Determination of the Curcuminoid Pigments in Turmeric (*Curcuma domestica* Val) by Reversed-Phase High-Performance Liquid Chromatography

S. J. Taylor* / I. J. McDowell

Natural Resources Institute, Central Avenue, Chatham Maritime, Chatham, ME4 4TB, UK

Key Words

Column liquid chromatography
Curcuminoid pigments
Turmeric, *Curcuma domestica* Val.

Summary

A rapid, simple and reproducible reversed-phase high-performance liquid chromatographic method was developed for the quantitative determination of curcumin, demethoxycurcumin and bis-demethoxycurcumin in ethanolic extracts of turmeric. The pigments were separated on a styrene-divinylbenzene copolymer column (Hamilton PRP-1), using an acetonitrile-water (55:45, v/v) mobile phase. The pigments were monitored with a diode-array detector at 425 nm. The limit of detection was 10.2 ng curcumin, 11.1 ng demethoxycurcumin and 6.2 ng bis-demethoxycurcumin. Comparison of HPLC and spectrophotometric results for the determination of the total curcuminoid content for a number of turmeric samples, reveal that the spectrophotometric method invariably yielded higher results, indicating an overestimation of curcuminoids.

Introduction

The rhizomes of turmeric, *Curcuma domestica* Val. (syn. *C. longa* Koenig *non* L.), possess an intense yellow-orange pigmentation which is attributed to three curcuminoids [1]: curcumin, demethoxycurcumin (DMC), and bis-demethoxycurcumin (BDMC; Figure 1). A substantial trade exists in turmeric fingers, the boiled and dried lateral branches of the rhizome, which are employed in the ground form as a spice and colouring agent in food preparations or for the industrial extraction of the curcuminoid pigments. In trade, turmeric fingers are classified into two types, 'Madras' and 'Alleppey', which differ in their curcuminoid content and colour tone [2]. In all end-use applications, the curcuminoid content of the fingers or the solvent extracts is a major, if not the main, quality criterion.

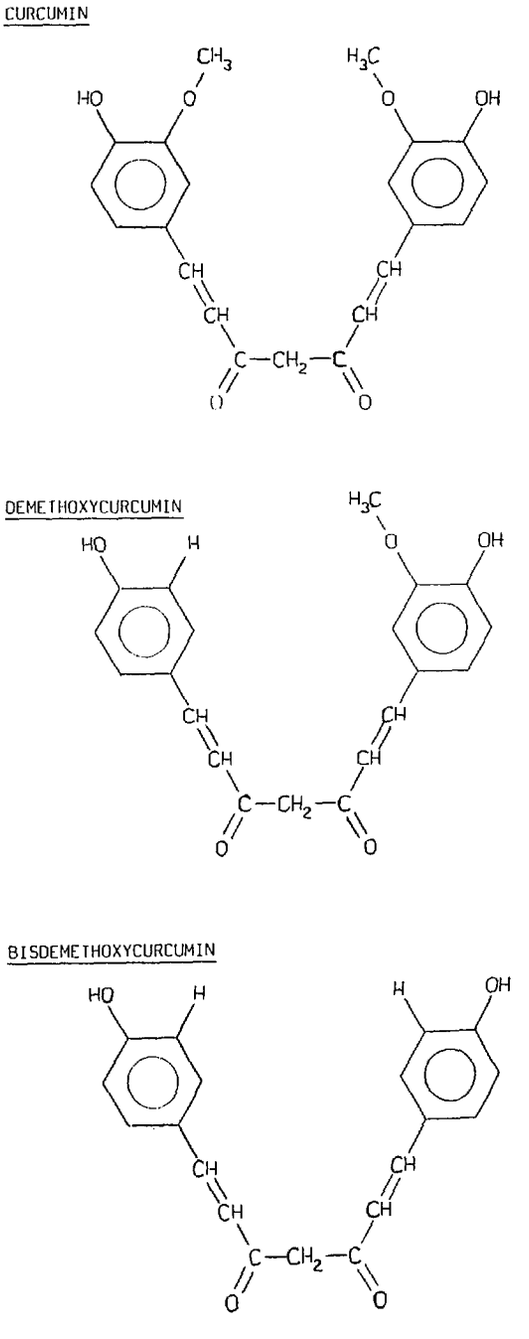

Figure 1
The structures of the curcuminoid pigments.

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A variety of procedures have been published for determining the colour value of turmeric. The most commonly used method is the determination of total curcuminoids by a spectrophotometric measurement at 425 nm of an ethanolic extract of the ground spice [3, 4]. The method, however, gives no information on the relative proportions of curcumin, DMC and BDMC, which could be useful in studies of the effects of cultivar, environment and, agronomic and processing treatments on turmeric quality. Paper or thin layer chromatography methods followed by spectrophotometric analysis [5, 6] have been described for determining the relative amounts of the three curcuminoid pigments, but these procedures are time-consuming and lacking in precision. Recently, two high-performance liquid chromatographic (HPLC) methods [7, 8] have been reported that offer a more convenient and accurate means of separating and estimating individual curcuminoids. The first publication [7] reported the separation of curcuminoid pigments on a bonded amine column with ethanol as the mobile phase. The method gave near-baseline resolution of the pigments, however, there was noticeable tailing of all the peaks, indicating adsorption of curcuminoids onto the column. In the later publication [8], a C_{18}-reversed phase column was used with acetonitrile-0.05 M phosphate buffer (pH 4.4; 50:50, v/v) as the eluent. Although peak tailing was reduced using this method, baseline resolution of the peaks was poor.

The present work was undertaken to develop a stable and reproducible HPLC method for the analysis of curcuminoid pigments. Two different types of reversed-phase columns were evaluated for their ability to resolve the curcuminoid pigments in turmeric. Hypersil ODS, a hydrocarbon (C_{18}) bonded silica column, which has previously been used to analyse curcumin pigments [8] was studied using various mobile phase modifiers. This was compared with an alternative reversed-phase system based on a non-silica polymeric column (Hamilton PRP-1). A comparison was also made between the HPLC and the ISO 5566-1982 spectrophotometric methods in the determination of the total curcuminoid content of turmeric.

Experimental

Solvents and Standards

Acetonitrile (HPLC grade), water (HPLC grade) and ethanol (AR and HPLC grade) were obtained from FSA Laboratory Supplies (Loughborough, UK).

Curcumin and BDMC were synthesized according to the procedures of Roughley and Whiting [9], and recrystallized from 96 % ethanol. Each standard yielded a single peak on HPLC analysis under the conditions described here. The melting points were 216–218 °C and 177–184 °C for curcumin and BDMC respectively. It was not possible to synthesize DMC on a small-scale using the methods of Roughley and Whiting [8]. It was therefore decided to use a natural mixture of curcuminoids (Aldrich Chemical Company Ltd, Gillingham, Dorset, UK) containing DMC, curcumin and BDMC to estimate the DMC content of turmeric samples. The amount of DMC present in the mixture was calculated by difference using the synthesized curcumin and BDMC. Prior to use the mixture was recrystallized from ethanol, and the purity of the standard was ascertained following chromatography by the HPLC method described here.

Preparation of External Standard Solutions

Standard solutions for the synthesized curcumin and BDMC, containing 50, 100, 250, 500 and 1000 ng of each standard were prepared in HPLC grade ethanol. Ethanolic solutions of the purified natural curcuminoid mixture containing 50, 100, 250, 500 and 1000 ng of DMC were also prepared. The solutions were stored at –10 °C until required for analysis.

Preparation of Curcumin Extract

Commercial finger turmeric, originating from India (Alleppey and Madras types) and Jamaica were employed to prepare curcumin extracts. The whole fingers were ground to pass a sieve with 0.5 mm apertures. The ground material (0.5 g) was extracted by refluxing for 2.5 h with ethanol, according to the procedures described in ISO 5566-1982 [3]. After cooling, the extract was filtered and made up to 100 ml with ethanol rinsings in a volumetric flask. This stock solution was used directly for HPLC analysis of curcuminoid pigments. The extract was diluted (1:50, v/v) using ethanol prior to determination of the total curcuminoid content by the ISO 5566-1982 spectrophotometric method.

HPLC Equipment

The HPLC system used for the pigment analyses comprised of an ACS 352 tertiary gradient pump (Macclesfield, UK), a Spark Holland SPH 125 HPLC autosampler (ACS Macclesfield, UK) fitted with a 20 µl loop, and a Hewlett-Packard photo-diode array system (200–600 nm, Wokingham, Berkshire, UK). The equipment was interfaced with a Trivector data collector (NE Technology, Nottingham, UK). Data was stored and processed by LA 1000 chromatography software (NE Technology, Nottingham, UK).

HPLC Columns and Solvents

The following columns were used:

Hypersil ODS. Packing: Hypersil C_{18}, 5-µm, pore size 12 nm; column size: 25 × 0.49 cm; column source: Hichrom Excel grade (Woodley, Berkshire, UK).

Hamilton PRP-1. Packing: Hamilton PRP-1 styrene-divinylbenzene copolymer, 5-µm, pore size 7.5 nm; column size: 15 × 0.49 cm; column source: Anachem (Luton, Berkshire, UK).

The following mobile phases were assessed:

Acetonitrile-aqueous, (55:45, v/v). Three aqueous phases were used: 1 % citric acid (adjusted to pH 2.8 and 4.4 with solid sodium hydroxide) and water. Flow rates were 1.0 ml min^{-1}. The chromatography was performed at ambient temperature.

Identification and Quantitation

Individual pigments were identified by comparison of retention times and absorption spectra with authentic standards, and their concentrations determined by the external standard method. The HPLC method was compared with the ISO spectrophotometric method [3] in the determination of total curcuminoid content. The spectrophotometric measurements were made at 425 nm using a Pye-Unicam SP8-400 spectrophotometer (Unicam, Cambridge, UK). Ethanol was used as the reference liquid. The total curcuminoid content was expressed as curcumin on the dry basis by means of the formula [3]:

$$\frac{A \times 50 \times 100 \times 100}{E^{1\,\%}_{1\,cm} \times m \times (199 - n)}$$

where A is the measure absorbance

$E^{1\,\%}_{1\,cm}$ is the specific absorbance of a 1 % solution of curcumin measured at 425 nm in cells of optical path length 1 cm, i.e. 1,607

H is the moisture content of the sample, expressed as a percentage by mass.

Statistical Analysis

All statistical analyses were performed using the Statgraphic software package (Statistical Graphics Corporation, Rockville, Maryland, USA).

Results and Discussion

Comparison of Analytical Columns

The adsorption effects previously reported [7, 8] when using silica-based reversed-phase columns to separate the curcuminoid pigments in turmeric [7, 8] were observed in the present study. A chromatogram of a turmeric extract run on a Hypersil ODS column with a mobile phase of acetonitrile-water (55:45, v/v) is shown in Figure 2a. The poor baseline resolution and the pronounced tailing of peaks could be due to interactions between the polar parts of the curcuminoid molecules i.e. phenolic hydroxyls and the keto-enol group, and the surface hydroxyls of these columns. Furthermore, metals bound on the surface of silica-based reversed-phase columns and/or present in the sample could also be implicated in the peak broadening and peak tailing of the curcuminoid compounds. This phenomena has previously been described by Bailey et al. [10] for the poor separation of phenolic compounds on silica-based reversed-phase columns. Citric acid which is a strong metal chelator, was therefore introduced in this study in order to prevent any absorption effects due to these mechanisms. Although there was a marked improvement in resolution and a reduction in tailing, these effects were not completely eliminated (Figure 2b). Furthermore a comparison of the chromatograms obtained when eluting the column with the aqueous phase buffered at pH 2.8 and 4.4 (Figure 2b and 2c) indicate that the separation of curcuminoid pigments on the silica-based column is not pH dependent.

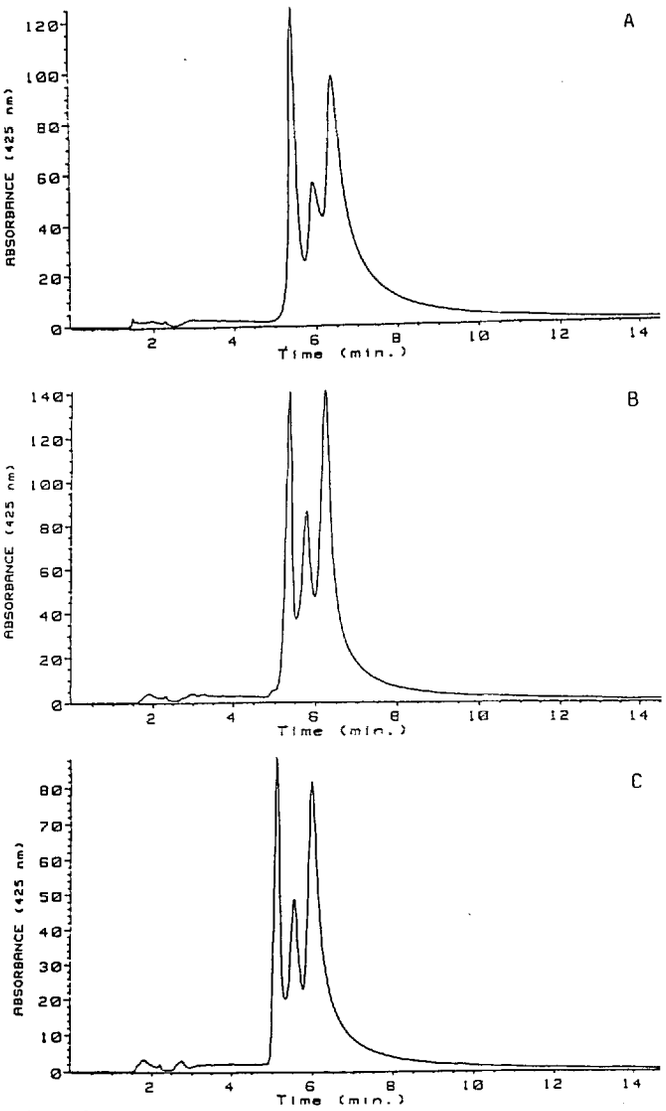

Figure 2

Chromatogram (425 nm) of 'turmeric extract' on the Hypersil ODS column. Acetonitrile-aqueous (55:45, v/v). (a) Aqueous solvent: water. (b) Aqueous solvent: 1 % (w/v) citric acid adjusted to pH 2.8 with solid sodium hydroxide. (c) Aqueous solvent: 1 % (w/v) citric acid adjusted to pH 4.4 with solid sodium hydroxide.

On changing to the Hamilton PRP-1 column, which has no silica present, a baseline separation of the curcuminoid pigments was obtained with a further reduction of tailing. Furthermore, it was found that this column required no buffer, simplifying the analysis (Figure 3). Data on the peak asymmetry of curcumin and the efficiency (N) of the two analytical columns are presented in Table I. Although the values for the column efficiencies are similar, the chromatographic performance of the curcuminoids on the Hamilton PRP-1 column is superior when the test efficiencies of the two columns are taken into account i.e. 20,000 and 3,300 plates for the C-18 and PRP-1 columns respectively.

Different organic phases i.e. methanol and tetrahydrofuran have previously been evaluated in the separation of curcuminoid pigments on silica-based columns [8], but were found to give poorer selectivity than acetonitrile. In the present study, the employment of methanol or

tetrahydrofuran as the organic phase, offered no improvement in selectivity for the Hamilton PRP-1 column.

Comparison of Peak Areas and Heights on the Hamilton PRP-1 Column

The precision of peak area and peak height measurements were compared for the analysis of the curcuminoid pigments using five replicates over a range of amounts (50 to 1000 ng). A comparison of the coefficients of variation (CV; Table II) for both peak height and peak area measurements reveal that the height measured peaks offered improved precision over the range of amounts tested for all three curcuminoids.

Table III shows the linear regression equations, correlation coefficients (r) and limits of detection (l.o.d) of the peak height measurements. A linear relationship was observed for each of the curcuminoids between 50 and 1000 ng. The correlation coefficients of 0.991 to 1.000 confirming the closeness of fit of the experimental data to the regression curve.

The limit of detection (l.o.d) was found to be 10.2 ng curcumin, 11.1 ng demethoxycurcumin and 6.2 ng of bis-demethoxycurcumin.

Table I. Comparison between the C-18 and the PRP-1 columns for peak asymmetry and efficiency. (Calculated from curcumin).

Column	Curcumin Ret. Time (min)	Peak Asymmetry (A_s)	Peak Efficiency (N)
C-18	6.08	4.46	1783
PRP-1	8.15	2.08	1471

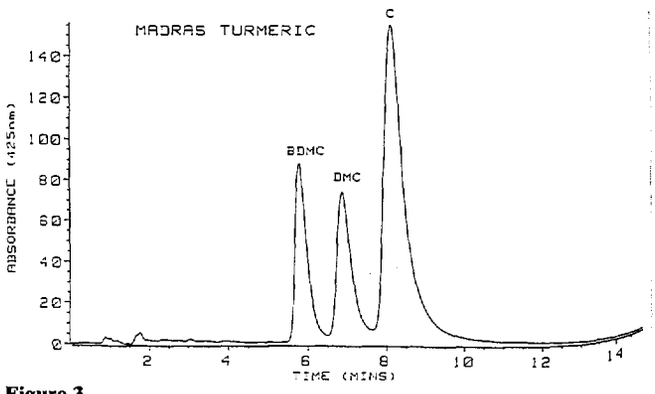

Figure 3
Chromatogram (425 nm) of 'turmeric extract' on the Hamilton PRP-1 column. Acetonitrile-water (55:45, v/v).

HPLC Elution Profile of Curcuminoids Extracted from Madras-Type Turmeric

An HPLC chromatogram (425 nm) of a Madras type turmeric extract run on a Hamilton PRP-1 column is shown in Figure 3. The three peaks at retention time (RT) 5.811 min, 6.896 min and 8.139 min, were identified as BDMC, DMC and curcumin respectively, following comparison of retention times and absorption spectra with those of authentic standards (Figure 4).

Some peaks were observed at the void volume, but as a result of their small size and unresolved nature it was impossible to obtain UV/VIS spectra. These were followed by a further group of seven resolved and partially resolved minor peaks which accounted for less than 0.5 % of the total absorbance at 425 nm (Figure 5). The photodiode-array UV/VIS spectra for these peaks were similar with absorption maxima between 350–360 nm. The identities of these compounds are unknown.

Table II. Comparison between the coefficient of variation (CV) for peak area and peak height measurements for amounts of curcuminoid pigments between 50 and 1000 ng.

Amount (ng)	Coefficient of Variation (CV)					
	Curcumin		DMC		BDMC	
	Height	Area	Height	Area	Height	Area
50	1.71	7.89	2.22	5.21	1.21	4.87
100	0.92	1.68	1.01	2.52	1.47	3.44
250	0.66	4.03	0.22	0.45	0.08	0.28
500	0.34	2.86	1.42	1.11	0.76	1.82
1000	0.75	0.70	0.60	0.76	0.63	1.94

Table III. Regression equations and limits of detection (l.o.d) for the analysis of curcuminoid pigments at 425 nm on a Hamilton PRP-1 column.

Pigment	Intercept (a)	S.E.	Slope (b)	S.E.	Correlation (r) coefficient	l..o.d (ng)
Curcumin	-5.969	1.797	0.142	3.823×10^{-3}	0.989	10.2
DMC	-3.257	1.262	0.150	4.086×10^{-3}	0.991	11.1
BDMC	-9.528	2.469	0.336	5.253×10^{-3}	0.996	6.2

S.E = Standard error
l.o.d = limit of detection

Table IV. Curcuminoid content (% m/m) of turmeric, as determined by the HPLC method and ISO spectrophotometric method.

Origin		Individual Curcuminoids*			Total Curcuminoids	
		BDMC	DMC	Curcumin	By HPLC	By spectro-photometry
'Madras'	(India)	0.22	0.48	0.84	1.54	1.69
'Alleppey'	(India)	0.89	1.75	3.16	5.80	6.26
Jamaica		0.62	0.92	1.56	3.10	3.67

* Determined by HPLC.

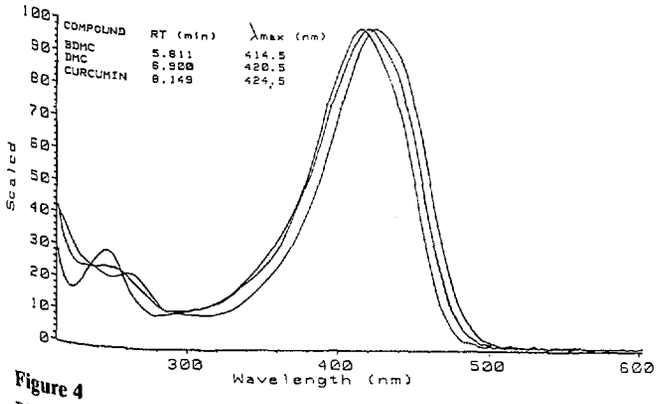

Figure 4
Photodiode-array UV/VIS spectra of the curcuminoids.

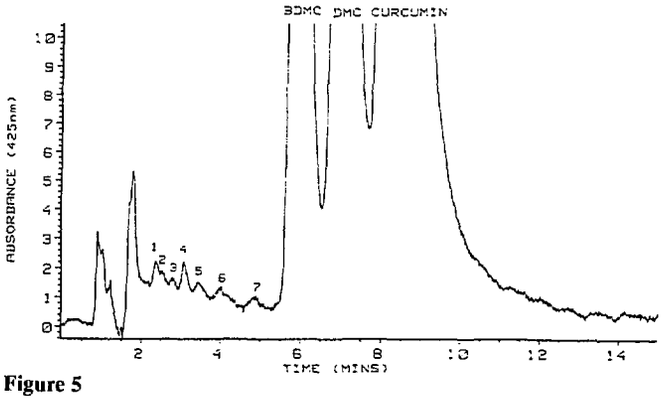

Figure 5
Chromatogram (425 nm) of 'turmeric extract' on the PRP-1 column showing the group of seven resolved and partially resolved minor peaks.

Quantitative Determination of Curcuminoid Pigments in Turmeric

Table IV reports the amounts (% m/m) of individual curcuminoid pigments and the total curcuminoid content for the three samples examined, as determined by HPLC. The results are comparable to those previously reported in the literature for *C. domestica* [1, 6, 11–13].

Comparison of the HPLC and spectrophotometric results (Table IV) for the analysis of total curcuminoid content, reveal that for the three turmeric samples analysed in the present study, the spectrophotometric results yielded higher results. This is attributable to the very selective and accurate HPLC assay. The spectrophotometric method measures all coloured materials, including any non-curcuminoids present and absorbing at the wavelength, and expresses the result as 'curcumin'.

Conclusions

The HPLC method described here, gives a baseline separation of curcumin, DMC and BDMC. There is little or no adsorption of curcuminoids onto the column. The method is simple, reproducible, requires no additives to the aqueous phase and is rapid, the analysis was achieved in less than 10 minutes. Height measured peaks gave a linear response for sample amount between 0 and 1000 ng and offered improved precision than area measured peaks. The HPLC method offers a more accurate means of determining the total and individual curcuminoid content of turmeric than the ISO spectrophotometric method currently employed.

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