Determination of Curcuminoids by Liquid Chromatography with Diode Array Detection: Application to the Characterization of Turmeric and Curry Samples

Òscar Vidal[a], Xavier Castilla[a], Núria Aliaga-Alcalde[b,c], Ana M. López-Periago[b], Concepción Domingo[b], Sonia Sentellas[a] and Javier Saurina[a,*]

[a]*Department of Chemical Engineering and Analytical Chemistry, University of Barcelona, Martí i Franquès 1-11, 08028, Barcelona, Spain;* [b]*Institut de Ciència de Materials de Barcelona (CSIC), Campus UAB, 08193 Bellaterra, Spain;* [c]*ICREA (Institut Català de Recerca i Estudis Avançats), Passeig Lluis Companys 23, 08010 Barcelona, Spain*

Abstract: ***Background*:** A simple, rapid and efficient method for the determination of curcumin and other polyphenols in turmeric and curry samples was here developed. The method relied on sample extraction with methanol and extract analysis by liquid chromatography with diode array detection (HPLC-DAD).

***Method*:** The separation of components was carried out in reversed-phase mode using an elution gra-dient based on 0.1% (v/v) formic acid aqueous solution and acetonitrile as the components of the mo-bile phase. Chromatograms were recorded at 420 nm for specific monitoring of curcumin and related compounds.

***Results*:** Extraction and separation conditions were optimized by experimental design and multicriteria response functions. Figures of merit were established under the selected experimental conditions. In general, repeatability of peak areas were better than 0.4%, detection limits were below 0.006 mg L^{-1} and quantitative recoveries expressed as a percentage were about 100 ± 2. The method was applied to quantify curcuminoids in commercial samples. It was found that apart from curcumin, demethoxycur-cumin and bisdemethoxycurcumin, other related molecules also occurred in the samples. In this re-gard, a tentative elucidation of possible unknown curcuminoids was attempted by liquid chromatog-raphy coupled to mass spectrometry.

***Conclusion*:** Differences in the compositional profiles among samples were encountered to be rele-vant, so that the resulting HPLC-DAD data was exploited for chemometric characterization of turmer-ic and curry samples. Samples were successfully discriminated according to matrix types, species va-rieties and origins.

Keywords: Curcumin determination, curcuminoids, experimental design, food sample characterization, liquid chromatography, principal component analysis.

1. INTRODUCTION

Turmeric (*Curcuma longa*) is a plant related to ginger family that has been used for centuries as a remedy in traditional Asian pharmacy due to its antioxidant and anti-inflammatory activities [1-3]. Nowadays, new applications as an anti-cancer agent have stirred up the interest of the scientific community [4, 5]. Apart from medical uses, turmeric is appreciated worldwide as a condiment and colorant in cuisine [6]. The turmeric rhizome awakens great commercial interest as it contains the highest amounts of active molecules. Turmeric is typically sold as dried portions or powdered products, also in combinations with other species such as ginger, garlic, cinnamon, clove, mustard seed, cardamoms and peppers for the preparation of curries. Typical contents of turmeric in curry samples are about 30 wt% [7].

Among other components, curcumin (ccm) has been recognized as the most relevant molecule of turmeric, providing both color and pharmacological activity to the product [1-6]. Various related molecules, the so -called curcuminoids (*e.g.*, demethoxycurcumin –dmc– and bisdemethoxycurcumin –bdmc–), are concomitant to the presence of curcumin. Any-way, curcuminoids display similar physicochemical and pharmaceutical properties [4, 5]. The wide range of curcumin bioactivities has attracted the interest of the scientific community to elucidate its structure-activity relationship [8], as a

*Address correspondence to this author at the Department of Chemical Engineering and Analytical Chemistry, University of Barcelona, Martí i Franquès 1-11, 08028, Barcelona, Spain; Tel: +34 4021277;
E-mail: xavi.saurina@ub.edu

starting point to design and synthesize new structurally related drugs with improved properties [5].

The recovery of curcuminoids from turmeric and other food samples is commonly carried out by liquid extraction using organic solvents such as methanol, ethanol, dichloromethane, *etc*. [9]. Standard procedures for quantitative extraction consist of solvent reflux with or without further solvent evaporation [10, 11]. Complementarily, solid phase extraction has been introduced for further clean-up and preconcentration of extracts [12]. Recent publications rely on novel approaches, such as ultrasonic-assisted extraction [13] and pressurized fluid extraction [14] for more straightforward and efficient procedures. Another exhaustive study of factors influencing on the extraction has been conducted by Mudge *et al.* using design of experiments (DOE) [15].

The quantification of curcumin and related molecules in food samples entails an efficient separation of components in order to avoid mutual interferences. High performance liquid chromatography (HPLC) with UV-Vis spectroscopic detection (often with a diode array detector, DAD) is the current technique of choice for a rapid, feasible and accurate determination of curcumin in food samples. Curcuminoids are typically monitored at 420 nm on the basis of their intense yellow color. As reviewed by Ali *et al* . [11], reversed-phase HPLC with C18 column is commonly used, although anion exchange [16] and micellar chromatography [17] have also been introduced. Some recent modifications to enhance the separation performance are based on phenyl, monolithic and fused-core columns [11, 18, 19]. Apart from UV-vis detection, liquid chromatography coupled to mass spectrometry (LC-MS) has been used for a more selective and sensitive detection [11]. For instance, Ashraf *et al*. proposed an ultra-high performance LC-Quadrupole-Time of flight-MS (UHPLC/Q- TOF-MS) method for rapid determination of curcumin in turmeric cultivars [20]. As an alternative separation technique to resolve curcuminoid mixtures, in the last years, capillary electrophoresis (CE) has also been proposed. Various CE modes, such as capillary zone electrophoresis, non-aqueous capillary electrophoresis, micellar electrokinetic chromatography and microemulsion electrokinetic chromatography, have been applied to determine curcumin-related molecules, including degradation products such as vanillic and ferulic acids and 4-hydroxybenzaldehyde, in food samples [13, 21-24].

As pointed out elsewhere, naturally occurring food components can be used as sample descriptors for characterization, classification and authentication purposes [25-17]. Recently, some researchers explored the possibilities of the curcuminoids as a source of analytical information. For instance, Kulyal and coworkers applied principal component analysis (PCA) to the characterization of turmeric cultivars. Authors concluded that curcuminoid levels could be used to assess the turmeric quality and to discriminate among the plant varieties [28]. In another study, gas chromatography-MS (GC-MS) and LC- MS platforms were used to obtain the compositional profiles of curcuminoids and terpenoids as the data for PCA and cluster analysis. Results revealed differences among cultivars and tissues [29]. Analogously, curcuminoid and terpenoid profiling was exploited to discriminate curcuma samples according to varieties and origin, using PCA and partial least square-discriminant analysis (PLS-DA) for data treatment [30]. Beyond these targeted approaches, untargeted counterparts exploring complex instrumental fingerprints related with curcumin, polyphenols and other organic components were also considered. Ni *et al*. analyzed HPLC -DAD fingerprints with PCA and K-nearest neighbor for the assessment of origin and processing patterns of turmeric rhizomes [31]. In the study by Rohaeti *et al.*, various traditional Indonesian remedies, including various turmeric species, were classified from infrared spectroscopic fingerprints using PCA and other multivariate methods [32].

In this work, a new method for the determination of curcumin and related compounds based on compound extraction with methanol and HPLC-DAD analysis was developed. Although several papers have already been published on the determination of curcuminoids by liquid chromatography (see ref. 12 and therein cited papers), our approach is simple and straightforward from the point of view of both extraction and separation steps. The application of experimental design to method optimization was especially concerned in achieving rapid analyses and high performance, features greatly appreciated when dealing with quality control and routine analysis of large series of food samples. The data resulting from the analysis of commercial samples was further treated by chemometric methods to tackle the characterization of turmeric and curry samples. As a novel aspect, additional data from unknown related compounds and curcuminoid ratios was used to enrich the data set for a most fruitful description of sample patterns. In order to delve into the composition of unknown minor curcuminoids LC-MS was used. Results pointed out the complexity of minor curcuminoid molecules as well as their relevance as descriptors to tackle classification and authentication issues.

2. MATERIALS AND METHODS

2.1. Chemicals and Solutions

Curcumin (ccm, 98%, Sigma-Aldrich, St. Louis, Missouri, USA), desmethoxycurcumin (dmc, >95%, Biopurify Chemicals Ltd., Chengdu, Sichuan, China) and bisdemethoxycurcumin (bdmc, >95%, Biopurify Chemicals Ltd., Chengdu, Sichuan, China) were used for the preparation of pure stock standards at a concentration of 1 g L^{-1} in methanol (MeOH, UHPLC- Supergradient, Panreac, Barcelona, Spain). Working standard solutions for HPLC consisted of a mixture of the 3 analytes at concentrations ranging from 20 to 0.01 mg L^{-1} in MeOH:water (1:1, v:v). Formic acid (>96%, Sig-ma-Aldrich), acetonitrile (ACN, UHPLC -Supergradient, Panreac, Barcelona, Spain) and water (Elix 3 Milli-Q purifi-cation system, Millipore, Bedford, MA) were used to prepare the mobile phase.

2.2. Samples and Sample Treatment

21 turmeric and 9 curry samples were purchased from different retail stores (see Table **1**). Samples (ca. 5 mg) were subjected to ultrasound-assisted extraction (ultrasonic bath Branson 5510, Branson Ultrasonics, Danbury, Connecticut, USA) with 10 mL of MeOH for 30 min at room temperature. The resulting extracts were centrifuged for 10 min at 4000 r.p.m with a Rotanta centrifuge 460 RS (Hettich, Tuttlingen, Germany), filtered through a nylon membrane of 0.45 µm of pore size (Scharlab, Barcelona, Spain) and stored in a dark

Table 1. List of samples analyzed and their characteristics.

Sample Type	Brand	Code	Composition
Turmeric	Hacendado	*t_HacA*	*c. longa* (Erode)
		t_HacB	
		t_HacC	
		t_HacD	
		*t_HacE**	
	MG	*t_MG*	*c. longa* (Allepey)
	Burriac	*t_Bur*	*c. longa*
	Carmencita	*t_Car*	*c. longa* (Erode)
	Ducros	*t_Duc*	*c. longa* (Erode)
	Artemis Bio	*t_Art*	*c. longa*
	Natco	*t_Nat*	*c. longa*
	Pelotari	*t_Pel*	Unknown
	Dani	*t_DanA*	*c. zedoaria*
		t_DanB	
	Natural diet herb	*t_Ndh*	*c. longa* (Madras)
	Onena	*t_One*	*c. longa* (Madras)
	Tata Sampann	*t_Tat*	Unknown
	Herbalist	*t_Her*	*c. longa* (Madras)
	Street market	*t_Mrk*	*c. longa* (Allepey)
	Biospirit	*t_Bsp*	*c. longa*
	NAA1	*t_Naa*	Unknown
Curry	Hacendado	*c_HacA*	white pepper, coriander, ginger, cardamom, clove, cinnamon, anise, mustard
		c_HacB	
	Carrefour	*c_Car*	pepper, coriander, ginger, cumin, fenugreek, laurel, fennel, mustard
	Species Kania	*c_Kan*	pepper, coriander, cumin, fenugreek, parsley, chili, garlic, fennel
	Condis	*c_Con*	pepper, coriander, fennel, cumin, cayenne, garlic, anise
	Burriac	*c_Bur*	white pepper, coriander, ginger, cardamom, clove, cinnamon, anise, mace
	Eroski	*c_Ers*	coriander, cardamom, ginger, fenugreek, anise, garlic, clove, mustard
	Ducros	*c_Duc*	pepper, coriander, cumin, ginger, laurel, anise, garlic, clove, cinnamon, mace
	Street marked	*c_Mrk*	unknown

vial at 4°C until chromatographic analysis. Quality controls (QCs) were prepared by mixing equal volumes (100 µL) of all sample extracts.

2.3. Chromatographic Analysis

The chromatographic system consisted of an Agilent Series 1100 HPLC Chromatograph (Agilent Technologies, Palo Alto, California, USA) with a quaternary pump (G1311A), a degasser (G1322A), an automatic injection system (G1392A) and a diode array detector (G1315B). The Agilent ChemStation software was used for instrument control and data processing. The separation was carried out in a core- shell reversed phase column Kinetex (100 mm x 4.6 mm I.D. 2.6 µm particle size) equipped with a C18 guard column (4.0 mm x 6.0 mm internal diameter), all of them from Phenomenex (Torrance, California, USA). The components of the mobile phase were 0.1 % formic acid in water (v/v) (solvent A) and acetonitrile (solvent B). The elution gradient for the separation of curcuminoids was as follows: from 0 to 7 min, B(%) 40→54 lineal increase. For the analysis of turmeric and curry samples, the gradient was extended up to 20 min maintaining the gradient slope (*i.e*. 0 to 20 min, B(%) 40→90 lineal increase) to elute other compounds also pre-sent in the samples. Subsequently, the column was cleaned for 2 min at 90% B and conditioned for 3 min at 40% B. The injection volume was 10 µL and the flow rate was 1 mL min^{-1}. Chromatograms were acquired at 280, 310, 370 and 420 nm.

A complementary characterization was carried out by LC-MS to try to establish the composition of unknown curcuminoids that play an important role in sample description. An Accela UHPLC system (Thermo Fischer Scientific, Waltham, MA USA), equipped with a quaternary pump and an Accela autosampler, was coupled to a Q-Exactive q-Orbitrap mass spectrometer (Thermo Fischer Scientific) equipped with a heated-electrospray ionization (H- ESI) source. Chromatograms were acquired in the negative mode in the range 100 to 1000 m/z at a resolution of 70000 Full Width at Half Maximum (FWHM) for high resolution MS spectra. H -ESI conditions were as follows: electrospray voltage, 2.5 kV; vaporizer temperature, 350°C; sheath gas flow rate, 60 a.u; auxiliary gas flow rate, 10 a.u; capillary temperature, 320°C. The injection volume was 5 µL and the flow rate was 0.3 mL min^{-1}. The separation was performed in a C18 reversed-phase column Syncronis (100 x 2.1 mm, 1.7 µm particle size) under gradient elution with 0.1% formic acid aqueous solution and ACN (0 to 20 min, B(%) 5→90 lineal increase).

2.4. Data Analysis

MATLAB was used for calculations. Principal Component Analysis (PCA) and other chemometric methods were from the PLS-Toolbox [33] following a similar approach to that given elsewhere [34].

For exploratory studies by PCA, the data matrix consisted of concentration values of quantified curcuminoids and peak areas at 420 nm of unknown curcumin-related components in the different samples under study (see Table **1**). Additional information consisting of dmc/ccm, bdmc/ccm and bdmc/dmc concentration ratios was also included in order to enrich the data matrix to facilitate the discrimination among turmeric classes. In order to improve the quality of the model, a precision filter based on the reproducibility of QC data was applied on the variable domain from which variables with RSD values higher than 20 % were discarded. Data was autoscaled to provide similar weights to major and minor variables. The plot of scores, showing the distribution of the samples on the principal components (PCs), was used to reveal patterns of sample characteristics, such as variety or origin. The location of QCs in this graph was used to evaluate robustness of the model. The plot of loadings showed the distribution of variables and their correlations. It was also used to deduce their influence on the sample properties.

3. RESULTS AND DISCUSSION

3.1. Optimization of the Extraction

First studies were focused on the selection of the extraction solvent for a quantitative recovery of analytes from turmeric and curry samples. Various organic solvents and hydro-organic mixtures were assayed including CH_2Cl_2, ACN, MeOH, ethanol (EtOH), dimethyl sulfoxide (DMSO) MeOH/water (50% v/v) and DMSO/water (50% v/v). Other experimental conditions were as follows: extraction time, 24 h; extraction method, sample maceration with occasional shaking; solvent volume, 10 mL; sample amount, 5 mg. The resulting extracts were centrifuged for 10 min at 4000 r.p.m and supernatant solutions were injected into the HPLC system. As shown in Fig. (**1**), MeOH was the most efficient extracting agent of curcuminoids. Other polar organic solvents such as ethanol and dimethyl sulfoxide, and hydro-organic mixtures provided lower yields (30 to 40 % lower) than those obtained with MeOH. The most apolar solvents, especially CH_2Cl_2, were even less suitable.

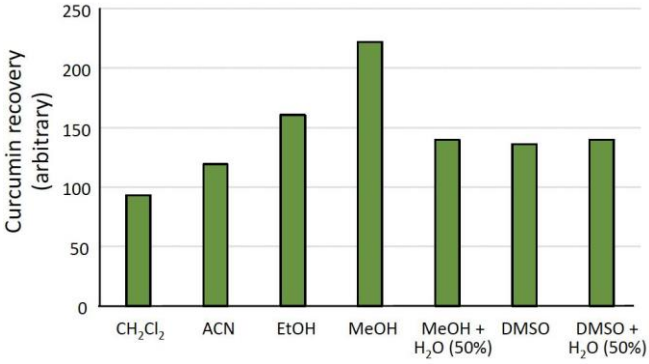

Fig. (1). Influence of the extraction solvent on the recovery of curcuminoids. ACN, acetonitrile; EtOH, ethanol; MeOH, methanol; DMSO, dimethyl sulfoxide.

The influence of the extraction method on the analyte recovery was evaluated under various approaches, including (i) sample maceration (leaving the sample and solvent in con-tact, with only occasional shaking), (ii) rotary -assisted ex-traction (SBS rotary system, Barcelona, Spain operating at 60 rpm) and (iii) sonication. In the experiments, 5 mg of sample were treated at two solvent levels (5 or 10 mL of MeOH) using various extraction times depending on the pro-cedure. Sample volumes of 250 µ L were withdrawn at prese-lected times throughout the assay to follow the process kinet-ics. As the optimization criterion, the extraction performance relied on achieving a good compromise among simplicity, processing time and yield. Results indicated that maceration

was less efficient in comparison with rotary or ultrasonic approaches as longer processing times were required (see Fig. **2**) . The ultrasound-assisted extraction using 10 mL MeOH was the best procedure which allowed a high recovery of curcuminoids in 30 min.

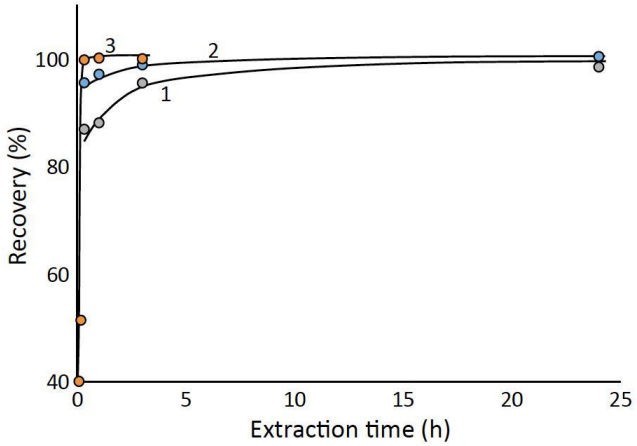

Fig. (2). The influence of the extraction method on the recovery curcuminoids. 1 = sample maceration; 2 = rotary-assisted extraction; 3 = sonication.

3.2. Optimization of the Separation

The chromatographic method was optimized by experi-mental design to achieve satisfactory separation perfor-mance. In particular, resolution of close peaks (*e.g.*, bdmc and dmc $Rs_{bdmc/dmc}$, and dmc and ccm $Rs_{bmc/ccm}$) and retention

R_{ccm}

sidered as the objectives to express mathematically the overall suitability criterion. The overall objective function *D* was defined as follows: $D = (d_{Rsbdmc/dmc} \times d_{Rsdmc/ccm} \times d_{tRccm})^{1/3}$, being $d_{Rsbdmc/dmc}$, $d_{Rsdmc/ccm}$, d_{tRccm} the desirabilities of $Rs_{bdmc/dmc}$, $Rs_{bmc/ccm}$and t_{Rccm}, respectively. Experimental results were transformed into desirability in the following way: for resolution, when Rs > 1.5 (*i.e.*, excellent separation) *d* was 1; when Rs < 0.9 (*i.e.*, unacceptable separation) *d* was 0; when 0.9 < Rs < 1.5, *d* was between 0 and 1. In a similar way, for retention time, limits of optimal (fast) and unacceptable (too time-consuming) were set to 5 and 20 min, respectively.

ACN percentage (ACN%) in the mobile phase and gradient time were chosen as the experimental factors under study, which were evaluated at 4 levels (*i.e.*, 16 runs to complete the design). ACN% were 20, 30, 40 and 50%, and gra-

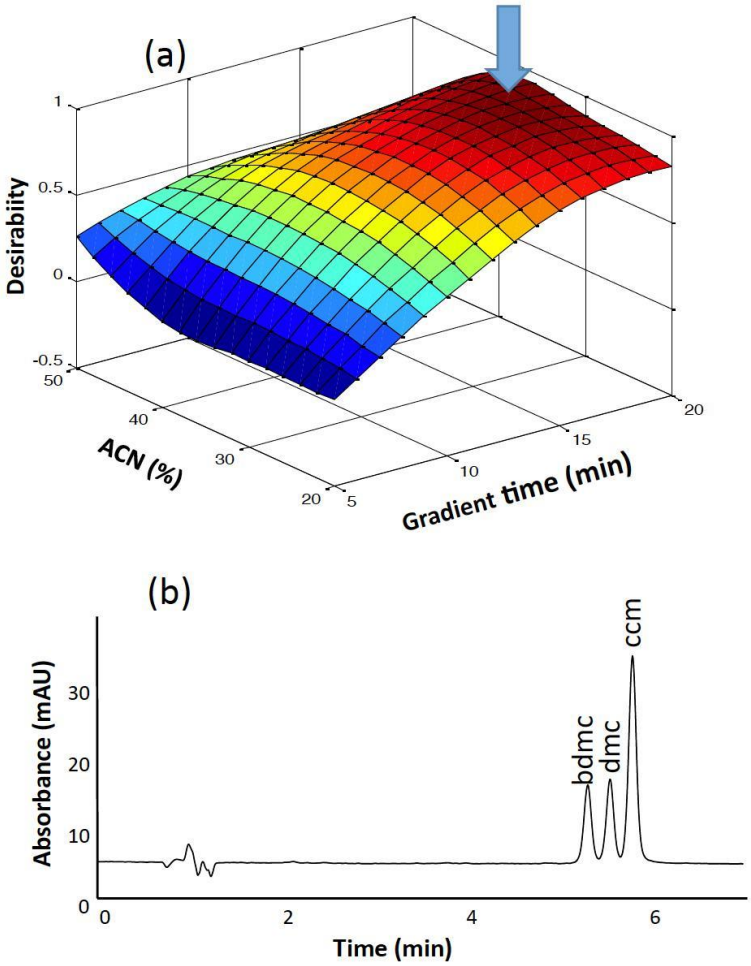

Fig. (3). Optimization of the HPLC separation as a function of ACN% in the mobile phase and analysis time. (**a**) Overall objective function D based on the best resolution of bdmc-dmc dmc and dmc-ccm peaks and minimum analysis time. (**b**) Chromatogram of a standard solution of 10 mg L^{-1} bisdemethoxycurcumin, 10 mg L^{-1} desmethoxycurcumin and 20 mg L^{-1} curcumin recorded at 420 nm under optimal conditions. Peak assignment: bdmc= bisdemethoxycurcumin, dmc = desmethoxycurcumin, ccm= curcumin.

Table 2. Figures of merit of the proposed method.

Compound	Retention Time (min)	Peak Area Repeatability RSD%	Linear Range (mg L^{-1})	Sensitivity (A.U. min L mg^{-1})	r^2	LOD (mg L^{-1})
bdmc	5.27±0.01	0.26	0.02 - 50	74.43	0.9998	0.003
dmc	5.51±0.01	0.21	0.02 - 50	67.64	0.9998	0.002
cur	5.75±0.01	0.36	0.02 - 50	61.90	0.9998	0.005

dient time ranges were 5, 10, 15 and 20 min. In all the cases, the final ACN% was 90%. The best separation was obtained using 40% ACN as the initial mobile phase and 20 min as the gradient time (see 3D plot of Fig. **3a**). The chromatogram of a standard mixture of bdmc, dmc and ccm under optimal conditions is shown as an example in Fig. (**3b**).

3.3. Figures of Merit

Figures of merit were assessed at 420 nm using mixture standards of ccm, dmc and bdmc prepared in MeOH/water (1/1, v/v). Retention times were 5.27 ± 0.01 for bdmc, 5.51 ± 0.01 for dmc and 5.75 ± 0.01 min for ccm, and peak resolutions $Rs_{bdmc/dmc}$ = 1.32 and $Rs_{dmc/ccm}$ = 1.31. The linearity of the calibration was evaluated in the working range 0.01 to 50 mg L^{-1}. Results given in Table **2** indicated that the model was linear within the range 0.02 to 50 mg L^{-1} (maximum assayed), with regression coefficients better than 0.9998. The sensitivities were 74.4, 67.6 and 61.9 AU min L mg^{-1} for bdmc, dmc and ccm, respectively. Repeatability values of both retention time and peak area were assessed at 2 mg L^{-1} from 10 assays (n = 10). For instance, intra-day precisions of ccm expressed as RDS% were 0.12 and 0.36% in terms of retention time and peak areas, respectively. For the other components, precision data were similar (see Table **2**) . Detection limits estimated at a signal-to-noise ratio of 3 were better than 0.006 mg L^{-1}.

The precision of the extraction step was evaluated from 6 independent assays using a commercial turmeric product, using ca. 5 g of sample, 10 mL MeOH and sonication for 30 min (see additional details in experimental section) . RSD values of peak areas were 3.54, 3.98 and 2.48 % for bdmc, dmc and ccm, respectively. These results were highly satisfactory and suggested that this step resulted in the main source of variability of the method.

Matrix effects were assessed from the comparison of the slopes of calibration curves of synthetic MeOH/water (1/1, v/v) and matrix -based standards within the working range 1 to 20 µg mL^{-1}. The sensitivities in the two matrices were similar, with standard/matrix slope ratios (in percentage) of 98.5, 101.7 and 98.3 for bdmc, dmc and ccm, respectively. It was thus concluded that matrix effects were negligible, so that calibration with pure standards prepared in MeOH/water (1/1, v/v) was appropriate for this application.

The accuracy of the determination was assessed by a spiking/recovery approach from 6 independent turmeric replicates spiked at 2 mg L^{-1} bdmc, dmc and ccm. Quantitative recoveries, expressed as the percentages of added/calculated concentrations, were 100±2, 98±2 and 99±1 for bdmc, dmc and ccm, respectively. Results suggested that calibration models build with MeOH/water standards provided excellent quantifications of target curcuminoids.

3.4. Determination of Curcurmin in Food Samples

The proposed method was applied to determine ccm and related curcuminoids in turmeric and curry samples. Commercial samples were analyzed in triplicate from independent extractions of ca. 5 mg sample with 10 mL of MeOH, as specified in the experimental section. Fig. (**4**) shows several representative chromatograms of a curry extract (*c_Hac*), a *c. longa* (Madras var., *t_Hrb*) extract, a *c. longa* (Allepey var., *t_Mrk*) extract, a *c. longa* (Erode var., *t_HacA*) extract. As it can be seen, curry extracts contain additional colored compounds (marked with *) coming from the other ingredients and curcuminoid concentrations are notably lower. Chromatograms of turmeric samples are more similar on components although they differ in their proportions, especially in the cases of unknown 3 and 4.

Various illustrative cases of quantification results are depicted in Fig. (**5**). In general, turmeric samples contained between 2 to 2.5% ccm and 3 to 4% curcuminoids (including dmc and bdmc), with the exception of *t _Pel* and *t_Mrk* samples with significantly lower amounts. Besides, the ratio of components was similar with relative percentages of ca. 15, 22, 63% for bdmc, dmc and ccm, respectively.

Regarding curry samples, turmeric is one of the multiple ingredients (see Table **1**), which is added in different proportions depending on the manufacturer. Other common species of curry are coriander, cumin, fenugreek and pepper. It was found that the overall contents of curcuminoids in curry were, in general, below 1%, in the range 0.4 to 0.6% for most of the samples. The relative percentages of curcuminoids were similar to those found in turmeric.

3.5. Sample Characterization by PCA

Sample extracts were analyzed randomly in order to minimize (if existed) the influence of any systematic drift. The QC was injected every 10 samples in order to evaluate the stability and repeatability of the results. As mentioned above, chromatograms recorded at 420 nm showed that, apart from bdmc, dmc and ccm, other components were also detected at this wavelength (Fig. **4**). Data from known and unknown absorbing compounds was here used for descriptive purposes as a source of analytical information. The data matrix to be treated by PCA consisted of peak areas of 99 samples (*i.e.*, 3 independent extraction replicates of 21 turmeric and 9 curry extracts, and 9 QCs) and 10 absorbing features namely: bdmc, dmc, ccm and 7 unknown. Additional dmc/ccm,

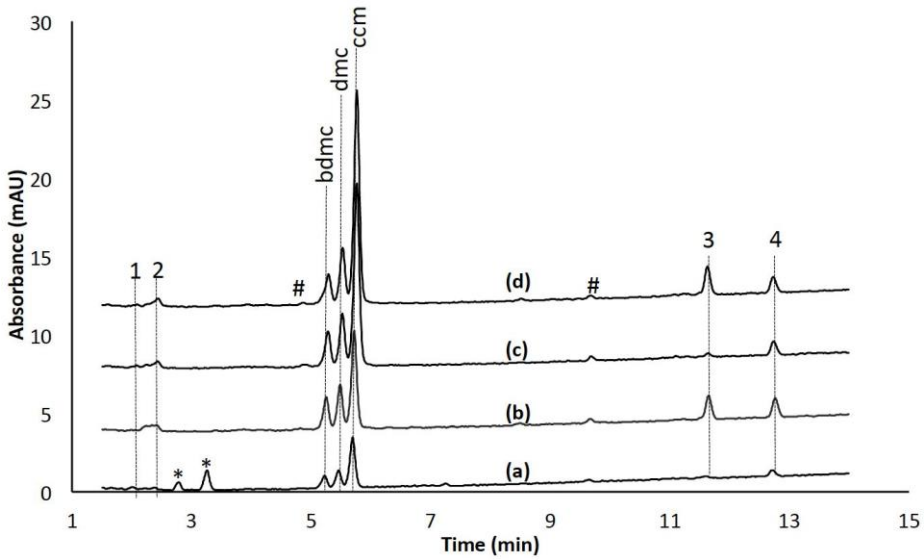

Fig. (4). Chromatogram of representative turmeric and curry samples recorded at 420 nm under optimal conditions. (a) *c_Hac*; (b) ***t_Hrb***; (c) ***t_Mrk***; (d) ***t_HacA***. Sample assignment: see Table **1**. Peak assignment: * = not from turmeric; 1 to 4 unknowns; # = minor curcuminoid components not considered for PCA.

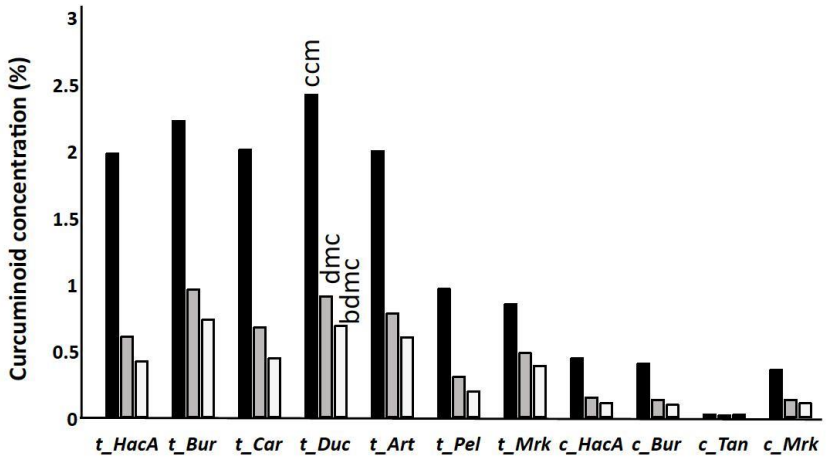

Fig. (5). Determination of curcuminoids in several turmeric and curry samples. Sample assignment: see Table **1**.

bdmc/ccm and bdmc/dmc concentration ratios were included in the data set to enrich the descriptive model. In order to remove noise contributions and improve the quality of the model, variables displaying RSD values higher than 20% in the QCs were discarded. As a result, ccm, dmc, bdmc, their ratios dmc/ccm, bdmc/ccm and bdmc/dmc, and 4 unknown components (see Fig. **4**) were finally retained for PCA.

A first PCA model was built working with mean values of replicates as a way to increase the reliability of the description. Data was autoscaled to equalize the influence of each variable in the model. The scatter plot of scores of PC1 *vs* PC2 depicted in Fig. (**6a**) showed that curry and turmeric samples were clearly segregated as a function of curcuminoid contents, with curries located on the bottom left quadrant and turmeric samples were spread throughout the other quadrants. QCs appeared in a compact group in the center of the model, thus indicating an excellent stability and reproducibility of results. PC1 mainly described quantitative patterns, with samples richer in curcuminoids to the right and those with lower contents to the left. Turmeric samples were distributed according to plant varieties, with most of Erode in the top right corner, Allepey in the bottom right corner, and samples labeled as Madras in the top left corner.

The study of the loading plot showed that curcuminoids appeared in the bottom right corner, closely located which indicated that they were highly correlated. Unknown components 1 and 2 were also highly correlated with ccm, dmc and bdmc, being thus quite redundant form the point of view of descriptive features. Other relevant compounds (those with retention time 11.6 and 12.9 min, labeled as peaks 3 and 4, respectively) appeared on the top left corner, thus providing complementary information to curcuminoid levels. The simultaneous analysis of scores and loadings revealed that samples located on the top right quadrant, mainly associated with the Madras variety, were characterized by increased levels of components 3 and 4 and relatively low levels of ccm, dmc and bdmc. Regarding to concentration ratios, it was found that samples belonging to Erode class displayed low dmc/ccm, bdmc/ccm and bdmc/dmc ratios while in the Allepey type the proportion of dmc/ccm seemed to be higher.

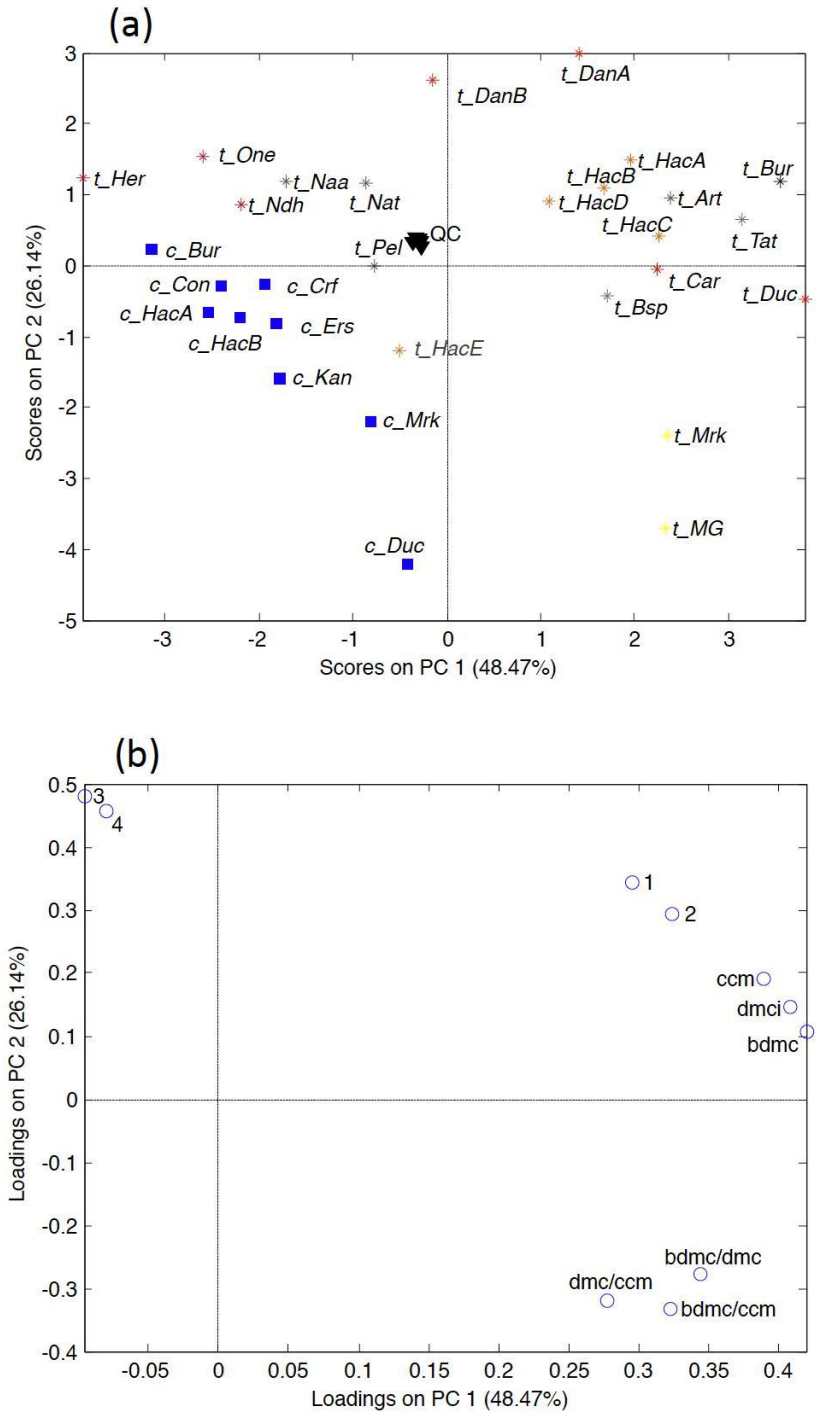

Fig. (6). Characterization of turmeric and curry samples PCA results. (**a**) Plot of scores; (**b**) Plot of loading. Sample assignment: see Table **1**.

A tentative elucidation of unknown curcuminoids was here attempted on the basis of previous results and LC- MS experiments. In a comprehensive characterization study using NMR, Li *et al.* identified 13 different curcuminoids (ccm, dmc, and bdmc plus 10 new ones) as natural components of turmeric [35]. Compounds were extracted from raw turmeric by overnight percolation with 95 % ethanol and were further purified by column chromatography on a XAD-4 resin. Fractions eluted from different ethanol percentages (from 40 to 95) were collected, purified by preparative HPLC, solvent-evaporated and the resulting solid residues were characterized by ^{1}H and ^{13}C NMR. Some compounds occurred in high amounts (*e.g.*, ccm and 1,5-dihydroxy-1,7-bis(4 -hydroxyphenyl)-4,6-heptadien-3-one), while others were less abundant. Structures proposed there were mainly based on the C6(phenyl)-C7(alquil)-C6(phenyl) skeleton of curcumin with some modifications regarding hydroxylation on C7 or phenyl carbons, reductive hydrogenation of a double bond in the C7 chain or loss of O in the ketone. Here, the exact *m/z* values of ccm, dmc and bdmc using negative ionization (see experimental section) were 367.1187, 337.1081 and 307.0976, respectively, which corresponded to deproto-

nated molecular ions $[M-H]^-$. The same ions were also de-tected at quite high intensity (c.a. 10% of ccm, dmc and bdmc) in peaks eluted at lower retention time (not elucidated yet) . For unknowns 1 and 2, the main peaks at *m/z* 353.1030 and 323.0925 suggested that they differed from ccm in the loss of a CH_2 (O-demethylation) and the loss of CH_2 + OCH_3 (from O-demethylation + demethoxylation of methoxy groups of curcumin), respectively, thus resulting in 1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)-1,6-heptadien-3,5-dione and 1-(4-hydroxyphenyl)-7-(3,4-dihydroxyphenyl)-1,6-heptadien- 3,5-dione. These transformations resulted in more polar compounds eluting before ccm. Compounds 3 and 4 were detected as quite intense peaks in most of the turmeric samples. Ions at *m/z* 291.1026 and 351.1238 suggested the loss of O (ketone) from bdmc and ccm leading to 1,7-bis-(4-hydroxyphenyl)-1,4,6-heptatrien-3-one and 1,7-bis-(4-hydroxy-3- methoxyphenyl)-1,4,6- heptatrien-3 -one, respectively. Their polarities were lower than that of ccm and dbmc, so they were more retained in the reversed phase column. Several additional ions tentatively assigned to curcuminoids were detected, possibly at concentrations 100- to 1000 -fold lower than those of ccm. Some of them matched with structures proposed by Li *et al*. [35] and others were in agreement with new isomers and derivatives of curcumin. LC-MS results are just preliminary and need to be complemented in further studies. Anyway, it is obvious that the complexity in curcuminoids is much higher than that given in previous studies and the role of minor curcuminoids as descriptors for classification and authentication issues should not be underestimated.

CONCLUSION

The reversed phase HPLC-DAD method developed here resulted in an excellent approach for a rapid, simple and feasible determination of curcumin and its derivatives in turmeric and curry samples. Experimental conditions affecting to both extraction and separation processes were optimized *via* experimental design to achieve an efficient quantification of curcuminoid components. Selected conditions for the extraction were focused on a fast and quantitative recovery of curcuminoids, while minimizing the occurrence of side components. The criterion for the HPLC optimization relied on reaching the full separation of curcuminoids in a minimum analysis time. For such a purpose, a multi-objective response was designed for achieving rapid analyses and high performance to deal with quality control and routine analysis of sample sets. Besides, the concept of multi-objective responses could easily adapted to new circumstances. Figures of merit in terms of repeatability, accuracy, detection limits, *etc*., were highly satisfactory and fully compatible with an accurate determination of analytes in the sample matrices under study. Apart from quantification issues, characterization of turmeric and curry samples was also addressed using peak areas of colored components (at 420 nm) and curcuminoid concentrations as the data. Apart from ccm, dmc and bdmc, additional data from unknown minor compounds and dmc/ccm, bdmc/ccm and bdmc/dmc, ratios were used to enrich the data set. Principal component analysis resulted in a good strategy to gain information on turmeric and curry classes as they were located in different areas of the scores plot. Besides, turmeric samples could be discriminated according to their varieties. LC-MS suggested a great complexity in curcuminoid composition, showing a variety of components to be elucidated. It was believed that these minor curcuminoids could be relevant descriptors for classification and authentication issues to be addressed *via* profiling or fingerprinting strategies using LC-DAD and/or LC/MS data.

LIST OF ABBREVIATIONS

ACN	=	Acetonitrile
bdmc	=	Bisdemethoxycurcumin
ccm	=	Curcumin
DAD	=	With diode array detection
dmc	=	Demethoxycurcumin
DMSO	=	Dimethyl sulfoxide
DOE	=	Design of experiments
FWHM	=	Full width at half maximum
GC	=	Gas chromatography
H-ESI	=	Heated-electrospray ionization
HPLC	=	Liquid chromatography
LC	=	Liquid chromatography
MeOH	=	Methanol
MS	=	Mass spectrometry
NMR	=	Nuclear magnetic resonance
PCA	=	Principal component analysis
PLS-DA	=	Partial least square-discriminant analysis
Q	=	Quadrupole
QC	=	Quality control
RSD	=	Relative standard deviation
TOF	=	Time of flight
UHPLC	=	Ultrahigh performance liquid chromatography

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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