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Analytical Methods

Flow injection analysis of total curcuminoids in turmeric and total antioxidant capacity using 2,2′-diphenyl-1-picrylhydrazyl assay

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ABSTRACT

A simple flow injection analysis procedure is proposed for the determination of curcuminoids content in turmeric extracts. The method is based on the formation of a coloured complex between 4-aminoantipyrine and curcuminoids, in the presence of an oxidising reagent such as potassium hexacyanoferrate (III) in alkaline media. Conditions selected as a result of these trials were implemented in a flow injection analytical system in which the influence of injection volume, flow rate, reagent concentration and mixing coil length, was evaluated. Under the optimum conditions the total amount of curcuminoids could be determined within a concentration range of 5–50 μg mL^{-1} which can be expressed by the regression equation $y = 0.003x - 0.0053$ ($r^2 = 0.9997$). The limits of detection and quantitation were found to be 0.6 μg mL^{-1} and 1.8 μg mL^{-1}, respectively. The reproducibility of analytical readings was indicative of standard deviations <2%. The sample was extracted and analysed by using the proposed method. The percentage recoveries were found to be 94.3–108.0. The proposed system was applied to the determination of curcuminoids content in turmeric. The total curcuminoid contents in turmeric extract were found to be 0.9–4.3% (w/w). The development method is simple, economic, rapid and especially suitable for quality control in pharmaceutical plants.

1. Introduction

Turmeric (*Curcuma longa* L.) has been used as an orange–yellow colouring agent, and has been found to be a rich source of phenolic compounds, namely, curcuminoids (Govindarajan, 1980). Turmeric extracts contain three different diarylheptanoids, curcumin, demethoxycurcumin and bis-demethoxycurcumin. Commercially available curcumin consists of a mixture of three naturally occurring curcuminoids with curcumin as the main constituent (~77%) (Ahsan, Parveen, Khan, & Hadi, 1999). Curcuminoids are recognised for their broad spectrum of biological activities and safety in foods or pharmaceuticals (Shankaracharaya & Natarajan, 1974). Curcumin, the principal natural yellow pigment, is widely used for foods and dye which shows many biological activities, for examples: antioxidant (Reddy & Lokesh, 1992), anti-inflammatory, antimicrobial, antiparasitic, antimutagenic, anticancer and antivirus properties (Srimal, 1997). Recently, the effect of curcuminoids has been examined on the proliferation of MCF-7 human breast tumor cells. It was reported that demethoxycurcumin was the best inhibition of MCF-7 cells (Simon et al., 1998). Kim reported the strong antioxidant activity of demethoxycurcumin and bis-demethoxycurcumin (Kim, Park, & Kim, 2001) and Ahsan reported the antioxidant and pro-oxidant activities of curcumin and the structure relationship between curcumin, demethoxycurcumin, and bis-demethoxycurcumin (Ahsan et al., 1999).

A variety of methods for the quantification of the curcuminoids have been reported. Most of these are spectrophotometric methods, expressing the total colour content of the sample (ASTA Method, 1985). Commercial turmeric products contain mixtures of curcumin, demethoxycurcumin and bis-demethoxycurcumin (Ahsan et al., 1999). Gupta described a simultaneous determination of curcuminoids in *curcuma* samples using high performance thin layer chromatography. Samples and standard were applied on a silica gel $60F_{254}$ plate and the separation was performed using chloroform–methanol (95:5 v/v) follow by scanning of the spots at 366 nm using a UV detection mode (Gupta, Gupta, & Sushil, 1999). Ramussen developed a simple and efficient column chromatographic method for the separation of three phenolic diketones: curcumin, demethoxycurcumin and bis-demethoxycurcumin from the rhizomes of *Curcumar longa* (Ramussen, Christensen, Kvist, & Karazmi, 2000). A direct fluorimetric method for the analysis of curcumin and its structural isomers in food materials was proposed by Karasz and Tounesen based on high performance liquid chromatography (HPLC) with UV or visible or fluorescence detection (Karasz, DeCocca, & Bokus, 1973; Tonnesen & Karlsen, 1986). HPLC was carried out on a Nucleosil NH_2 column using ethanol as mobile phase (Tonnesen & Karlsen, 1983). Smith

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compared the use of UV spectrometric and electrochemical detectors for the HPLC determination of curcumin in turmeric powder using ODS-Hypersil column (Smith & Witowska, 1984). Rouseff developed an isocratic HPLC system and a gradient water/THF HPLC system for quantitative analysis of the pigments in food colorants, annatto and turmeric with visible spectrometric and fluorescence detections (Rouseff, 1988). Khurana determined curcuminoids and their photo-oxidative decomposition compounds such as curcumin, bis-demethoxycurcumin, demethoxycurcumin in methanolic and ethanolic extracts of dry powder of *Curcuma linga* L. root by HPLC (Khurana & Ho, 1988). He described an on-line high performance liquid chromatography for simultaneous analysis of curcuminoids and sesquiterpenoids in fresh turmeric (He, Lin, Lian, & Lindernmaier, 1998). Taylor developed a rapid, simple and reproducible reversed-phase high performance liquid chromatographic method for determination of curcumin, demethoxycurcumin and bis-demethoxycurcumin in ethanolic extract of turmeric, using an acetonitrile-water (55:45 v/v) as mobile phase with diode array detection at 425 nm (Taylor & McDowell, 1992). Hiserodt characterised turmeric powder using liquid chromatography-mass spectrometry. These involve an octadecyl stationary phase using a mobile phase consisting of ammonium acetate with 5% AcOH and acetonitrile. The presence of inorganic salt may interfere with the mass spectrometer ion source (Hiserodt, Hartman, Ho, & Rosen, 1996).

In the present work, an FIA spectrophotometric procedure was developed for curcuminoids determination based on the reaction between curcuminoids and 4-AP in an alkaline potassium hexacyanoferrate (III) solution. The method is based on the condensation reaction of 4-AP with phenolic moieties in the presence of an alkaline oxidising agent yielding a red coloured product. According to their report the probable mechanism is oxidation of 4-AP with potassium hexacyanoferrate (III) in alkaline medium causing lose of two protons from the former leading to the formation of a nucleophilic intermediate that further undergoes nucleophilic substitution with the phenolic moieties of compounds that in turn results in the coloured product (Mueller & Hackenberg, 1959; Barsoom, Abdelsamad, & Adib, 2006). 4-AP does react with phenol, *ortho*- and *meta*- substituted phenols. Furthermore, phenols in which a carboxyl, halogen, methoxyl or sulphonic acid group is located in the para- position will react under appropriate pH conditions. Because the relative amounts of various phenolic compounds in a given sample are unpredictable, it is not possible to provide a universal standard containing a mixture of phenols. Hence, phenol (C_6H_5OH) has been selected as a standard for FIA spectrophotometric procedures and all color produced by the reaction of other phenolic compounds is reported as phenol (Van Staden & Britz, 1997). In general, functional groups on the aromatic ring reduce the response thus; this value represents the minimum concentration of phenolic compounds. Phenolic compounds react with buffered alkaline potassium hexacyanoferrate (III) and 4-AP to form a red coloured complex. Selectivity may be increased by means of a chemical reaction producing a chromophore. As stated before, the curcuminoids molecule has a phenolic group that can be used to generate a chromophore, thus enabling a quantitative method to be developed. The reaction with 4-AP in the presence of an oxidant, such as potassium hexacyanoferrate (III) in alkaline solution, is one of the most widely used reactions for the FIA determination of phenols. No articles on flow injection spectrophotometric analysis of curcuminoids were reported in the literature. In general, flow analysis is known to be fast, precise, inexpensive (due to small sample and reagents volume needed), to enhance selectivity and sensitivity, to allow multiple analysis and easy to automate as compared to manual or batch methods. The use of FIA should overcome the need for such separation by virtue of the inherently accurate timing. Besides, the use of a transient signal measured from a baseline, characteristic of FIA may contribute to minimise the influence of a coloured reagent such as 4-AP in the presence of an oxidant such as potassium hexacyanoferrate (III) in alkaline solution.

The present paper describes a simple, rapid, inexpensive and reliable FIA method with spectrophotometric detection for the quantitation of total curcuminoids using 4-AP in the presence of potassium hexacyanoferrate (III) as chromogenic reagent. The method has been successfully applied to determination of total curcuminoids in the powdered turmeric (*Curcuma longa* L. and *Curcuma zedoaria* (Berg) Roscoe) collected from Chiang Mai Province and Phitsanulok Province. This method is considered to be greener analytical method due to its low sample and reagent consumption with minimum waste released.

2. Experimental

2.1. Apparatus and instruments

A schematic diagram of the flow system used for the determination of total curcuminoids content is shown in Fig. 1. It is a three channel flow injection manifold. A peristaltic pump with three channels and variable speed (Pharmacia, Sweden) was used to deliver both the carrier (borate buffer solution) and the reagent streams (4-AP solution and potassium hexacyanoferrate (III) solution) through the flow system. Each stream was pumped at a constant flow rate (0.8 mL min^{-1}) using tygon tubing with 0.80 mm i.d., and PTFE tubing with 0.635 mm i.d. (Cole-Parmer Instrument Company, IL) as flow lines. The standard and/or sample solution containing curcuminoids was injected by using a 20 μL disposable micro syringe (SGE, Australia) into the borate buffer solution stream via a laboratory-made, low-cost injection valve which was then merged with the reagent (4-AP solution) stream at the Y-shaped connector and subsequently merged with an oxidant stream of potassium hexacyanoferrate (III) solution. The merged stream was passed through a mixing coiled reactor. The coloured complex formed was passed through a 10 mm path length flow-through cell (cell volume 100 μL, Perkins Elmer, USA) in a spectrophotometer model Speckol 1200 (Jena Analytic, Germany) connected to a software LDR computer-controlled by means of a homemade program written in Microsoft Visual Basic 6.0. An absorbance signal could be retrieved directly from a Speckol 1200 spectrophotometer via the RS-232 interface. The absorbance was measured at 456 nm and displayed as a peak as a function of time.

An UV–Visible spectrophotometer (model Speckol 1200, Jena Analytic, Germany) was used to scan the spectra of curcuminoids.

2.2. Materials

Fresh turmeric was purchased from commercial sources in Chiang Mai Province, Thailand (sample codes; T1–T2: purchased from Tonpayorm market and T3: purchased from Warorot market) and Phitsanulok Province, Thailand (sample codes; T4–T5: purchased from Watyai market and T6–T7: purchased from Kokma-

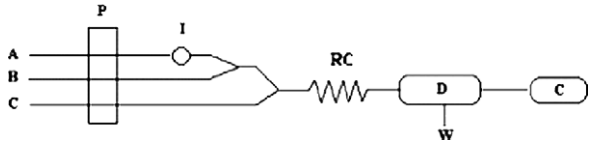


Fig. 1. Schematic configuration of the FIA system: A; carrier stream of borate buffer solution, B; 1.0×10^{-1} mol L^{-1} 4-AP solution, C; 1.0×10^{-3} mol L^{-1} potassium hexacyanoferrate (III) solution, P; peristaltic pump, I; injection valve, RC; reaction coil, D; detector, C; computer, W; waste.

toom market). A voucher specimen of the plant is stored in the herbarium of faculty of Pharmacy for reference. The samples were washed and dried in a hot air oven at 50 °C for 36 h. The dried material was ground to a fine powder, passed through a 60-mesh sieve and kept in an air-tight container at 4 °C until further use.

2.3. Chemicals and solutions

All chemicals used were of analytical reagent grade. Deionised distilled water was used throughout the experiment (Milli-Q water purification system, Millipour Co., USA). Standard curcuminoids was purchased from Wako Pure Chemical (Osaka, Japan, 99%). 4-aminoantipyrine (CH_3–$C_9H_7N_3O$–CH_3) was purchased from ACROS Organic (New Jersey, USA, 98%). Potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$) was purchased from May & Baker Ltd., (Dagenham, England, 99%). Hydrochloric acid, acetic acid and sodium borate ($Na_2B_4O_7$. $10H_2O$) were purchased from Aldrich (Poole, Dorset, UK). 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid were purchased from Sigma (St. Louis, USA). Quercetin was obtained from Aldrich (Milwaukee, USA). Methanol and acetonitrile were HPLC grade (E. Merck, Germany).

A stock solution (1000 μg mL^{-1}) of standard curcuminoids was prepared daily by dissolving 25 mg of the curcuminoids (accurately weighed) in ethanol and completing the volume up to 25 mL with ethanol. Working standard solutions of curcuminoids (5, 10, 20, 30 and 50 μg mL^{-1} curcuminoids) were prepared by appropriate serial dilution of the curcuminoids stock solution in ethanol.

The 4-AP solution 1.0×10^{-1} mol L^{-1} was prepared by dissolving 10.1625 g of 4-AP in 500 mL water and prepared daily. The potassium hexacyanoferrate (III) solution 1.0×10^{-3} mol L^{-1} in alkaline media was prepared by dissolving 0.1646 g of potassium hexacyanoferrate (III) in 0.5% Na_2CO_3 in 500 mL water and prepared daily.

The borate buffer solution pH 9.5 was prepared by dissolving the calculated amount of 0.025 M of $Na_2B4O_7 \cdot 10H_2O$ in deionised water and 0.1 mol L^{-1} HCl solution is used to adjust the pH at 9.5.

A stock solution (5.0×10^{-4} mol L^{-1}) of DPPH was prepared by dissolving the appropriate amount in ethanol. This solution was kept at 4 °C and protected from light, and it was stable during a week. The DPPH working solution containing 1.0×10^{-4} mol L^{-1} was prepared by measuring 50 mL of the stock solution, the volume was made up to 200 mL with ethanol. This working solution was prepared daily and protected from light. Ascorbic acid and Quercetin stock solution were prepared by dissolving the appropriate amount of the respective solid in ethanol. Working standard solutions containing either ascorbic acid or Quercetin in the concentration range 0.5–5 μg mL^{-1} were prepared by dilution of the respective stock solution using ethanol.

2.4. Sample preparation

Fresh *Curcuma longa* L. was purchased from Chiang Mai and Phitsanulok Province, 1 kg of the finger turmeric was chopped into small pieces and dried in a hot air oven at 50 °C for 36 h. Then the dried turmeric was powdered, about 0.5 g was accurately weighed and extracted with 10 mL of ethanol and sonicated for 30 min followed by centrifugation for 15 min at 2000 rpm. The supernatant was evaporated to dryness at 60 °C by means of a rotary evaporator (Buchi, Switzerland). Then the residue was reconstituted in 1 mL of ethanol. This solution was used for separation by preparative TLC. A Linomat IV sample applicator (Camag, Wilmington, NC) was used to apply 100 μL sample solution on a silica gel 60 GF TLC plate, 20 × 20 cm (Merck, Darmstadt, Germany). The plate was developed to a height 8 cm in the ascending direction in a chamber previously saturated with the dichloromethane–methanol (99:1 v/v) mobile phase. After separation, three fluorescence bands were observed under short wavelength UV (254 nm). They are curcumin, demethoxycurcumin and bis-demethoxycurcumin with the R_f values of 0.75, 0.63 and 0.55, respectively. Each band was scraped into a 25 mL conical flask. Then 10 mL of ethanol was added and sonicated for 10 min followed by centrifugation for 10 min at 16,000 rpm. The supernatant liquid was transferred into a 10 mL volumetric flask and made up to volume with ethanol. An aliquot of this solution was filtered through a 0.45 μm nylon membrane. Then 20 μL of this solution was injected into FIA system.

2.5. Procedure

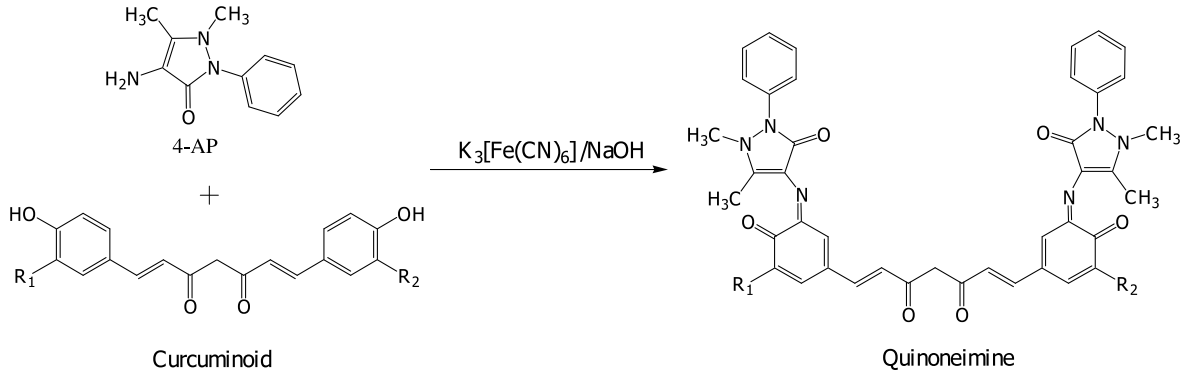
Using the fabricated FI manifold (Fig. 1) the experimental conditions for determining total curcuminoids were optimised by the univariate method. Under the optimum conditions the recommended FI procedure was applied. The standard and/or sample solution (20 μL) containing curcuminoids was injected into the carrier stream (borate buffer pH 9.5 solution) which was then merged with the reagent streams of 1.0×10^{-1} mol L^{-1} 4-AP solution. Subsequently, this merged stream was merged with the oxidant stream of 1.0×10^{-3} mol L^{-1} potassium hexacyanoferrate (III) solution with the same flow rate of 0.8 mL min^{-1}. Comparative experiment was also carried out using the expensive commercial flow-through cell. The solutions were mixed at a coiled reactor which was made from the PTFE tubing (i.d. 0.635 mm and 60 cm in length) forming the red color product that was then passed through the home-made and/or the commercial flow-through cell with the same path length (10 mm) and the same volume of 100 μL which was situated in the spectrophotometer where the absorbance was measured at 456 nm. The FI signals in a transient absorbance was displayed by a computer as a peak and the peak height corresponding to the maximal absorbance was proportional to the curcuminoids content in the sample.

2.6. Chemical reaction

It has been reported that phenols react with 4-AP in the presence of alkaline potassium hexacyanoferrate (III) as oxidising yielding *N*-substituted quinoneimine, which in turn is known to spontaneously react with phenolic compounds, leading to a red-coloured antipyrine dye. The phenolic hydroxyl group presence in curcuminoids renders it an extremely suitable substrate for the above coupling reaction. This method is based on the detection of the condensation reaction product of curcuminoids with 4-AP in the presence of potassium hexacyanoferrate (III) under alkaline medium. The possible reaction mechanism for the proposed FIA method is illustrated in Scheme 1.

2.7. Determination of DPPH radical scavenging activity

The stable free radical α, α′-diphenyl-β-picrylhydrazyl, which is generally available in laboratories in which electron spin resonance experiment are conducted, because of the paramagnetism conferred by its odd electron. It can be seen from its structure that while this compound can accept an electron of hydrogen radical to become a stable, diamagnetic molecule, it can be oxidised only with difficulty, and then, irreversibly. Because of its odd electron, 2, 2′-diphenyl-1-picrylhydrazyl shows a strong absorption band at 540 nm (in ethanol), its solutions appearing a deep violet color. As this electron becomes paired off, the absorption vanishes and the resulting decolorisation is stoichiometric with respect to the number of electron take up. The strong absorption is fortunate because the solubility of 2, 2′-diphenyl-1-picrylhydrazyl is not great, however, alcoholic solution covering concentration of approximately 5×10^{-4} mol L^{-1} are nevertheless densely coloured and



Scheme 1. Assumed scheme of reaction between curcuminoids and 4-AP, and formation of a highly coloured quinoneimine.

low concentration, the Lambert-Beer law is obeyed over the useful range of absorption. DPPH is a stable free radical compound and has been widely used to test the radical scavenging activity of various chemicals including the natural product.

In this study, the ethanolic extract of turmeric was evaluated for antioxidant activity *in vitro* using 2,2′-diphenyl-1-picylhydrazyl (DPPH) using quercetin and ascorbic acid as antioxidant reference standards (Suwipa et al., 2005).

2.8. Measurement of antioxidant activity

The test sample (20 μL) was added to 100 μL of 10 μM DPPH solution in a 96-well microtiter plate. The reaction mixture was incubated at 37 °C for 30 min, and then the absorbance of each well was measured at 540 nm. The DPPH solution was used as negative control. Quercetin and ascorbic acid were used as reference standards. For IC_{50} evaluation of turmeric extract, a graph showing concentration versus DPPH scavenging activity (%) reduction was plotted. The IC_{50} was then calculated from the calibration curve and activity was expressed as percentage DPPH scavenging relative to control using the following equation:

$$\text{DPPH scavenging activity } (\%) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100.$$

3. Results and discussion

3.1. Preliminary work

Initially, a three line FI manifold was used for testing the reactivity of curcuminoids and reagent solution. Standard solution of curcuminoids (5 μg mL^{-1}) was injected into a water carrier stream, whilst the reagent and oxidant streams were 4-AP (1.0×10^{-2} mol L^{-1}) and potassium hexacyanoferrate (III) (1.0×10^{-2} mol L^{-1}) in the alkaline media (0.5% Na_2CO_3) at constant flow rate 1.0 mL min^{-1} with equal flows in each channel. Curcuminoids reacted with 4-AP and potassium hexacyanoferrate (III) in the alkaline media (0.5% Na_2CO_3) to form a red coloured complex which led to the basic for the development of spectrophotometric determination of curcuminoids at 456 nm. Next, in FIA measurement that involves multiple reagents, it is necessary to optimise the FI manifold with suitable designs according to the reaction sequence in the batch-wise method. Therefore the order of introduction of sample carrier stream and reagent stream into the FI manifold is shown in Fig. 1. It is preferable to inject the sample into the buffer solution before merging with the reagent streams because it gave the better sensitivity.

The effect of alkaline media added to potassium hexacyanoferrate (III) solution was initially examined. Various alkaline media such as Na_2CO_3, NaOH and KOH solutions at the same concentration and various concentrations of all alkaline solutions (0.1–2.0%) were tested as 4-AP (1.0×10^{-2} mol L^{-1}) and potassium hexacyanoferrate (III) (1.0×10^{-2} mol L^{-1}) solution. The constant flow rate of three channel streams was 1.0 mL min^{-1}. It was found that 0.5% Na_2CO_3 gave the greatest sensitivity (slope of the calibration graph).

3.2. Effect of variables

The following variables were optimised in order to obtain greatest sensitivity. The conditions for the determination of curcuminoids were optimised by studying the influences of various parameters, such as injection volumes, reagent/carrier flow rates, and reagent/carrier concentrations of the respective measurements. The optimum conditions obtained by means of the univariate optimisation procedure (changing one variable in turn and keeping the others at their optimum values). The optimal value for each parameter was judging from maximum sensitivity of the detector response. The range of variables studied and the optimal values chosen are given in Table 1.

3.3. Optimisation of reagent concentrations

The effect of various 4-AP concentration was examined by measuring the absorbance at 456 nm for solutions containing a fixed concentration of potassium hexacyanoferrate (III) (1.0×10^{-2} mol L^{-1}) and varying the concentrations of 4-AP over the range 1.0×10^{-4}–1.0 mol L^{-1}. Curcuminoids standard solutions (1–15 μg mL^{-1}) were injected into a water carrier stream, whilst the oxidant streams were various concentration of 4-AP at constant flow rate 1.0 mL min^{-1} with equal flows in each channel. The optimum 4-AP concentration, leading to the maximum sensitivity, was 1.0×10^{-1} mol L^{-1}. Therefore, the 1.0×10^{-1} mol L^{-1} was chosen as optimum concentration and was used throughout.

The effect of various potassium hexacyanoferrate (III) concentrations was investigated. The concentration was studied over the range 1.0×10^{-4}–1.0 mol L^{-1}. Then, Curcuminoids standard solutions (1–15 μg mL^{-1}) were injected into a water carrier stream, whilst the oxidant streams were various concentrations of potassium hexacyanoferrate (III) and fixed concentration of 4-AP (1.0×10^{-1} mol L^{-1}) at constant flow rate 1.0 mL min^{-1}. The absorbance increases with increasing the oxidant concentration up to 1.0×10^{-3} mol L^{-1}. Thus, a concentration of 1.0×10^{-3} mol L^{-1} was selected for the next experiments to obtain the greatest sensitivity.

The effect of the pH of the carrier stream such as distillation water and borate buffer solution was investigated. The pH of carrier stream was studied over the range 8–10. Curcuminoids standard solutions (1–15 μg mL^{-1}) were injected into a water

Table 1
The optimum condition for determination of curcuminoids content

Parameters studied	Range studied	Optimum
Wavelength (nm)	200–700	456
4-AP concentration (mol L^{-1})	1.0×10^{-4}–1.0	1.0×10^{-1}
Potassium hexacyanoferrate (III) (mol L^{-1})	1.0×10^{-4}–1.0	1.0×10^{-3}
Type of alkaline media	Na_2CO_3, NaOH, KOH	Na_2CO_3
Sodium carbonate (%, w/v)	0.1–1.5	0.5
pH of Borate buffer solution	8.0–10.0	9.5
Mixing coil length (cm)	10–90	60
Mixing coil I.D (mm)	0.508–1.521	0.635
Injection volume (μL)	10–100	20
Flow rate of 4-AP (mL min^{-1})	0.5–2.5	0.8
Flow rate of potassium hexacyanoferrate (III) (mL min^{-1})	0.5–2.5	0.8
Flow rate of borate buffer solution (mL min^{-1})	0.5–2.5	0.8

carrier stream, whilst the oxidant streams were 1.0×10^{-1} mol L^{-1} 4-AP and 1.0×10^{-3} mol L^{-1} potassium hexacyanoferrate (III) at constant flow rate 1.0 mL min^{-1}. It was found that the borate buffer solution gave greatest sensitivity than the distillation water and when increasing pH values up to 9.5. For further increments of the pH, the absorbance increases very slightly and remains almost constant. Thus, a pH of 9.5 was chosen as appropriate pH for further investigations, due to reproducibility and sensitivity.

3.4. Optimisation of manifold parameters

The same procedure under the appropriate chemical concentrations was repeated to optimize the FI manifold parameters namely mixing coil (diameter and length), flow rate and injection volume. The effect of mixing coil diameter and length were investigated. The mixing coil is made from PTFE tubing wound around a plastic tube. The main function of this tubing is as a mixing reservoir into which sample and/or reagent is sequentially aspirated. The mixing coil volume should be large enough to prevent the stack of zones from being forwarded from the mixing coil to the detector before the chemical reaction is taken place. It is essential to investigate the optimum tubing size and length to assess the best sensitivity and precision. The effects of various tubing inner diameters for making the mixing coil on the sensitivity and precision for curcuminoids were investigated over the range 0.508–1.521 mm. A slight depression of sensitivity with increasing tubing inner diameter is observed. The 0.635 mm i.d. tubing was selected, owing to its high sensitivity and precision. The influence of the tubing length for making the mixing coil on the sensitivity and precision is also examined over the range of 10–90 cm. Thus, a-60 cm tubing is considered to be suitable due to its good sensitivity and precision.

The flow rate is one of the most important parameters to be optimised because it regulates the amount of final product (color red product) formed and hence the sensitivity together with the sample throughput. The effect of flow rate for carrier stream, 1.0×10^{-1} mol L^{-1} 4-AP and 1.0×10^{-3} mol L^{-1} potassium hexacyanoferrate (III) in 0.5% sodium carbonate were investigated from 0.5–2.5 mL min^{-1} and the flow rate was varied over the range 0.5–2.5 mL min^{-1}. The maximum sensitivity was obtained at a flow rate of 0.8 mL min^{-1}. Therefore, a flow rate of 0.8 mL min^{-1} was chosen for further investigations.

The influence of injection volume on absorbance sensitivity was studied by changing the sample loop over the range 10–100 μL. It was showed that initially, the absorbance sensitivity increased very rapidly with the increased sample injection volume up to 20 μL, above which the absorbance sensitivity decreased from 50–100 μL in sensitivity are nearly constant. Therefore, the 20 μL was considered to be optimum sample injection volume in the proposed FI system.

3.5. Analytical characteristics for curcuminoids determination

Under the selected experimental conditions (Table 1) and using the FI system as illustrated in Fig. 1, the linear calibration range, the detection limit (LOD) and the quantitation limit (LOQ) for the determination of curcuminoids were investigated.

3.6. Linearity of calibration curve

The linearity of calibration curve was determined using the optimal experimental parameters in Table 1. Linear calibration curve of curcuminoids over the concentration range of 5–50 μg mL^{-1} was established. Over this concentration range, linear regression analysis of the curcuminoids peak height versus curcuminoids concentration (C) ($n = 5$) yielded the following equation: $y = 0.003 \times -0.0053$ ($r^2 = 0.9997$).

3.7. Sensitivity, detection limit, quantification limit

The sensitivity value of the proposed FIA method, defined as the slope of calibration curve, was found to be 0.003 μg mL^{-1}. Linear calibration curve was obtained over the concentration range of 5–50 μg mL^{-1} with the correlation coefficient of 0.9997. The detection limit (3σ) and the quantification limit (10σ) were found to be 0.6 μg mL^{-1} and 1.8 μg mL^{-1}, respectively.

3.8. Precision and accuracy

The relative standard deviation of the proposed method (peak height in AU) calculated from 10 replicate injections of 5, 10 and 15 μg mL^{-1} of curcuminoids were found to be 2.1%, 1.1% and 0.8%, respectively. The percentage recoveries were determined with the standard addition method in herb samples. Curcuminoids (5, 10 and 15 μg mL^{-1}) were added and mixed with 0.5000 g of a fine powder of Turmeric, the samples was extracted and analysed using the proposed method. The mean percentage recovery of 5, 10 and 15 μg mL^{-1} ($n = 5$) of curcuminoids were found to be 96.6–108.0, 94.3–99.6 and 96.0–101.8, respectively, indicating that the proposed method could provide acceptable extraction efficiency and recovery of this method was good.

3.9. Analytical applications

The proposed FI method was successfully applied to the determination of curcuminoids in turmeric extracts. The extracts of *Curcuma longa* L. and *Curcuma zedoaria* (Berg) Roscoe were determined under the optimum conditions mentioned above, and the calculated contents of curcuminoids were shown in Table 2. The mean contents of curcuminoids from turmeric purchased from commercial sources in Chiang Mai province (three samples) were found to be 3.3, 4.1 and 2.7%, respectively, and turmeric purchased from Phitsanulok province (four samples) were found to be 0.9, 4.3, 3.8 and 1.1%, respectively, by using the proposed FIA method. Results were compared flavorable with the spectrophotometric method (ISO 5566, 1982). The assay results for total curcuminoids content in turmeric samples (Table 2) obtained using the proposed FIA method and the spectrophotometric method were compared by applying the paired *t*-test (Miller & Miller, 1993). The calculated *t*-value of 0.14 for total curcuminoids content is less than tabulated *t*-value 1.85 at the 95% confidence level. Therefore there is no significant difference in a determined content of curcuminoids by both methods.

Table 2
Comparative determination of total curcuminoids using the proposed FIA method and spectrophotometric method and antioxidant activity

Code	Turmeric samples	Total curcuminoids content (%,w/w), (n = 3)		Antioxidant activity (DPPH assay)[a], (n = 3)
		The proposed FIA method	Spectrophotometric method (ISO 5566)	IC_{50} (μg mL^{-1})
T1	Turmeric (*Curcuma longa* L.); Tonpayorm market, Chiang Mai	3.3	3.5	10.8
T2	Turmeric (*Curcuma longa* L.); Tonpayorm market, Chiang Mai	4.1	4.4	10.6
T3	Turmeric (*Curcuma longa* L.); Warorot market, Chiang Mai	2.7	2.7	26.3
T4	Turmeric (*Curcuma longa* L.); Watyai market, Phitsanulok	0.9	0.9	66.8
T5	Turmeric (*Curcuma longa* L.); Watyai market, Phitsanulok	4.3	4.4	10.0
T6	Turmeric (*Curcuma longa* L.); Kokmatoom market, Phitsanulok	3.8	3.8	10.7
T7	Turmeric (*Curcuma zedoaria* (Berg) Roscoe); Kokmatoom market, Phitsanulok	1.1	1.1	66.1

[a] Quercetin (IC_{50} = 1.7) and ascorbic acid (IC_{50} = 3.7) were used as reference standard in DPPH assay.

3.10. Determination of DPPH radical scavenging activity of turmeric extract

The DPPH assay was used to measure the free radical scavenging capacity of turmeric extracts. The IC_{50} of the turmeric extract samples are determined from the calibration curve. Results are shown in Table 2.

4. Conclusion

In this work, the proposed FI spectrophotometric method has presented to be a simple and uses inexpensive instrumentation for determination of curcuminoids contents. The curcuminoids is forming complex with 4-AP and potassium hexacyanoferrate (III) in alkaline medium as evidenced by the spectrophotometric system. The linearity of the calibration curve is in the useful concentration range for the curcuminoids quantitation in *Curcuma longa* L. and *Curcuma zedoaria* (Berg) Roscoe. The limits of detection and quantitation of this method were reasonable accepted. The development method is simple, economic, rapid and especially suitable for quality control in pharmaceutical plants.

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