Medicine. Volume 1. 1993. Aksara Buana Printing: Jakarta; 193–206.

Sun X, Gao C, Cao W, Yang X and Wang E. 2002. Capillary electrophoresis with amperometric detection of curcumin in Chinese herbal medicine pretreated by solid-phase extraction. *J Chromatogr A* **962**: 117–125.

Supinya T. 1993. Curcuminoids and volatile oil determination in turmeric from various location in Thailand. Masters thesis in Pharmacognosy. Chulalongkorn University: Thailand.

Taylor SJ and McDowell IJ. 1992. Determination of the curcuminoid pigments in turmeric (*Curcuma domestica* Val) by reversed-phase high-performance liquid chromatography. *Chromatographia* **34**: 73–77.

Thai Herbal Pharmacopoeia, Volume 1. 1995. Prachachon: Bangkok; 38–44.

Thamlikitkul V, Bunyapraphatsara N, Dechatiwongse T, Theerapong S, Chantrakul C, Thanaveerasuwan T, Nimitnon S, Boonroj P, Punkrut W and Gingsungneon V. 1989. Randomized double blind study of *Curcuma domestica* Val. for dyspepsia. *J Med Assoc Thai* **72**: 613–620.

Yegnanarayan R, Saraf AP and Balwani JH. 1976. Comparison of anti-inflammatory activity of various extracts of *Curcuma longa* (Linn). *Indian J Med Res* **64**: 601–608.

Copyright © 2008 John Wiley & Sons, Ltd.