PART I

DEVELOPMENT OF FLOW INJECTION ANALYSIS FOR THE DETERMINATION OF BIOACTIVE COMPOUNDS FROM MEDICINAL PLANTS AND PHARMACEUTICAL FORMULATIONS

CHAPTER I

INTRODUCTION

Flow injection is analytical techniques based on microfluidic manipulation of samples and reagents. Samples are injected into a carrier/reagent solution which transports the sample zone into a detector while desired chemical or biochemical reactions take place. Detector response (absorbance, fluorescence, mass spectra, etc) yield a calibration curve quantifying the target analyte.

1.1 The First Generation-Flow Injection Analysis (FIA)

FIA methodology was born and developed in analytical laboratories as a practical tool rather than a body of theoretical principles. Most FIA workers admit that the analytical and non-analytical performance of this methodology can still be further improved by the mere application of some empirical rules. Every experiment can readily alter FIA assemblies in order to optimize their results.

1.1.1 Principle of FIA

Flow injection analysis (FIA) is a kind of continuous was first introduced by Ruzika and Hansen in 1975[1, 2]. They have defined FIA as a method based on the injection of a liquid sample into a moving non-segmented continuous carrier stream of a suitable liquid. The injection sample forms a zone that is then transported towards a detector which records the absorbance, electrode potential or other physical parameters as it continuously changes as results of the passage of the sample material through the flow cell[1].

FIA is based on the combination of three principles[2] such as sample injection controlled dispersion of the injected sample zone and reproducible timing of its movement from the injection point toward and into the detector.

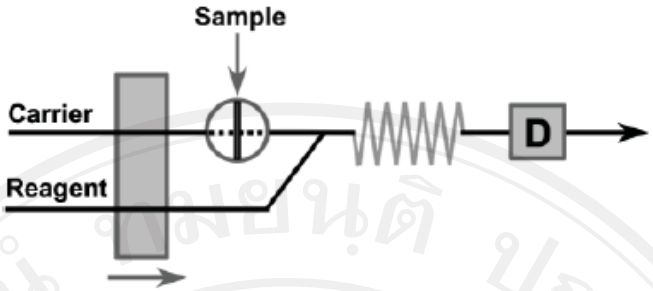


Figure 1 Typical representation of a FIA system, where a defined volume of sample is injected into a continuously flowing carrier stream which is merged with a stream of reagent. The ensuing transient generation of product is monitored by a suitable detector (D).

The design of FIA is simple, as represented in Figure 1, consisting of a pump for propelling the carrier (or reagent) stream through a narrow tube which is made of various materials according to the type of reagents and solvents used; an injection port through which a known volume of sample is injected into the carrier stream in a very reproducible manner; and a reaction coil in which the sample zone disperses and reacts with the components of the carrier stream, forming a species which is sensed by a flow-through detector and recorded, the output being a peak whose shape and magnitude reflects the concentration of the injected analyte. FIA is a technique characterized by its simplicity, economy, fast sampling rate and extreme versatility. It is an excellent tool for solving problems in various areas including clinical[3], environmental[4] and drug analysis[5-6].

1.1.2 FIA Instrumentation[7]

As described above the instrumentation used for FIA is very simple, and the design of the flow injection analyzer is depends on the particular requirements of the application used. A simple two line manifold is shown in Figure 2, using the common symbolic notation. The bottom half of the diagram shows optionally-used devices for reagent and carrier propulsion, sample injection, sample-reagent mixing and various detection modes.

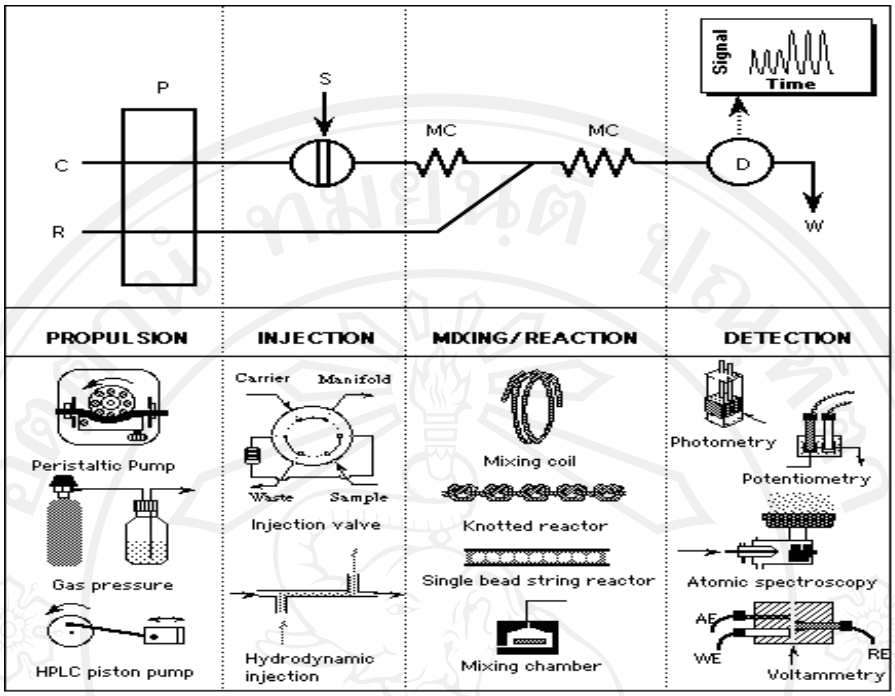


Figure 2 Schematic diagram of a typical flow injection analysis manifold. P is a pump, C and R are carrier and reagent lines respectively, S is sample injection, MC's are mixing coils, D is a flow through detector, and W is the waste line. The lower portion of the diagram indicates some typical instrument options available for reagent and carrier propulsion, sample injection, sample-reagent mixing, and various detection modes[7].

The major components of a flow injection analysis system are as follows:

(a) Propelling System

The propelling system or liquid delivery unit is a component used to drive or propel the carrier stream through the different elementary units of FIA in a perfectly pulse-free and constant flow. Peristaltic pumps[8] (set of a rollers on a revolving drum that squeezes flexible tubing to produce a constant, pulsing flow) are the most widely used type for propelling solutions in various flow injection systems (as shown in Figure 3). Various others means of propulsion units have been used in FIA system,

which include a syringe pump, a reciprocating piston pump, a gas-pressure reservoir and a gravity-based unit.

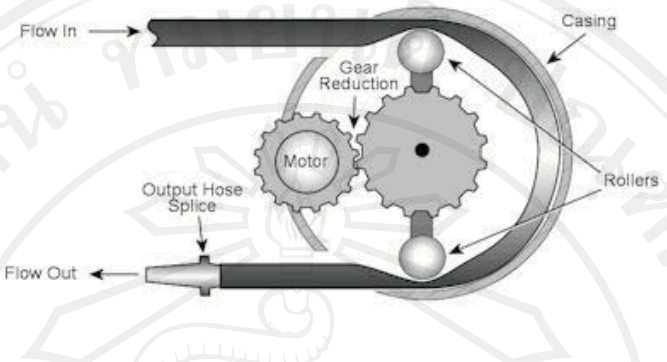


Figure 3 Schematic drawing of a peristaltic pump

(b) Sample Introduction or Injection System

The sample introduction system in FIA is intended to insert, in a fully reproducible manner which an accurate volume of sample solution into a carrier stream without altering its flow rate. The sample can be injected in a variety of ways. The earliest method of sample introduction was injection from a syringe through the wall of a tube or through a septum or flap valve. The injectors employed in FIA similar to those used in HPLC, in which they are furnished with an internal sample loop or more commonly, a dedicated FIA-valve comprising a rotor and stator having four, six or more individually accessible ports (Figure 4).

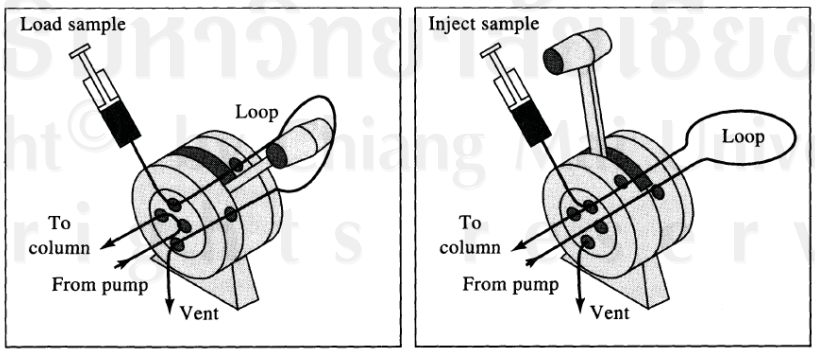


Figure 4 Schematic drawing of injection sample system[7]

(c) Transport and Reaction System

The transport system is an integral component of FIA system. The function of this part is to provide connections between the different components of the system. The conduits used in FIA manifolds mostly consist of narrow bore plastic tubes. The tubes are normally coiled or knotted in order to minimize the dispersion. But as a rule the tube lengths of the FIA manifold should be made as short as possible in order to avoid adverse dilution of the injected sample solution.

(d) Detection System

The detector system is a sensing part of FIA manifold, which allows continuous monitoring of a given property of the sample or its reaction product and provides qualitative and quantitative information of the analyte. Many different types of detector have been employed in FIA. Any detector that can be equipped with a flow-through cell can be adopted for use in FIA. FIA detectors should have a series of clear-cut attributes such as; small volume, low noise level, fast and linear response over a wide concentration range and high sensitivity. A variety of the detection systems have been used in FIA. These include the spectrophotometer, atomic absorption and inductively couple plasma spectrometer, fluorimeter, various electrochemical detectors and chemiluminescence detector.

(e) Data Processing Unit

In FIA, the analyte concentration is usually estimated from the peak heights or less frequently peak area. This signal output from detector is displayed on a chart recorder, microprocessor or a computer as a peak.

1.1.3 Dispersion in the FIA

As stated previously, the control of dispersion is the most important aspect of FI systems. The dispersion of a fluid zone reproducibly introduced into a non-segmented flow stream (carrier) during transport of the zone to the detector, is the most important physical phenomenon in all FI systems. The specific feature of

dispersion processes in FIA is that they are reproducible and controllable through the manipulation of flow parameters and geometrical dimensions of flow conduits. The driving forces active in dispersion of the injected zone into the carrier stream are molecular diffusion and convection, but the effects of convection dominate, and the effects of molecular diffusion may be neglected in most cases. Convection occurs both as result of linear flow-rate differences of fluid elements located at different points along the radial axis of the conduit and as a result of secondary flows created by centrifugal forces perpendicular to the flow direction in non-straight conduits. A convex parabolic front of the injected zone and a concave parabolic tailing edge are developed with penetration into the carrier stream, the extent increasing with the distance traveled. Thus, under the specific conditions applied in FIA and with a fixed conduit, the acting forces are well under control, so that no random turbulence occurs. The result provides perfectly reproducible concentration time relationships, recorded and superimposed, precisely overlap each other to form a single curve. This provides the basis for extracting reproducible readout under both physically and chemically non-equilibrium conditions. The dispersion process typical of FIA system is shown in Figure 5. The injected fluid zones in a non-segmented flow stream can be manipulated reproducibly to produce various degrees of dispersion. In order to provide a quantitative criterion evaluating the extent of dispersion, the term dispersion coefficient (D) was introduced that being defined as the ratio of the concentration of the constituent of interest in a fluid element of the injected zone before and after dispersion, expressed by:

$$D = C_0/C \qquad (1)$$

Where, C_0 is the original concentration of the constituent in the solution before dispersion, and C is the concentration of that fluid element of the dispersed fluid zone from which analytical readout is extracted. When the fluid element with the highest concentration is used for readout, equation 2 is expressed as:

$$D = C_0/C_{max} \qquad (2)$$

Where, C_{max} is the concentration of the constituent at peak maximum. D is a dimensionless value, which is equivalent to the dilution factor of the fluid element under consideration. For example, if the sample is diluted 1:1 by carrier, thus the dispersion coefficient is 2. FI systems are categorized into high, medium, and low dispersion systems depending on the degree of dispersion of the injected zone at the read out point. Systems with D above 10 are classified as high, those between 2 and 10 as medium, and those below 2 as low dispersion system.

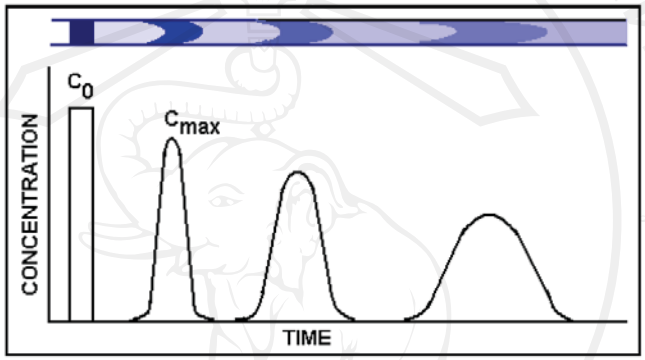


Figure 5 The dispersion process typical of FIA system

1.1.4 Optimization of an FIA System

Fine-tuning an analytical FIA method entails investigating the influence of a large variety of experimental variables that may have critical effects on the quality of the results (sensitivity, reproducibility and throughput). In principle, one may distinguish between chemical parameters (pH, temperature, reactant concentrations), which determine reactivity; FIA parameters (sample volume, reactor length, size and shape of the reagent bed, intensity of the light source, features of the mixing chamber and connector shapes, if used, etc.); and detection parameters (wavelength, window size, sensitivity scale, aspiration flow-rate, void volume of the flow-cell, electrode shape, size and arrangement, etc.)

The primary objective is to maximize the purely analytical properties: sensitivity, accuracy and reproducibility. Secondary to these, one should optimize such features as sample throughput, sample and reagent consumption and equipment running costs.

Finally, one should also consider automatability and the ability to perform comprehensive sample pretreatment and multi-determinations in a single sample.

1.2 Flow Injection Analysis of Arbutin in Medicinal Plants and Pharmaceutical Formulations

1.2.1 Arbutin

Arbutin is a naturally occurring glycoside of hydroquinone (Figure 6). It is found in the bark and leaves of various plants, usually occurring together with methyl-arbutin. Naturally occurring arbutin was first characterized by Kawalier[9], who obtained it from bearberry leaves. It is also found in the leaves of blueberry, cranberry and pear. Synthetic arbutin was first reported by Mannich[10], and later by others. Commercial arbutin is almost always synthetic in origin. Because of its antibacterial properties, arbutin is a constituent of the traditional medicine *uva ursi*, it is widely used in a variety of formulations[11]. The ability of arbutin to inhibit human melanin synthesis has given rise to it wide use in many cosmetic formulations[12]. Arbutin protect the skin against damage caused by free radicals. It is a skin whitening agent which is very popular in Japan and Asian countries for skin de-pigmentation. Arbutin inhibits the formation of melanin pigment by inhibiting tyrosinase activity[13]. Back in the 18th century arbutin was first used in medical areas as an anti-inflammatory and antibacterial agent. It was used particularly for cystitis, urethritis and pyelitis. It may be used to repress the virulence of bacterial pathogens and to prevent from contaminating bacteria. It is also used for treating allergic inflammation of the skin. More recently, arbutin has been used to prevent pigmentation and to whiten the skin. It can be used to whiten the skin, to prevent liver spots and freckles, to treat sunburn marks and to regulate melanogenesis[14].

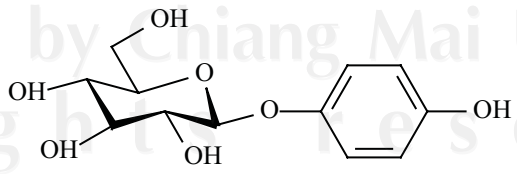


Figure 6 Chemical structure of arbutin

Arbutin give three main properties; whitening effect, anti-aging effect and UVB/UVC filter[15]. Arbutin is a very safe skin agent for external use which has low toxicity, has no odor or side effect as seen with hydroquinone. The encapsulation of arbutin constitutes a delivery system to potentialize the effect in time. It is a way to incorporate the hydrophilic arbutin in lipophilic media.

1.2.2 Previous Analytical Methods for Arbutin Determination

Different analytical methods have been reported for the determination of arbutin in medicinal plant extracts and cosmetic products. Kenndler *et al*[16] described the determination of arbutin in bearberry leaves based on capillary zone electrophoresis. The analysis was performed on a P/ACE^{TM} system 2000 instrument (Beckman) equipped with a separation capillary made from fused silica (75 μm, I.D. 50 cm length to the detector). The field strength was about 350 v cm^{-1} at an electric current about 70 μA; UV detection at 214 nm. Vanhaelen *et al*[17] determined arbutin from *Arctostaphyllos uva-ursi* crude extract by thin layer chromatography-densitometry. The plant extract was spotted on a silica gel 60 F_{254} plate, using EtOAc : MeOH : H_2O (85:17:13) as mobile phase, UV detection at 225 nm. Parejo *et al*[18] developed a fast and simple extraction procedure coupled with an HPLC method for the determination of arbutin content of leaves of *Arctostaphylos uva-ursi* plants, using a Nucleosil C_8 column, water-methanol (95:5) as mobile phase and UV detection at 280 nm. Bubenchikova *et al*[19] determined phenolic compounds e.g. luteolin, arbutin, rutin etc, in the ethanol extract of yellow sweet clover by HPLC. The sample fractions were separated on a platinum EPS C_{18} column. The mobile phase was acetonitrile-water-concentrated phosphoric acid (400:600:5), with UV detection at 254 nm. Messe *et al*[20] developed a simple and rapid method grouping TLC and HPLC/UV for separation, identification and determination of kojic acid and arbutin in skin-whitening cosmetics, using a polar stationary phase with diol groups and a polar buffered mobile phase at pH 2.5 consisting of methanol-0.05 M KH_2PO_4 (65:35, v/v), with UV detection at 286 nm. Chang *et al*[21] described a simultaneous high performance liquid chromatographic method for quantifying four of the most common used hydrophilic whitening agent e.g. glycolic acid, ascorbic acid, arbutin and magnesium ascorbyl phosphate, using a C_{18} column with ion-pair reagent as mobile

phase, UV detection at 220 and 240 nm, respectively. Kraus *et al*[22] determined arbutin from *folium uva-ursi* by UV spectrophotometry at 281 nm. Appropriate amount of sample solution was reacted with 10 mL of NH_4OH and 5.0 mL diazotized sulfanilic acid, and then determined colorimetrically at 570 nm. Franciszek *et al*[23] described a spectrophotometric method for the determination of arbutin in herbs, based on the reaction of Ce(IV)-arbutin-arsenazo III, Ce(IV) was reduced to Ce(III) by arbutin and the Ce(III) ions formed with arsenazo III as a colored complex with λmax = 655 nm at pH 3.5. Jiri *et al*[24] reported spectrophotometric methods for determining arbutin and hydroquinone. The sample solution was reacted with nitrous acid, a colored product; dinitroarbutin was obtained, whereas in the case of hydroquinone *p*-benzoquinone was the reaction product. Both compounds can be determined colorimetrically.

According to their report the probable mechanism is oxidation of 4-AP with potassium hexacyanoferrate in alkaline medium causing loss of two protons from the former leading to the formation of a nucleophilic intermediate that further undergoes nucleophilic substitution with the phenolic moieties of arbutin that in turn results in the colored product[25]. However, this method is completely manual and thus it is labour intensive, consumes large amount of reagents and generate the corresponding amount of waste. 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[26]. 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 ferricyanide and 4-AP to form a red colored complex. Selectivity may be increased by means of a chemical reaction producing a chromophore. As stated earlier, the arbutin molecule provides a phenolic group that can react with certain chromogenic reagent resulting in a chromophore, thus enabling a quantitative

method to be developed. The reaction with 4-AP in the presence of an oxidant, such as 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 arbutin were reported in the literature.

In general, flow injection 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 minimize the influence of a coloured reagent such as 4-AP in the presence of an oxidant, such as hexacyanoferrate (III) in alkaline medium solution.

This work describes a simple, rapid, accurate and inexpensive flow-injection method for the determination of arbutin. The method was applied to determination of arbutin in commercial whitening creams and medicinal plant extracts.

1.3 Flow Injection Analysis of Curcuminoids in Medicinal Plants and Pharmaceutical Formulations

1.3.1 Curcuminoids

Turmeric (*Curcuma longa* L.) has been used as an orange-yellow coloring agent, and has been found to be a rich source of phenolic compounds namely, curcuminoids[27]. 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%)[28]. Curcuminoids are recognized for their broad spectrum of biological activities and safety in foods or pharmaceuticals[29]. Curcumin, the principal natural yellow pigment, is widely used for foods and dye which shows many biological activities, for examples: antioxidant[30], anti-inflammatory, antimicrobial, antiparasitic, antimutagenic, anticancer and antivirus properties[31]. 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[32]. Kim reported that demethoxycurcumin and bis-demethoxycurcumin showed strong antioxidant activity[33]. The antioxidant and pro-oxidant activities of curcumin and the structure relationship between curcumin, demethoxycurcumin, and bis-demethoxycurcumin were investigated by Ahsan[28].

1.3.2 Chemical Constituents in Turmeric

Turmeric contains up to 5% essential oils and up to 3% curcumin, a polyphenol. It is the active substance of turmeric and it is also known as C.I. 75300, or Natural Yellow 3. The systematic chemical name is (1E, 6E)-1, 7-bis(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione. It can exist at least two tautomeric forms, keto and enol. The keto form is prefered in solid phase and the enol form in solution. The essential oil contains sesquiterpenes. Aroma is decided by the chemical like turmerone, ar-turmerone and zingiberene. The orange colour and the pungent taste is imparted to it by diarylheptanoids. Three major yellow pigments (3-5% of

raw plant) have been isolated from turmeric. These diarylheptane derivatives are curcumin, demethoxycurcumin and bis-demethoxycurcumin (Figure 7). The rhizome also contains 70% carbohydrates, 7% protein, 4% minerals, vitamins, other alkaloids, and resin.

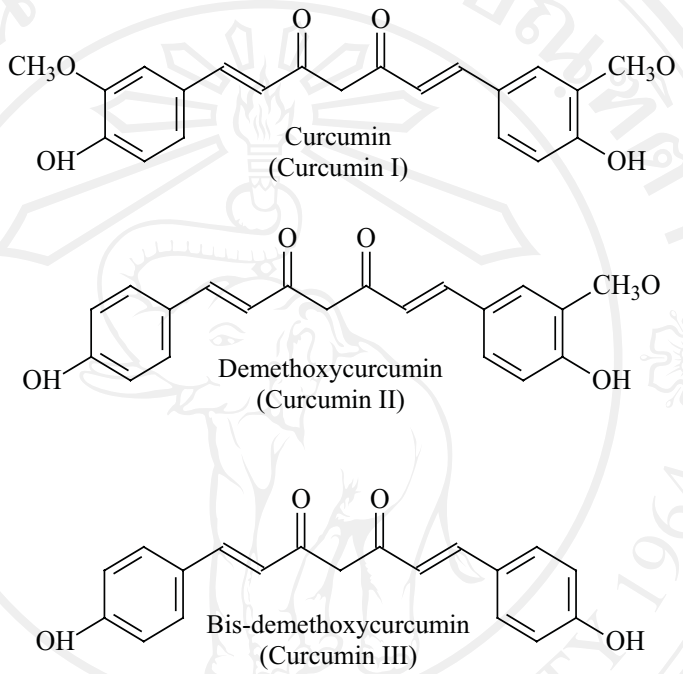


Figure 7 Chemical structures of curcumin, demethoxycurcumin and bis-demethoxycurcumin

1.3.3 Medicinal Uses and Pharmacology of Turmeric

Turmeric is reported to possess anticarcinogenic, anticoagulant, antihepatotoxic, antimutagenic/DNA protecting and anti-oxidative properties. This plant has been found to contain volatile oil and curcuminoids. These compounds are believed to be the active ingredients of turmeric. Recent research on turmeric has indicated that the rhizome is pharmaceutically active against a number of illnesses such as cancer, dermatitis, AIDS, inflammation, high cholesterol levels and dyspeptic conditions. A recent study involving mice has shown that turmeric slows the spread of breast cancer into the lungs and other body parts. Turmeric also enhances the effect of taxol in reducing metastasis of breast cancer.

1.3.4 Cosmetics Uses

The rhizome is used as a cosmetic by women. The rhizome is made into a paste with an oil base and applied to smoothen the skin and preserved a youthful complexion. Turmeric is currently used in the formulation of some sunscreens. Colourless compounds which called isolate tetrahydrocurcuminoids (THC) can be extracted from turmeric. These compounds might have antioxidant and skin-lightening properties and might be used to treat skin inflammations, and thus making these compounds useful in cosmetics formulations.

1.3.5 Previous Analytical Methods for Curcuminoids Determination

A variety of methods for the quantification of the curcuminoids have been reported. Most of these are spectrophotometric methods, expressing the total color content of the sample[34]. Commercial turmeric products contain mixtures of curcumin, demethoxycurcumin and bis-demethoxycurcumin[28]. Gupta described a simultaneous determination of curcuminoids in *curcuma* samples using high performance thin layer chromatography. Samples and standards were applied on a silica gel $60F_{254}$ plate and the separation was performed using chloroform-methanol (95:5 v/v) followed by scanning of the spots at 366 nm using an UV detection mode[35]. Rasmussen 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*[36]. A direct fluorimetric method for the analysis of curcumin and its structural isomers in food materials based on high performance liquid chromatography (HPLC) with UV or visible or fluorescence detection was proposed by Karasz and Tounesen[37-38]. HPLC was carried out on a Nucleosil NH_2 column using ethanol as mobile phase[39]. Smith compared the use of UV spectrometric and electrochemical detectors for the HPLC determination of curcumin in turmeric powder using ODS-Hypersil column[40]. 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[41]. 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[42]. He described an on-line high performance liquid chromatography for simultaneous analysis of curcuminoids and sesquiterpenoids in fresh turmeric[43]. 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[44]. Hiserodt characterized turmeric powder using liquid chromatography-mass spectrometry. These involved 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[45].

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 oxidizing agent yielding a red colored product. The probable mechanism is the 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 colored product[46-47]. 4-AP does reacts 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 an 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 [48]. 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 colored 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 colored reagent such as 4-AP in the presence of potassium hexacyanoferrate (III) in alkaline solution.

This research work 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 determine the total of 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 release.

1.4 Research Aims

In this research, the development of flow-based techniques will be undertaken for the determination of bioactive compounds in medicinal plant extracts. The aim of this research can be summarized as follows:

1.4.1 To develop two novel Flow Injection (FI) techniques for the determination of arbutin and curcuminoids from selected medicinal plants.

CHAPTER II

EXPERIMENTAL

Basic equipment used in this research work is presented in this chapter. All instruments and apparatus used are firstly exhibited. After that the list of chemical reagents and procedures are subsequently illustrated.

2.1 Flow Injection Analysis of Arbutin in Medicinal Plants and Pharmaceutical Formulations

2.1.1 Instruments and Apparatus

The instruments and apparatus used were as follows:

1. Micro pump: EYELA MP-3, Tokyo Rikakikai Co., Ltd, Tokyo, Japan
2. Tygon tubing (0.508-1.521 mm i.d.): Cole-Parmer Instrument Company, Chicago, IL, USA
3. Disposable plastic syringe: Nissho Nipre Corporation Ltd., Ayuttaya, Thailand
4. Three-way connector (T-shaped): Omnifit®, Cambridge, United Kingdom
5. Flow through cell: Hellma 1.0 cm, Suprasil I window, type 178, 711-GS. Hellma, Forest Hills, New York, USA
6. Spectrophotometer model 1000: Cecil Instruments Ltd., Milton Technical Centre Cambridge, United Kingdom
7. Glass tube (6.0 cm × 0.20 mm): Omnifit®, Cambridge, United Kingdom
8. Servograph REC-51 chart recorder: Radiometer Copenhegen, Denmark
9. UV-visible spectrophotometer (Model speckol 1200): Jena Analytic, Jena, Germany
10. Filter membrane (Whatman®, No. 41 filter paper): Whatman Company Ltd., Maidestone, United Kingdom
11. pH meter: Inolab WTW, Germany
12. Ultrasonicator: Model 889: Cole Parmer, USA

13. Analytical balance: Mettler Toledo AG 285, Switzerland

2.1.2 Chemicals and Reagents

Deionized water was used throughout all experiments. All chemicals used are of analytical reagent grade which are listed as follows:

1. 4-aminoantipyrine (4-AP, CH_3-$C_9H_7N_3O$-CH_3): ACROS Organic (98%); Morris Plains, New Jensey, USA
2. Potassium hexacyanoferrate ($K_3Fe(CN)_6$): May & Baker Ltd., (99%); Dagenham, United Kingdom
3. Boric acid (H_3BO_3): Sigma-Aldrich, Exeter, United Kingdom
4. Potassium chloride (KCl): Merck, Darmstadt, Germany
5. Sodium hydroxide (NaOH): BDH, Poole, United Kingdom
6. Sodium carbonate (Na_2CO_3): Sigma, St, Louis, MO, USA
7. Sodium phosphate (Na_2HPO_4): Sigma, St, Louis, MO, USA
8. Calcium carbonate ($CaCO_3$): Sigma, St, Louis, MO, USA
9. Lead (II) acetate ($Pb(CH_3COO)_2.3H_2O$): Sigma, St, Louis, MO, USA
10. Glucose: Sigma, St, Louis, MO, USA
11. Tween 80: Sigma, St, Louis, MO, USA
12. Sorbital: Sigma, St, Louis, MO, USA
13. Sodium metabisulphite: Sigma, St, Louis, MO, USA
14. Methyl paraben: Sigma, St, Louis, MO, USA
15. Propyl paraben: Sigma, St, Louis, MO, USA

2.1.3 Flow Injection Apparatus

A schematic diagram of the FI manifold system used for the determination of arbutin is shown in Figure 8. A micro-tube pump with tygon tubing to propel the carrier solution, 4-AP solution and hexacyanoferrate (III) solution were used.

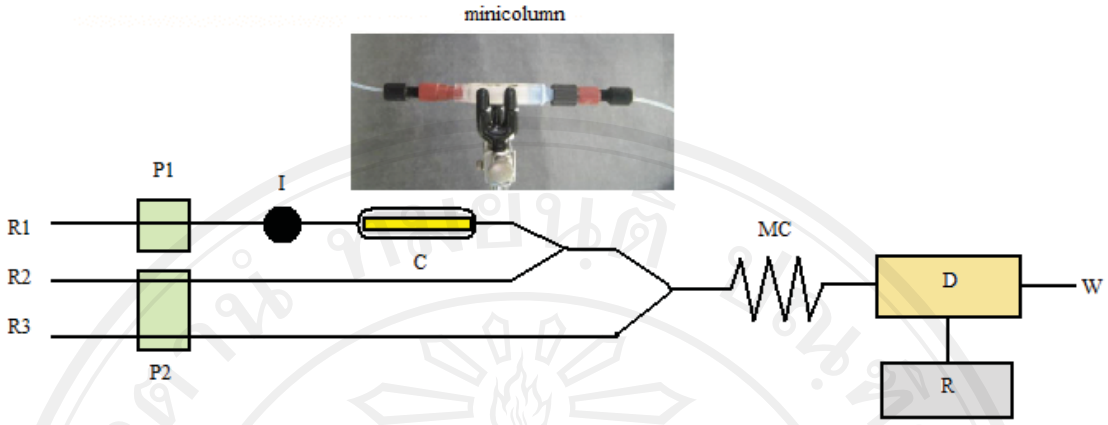


Figure 8 Proposed FI manifold; R1, Borate buffer solution; R2, 4-AP; R3, potassium hexacyanoferrate; P1 and P2, pump; I, injection valve; C, a glass tube packed with silica C_{18} as a mini-column; MC, mixing coil reactor; D, detector; R, recorder; W, waste.

The sample and/or standard solution containing arbutin was injected into the carrier stream via a laboratory-made, low-cost injection valve by using a 1.0 mL disposable plastic syringe. The sample in the 4-AP solution stream was then merged with the hexacyanoferrate (III) solution. The merged streams were passed through a mixing coil reactor. The coloured complex formed at this point was passed through the flow through cell in a spectrophotometer model 1000 instrument connected to a Servograph REC-51 chart recorder. Absorption measurements of the red coloured complex formed were made at 514 nm. A glass tube (6.0 cm × 0.20 mm) packed with silica C_{18} was used as a mini-column for separation of arbutin from sample matrices.

2.1.4 Standard, Reagents and Sample Preparations

a) Preparation of Standard Solutions

A 1000 µg mL^{-1} stock solution of arbutin was prepared daily by dissolving 25.0 mg of the standard in deionized water and making up the volume to 25 mL with deionized water. Working standard solutions (1.0, 5.0, 10.0, 20.0 and 30.0 µg mL^{-1} of arbutin) were obtained by appropriate dilution of the arbutin stock solution in deionized water.

b) Reagents

The 4-AP solution 1.0×10^{-2} mol L^{-1} was prepared by dissolving 1.0162 g of 4-AP in 500 mL deionized water and this solution was prepared daily. The hexacyanoferrate (III) solution (1.0×10^{-2} mol L^{-1}) in alkaline media was prepared by dissolving 1.6463 g of potassium hexacyanoferrate in 500 mL alkaline solution and this solution was prepared daily. The borate buffer (pH 9.4) was prepared by dissolving the calculated amount of boric acid and potassium chloride in deionized water and 0.2 mol L^{-1} sodium hydroxide solution was used to adjust the pH to 9.4.

c) Sample Pretreatments

Pharmaceutical Samples

About 0.10 g of each cosmetic sample; Cosmetic (1), Cosmetic (2) and Cosmetic (3) (commercially available in a drug store in Thailand) was accurately weighed, boiled for 30 min with 30 mL of deionized water in the presence of 0.10 g calcium carbonate and filtered through Whatman®, No. 41 filter paper. The residue was washed thoroughly with boiling water. Then 5 mL of 4% lead (II) acetate in water was added to the filtrate, cooled, diluted to 50 mL with deionized water, and filtered. Then 0.20 g of sodium phosphate was added to the filtrate, mixed and filtered again.

Medicinal Plant Samples

The peel of pear contains antioxidant compounds which are useful for health and cosmetic production. Galvis Sanchez *et al.*[49] reported that the main phenolic compounds in six pear cultivars were identified and quantified using HPLC/diode array detection and HPLC/electrospray ionization mass spectrometry. The peel contained higher concentrations of chlorogenic acid, flavonols and arbutin than the flesh, where only chlorogenic acid was detected. Total phenolics ranged from 1235 to 2005 mg kg^{-1} in the peel and from 28 to 81 mg kg^{-1} in the flesh. Ascorbic acid and dehydroascorbic acid were detected in the peel whereas only dehydroascorbic acid was present in the flesh. The vitamin C contents were over the ranges of 116 to 228 mg kg^{-1} and 28 to 53 mg kg^{-1} in the peel and the flesh respectively. The essential oil of the whole fruit and the peel of pear were also investigated by gas chromatography-

mass spectrometry. The predominant constituent of the two kinds of essential oils was butylated hydroxytoluene which is a typical antioxidant. Therefore, Ya pear, fragrant pear, earlymatured pear and Chinese pear were selected for this investigation. The medicinal plant samples are Ya pear, fragrant pear, early-matured pear and Chinese pear (commercially available from markets in Thailand).

d) Sample Preparation

Each sample was peeled and the peel was dried at 60 ^{o}C for 12 h and then powdered. About 2.0 g of each powdered plant material was weighed accurately, sonicated for 30 min with 30 mL of deionized water and 0.10 g calcium carbonate and filtered through Whatman® No. 41 filter paper. The residue was washed thoroughly with deionized water. Then 5 mL of 4% lead (II) acetate solution was added to the filtrate, cooled and diluted to 50 mL with deionized water and filtered. Then 0.20 g of sodium phosphate was added to the filtrate, mixed and filtered again.

2.1.5 Evaluation of the Proposed Flow Injection Manifold

The performance of this manifold was evaluated by oxidation of 4-AP solution and hexacyanoferrate (III) solution which were pumped at the flow rate of 1.2 mL min^{-1} (P2). The 0.2 mol L^{-1} borate buffer solution, pH 9.4, was pumped at the flow rate of 0.8 mL min^{-1} (P1). Comparative experiment was also carried out using the expensive commercial flow-through cell. The solutions were mixed at the coiled reactor (MC) and passed through the home-made and/or the commercial flow-through cell. The mixing coil was made from polyethylene tubing (0.635 mm i.d.) with 20 cm length. The selection wavelength was 514 nm. The flow diagram of the system is shown in Figure 8. In the position specified in the figure, a sample volume (I) of 300 μL was selected by the sample loop. After sample injection the arbutin zone was carried through separated silica C18 as a mini-column (C); (6.0 cm × 0.20 mm i.d.), and merged with 4-AP solution (R2) and hexacyanoferrate (III) solution (R3), respectively. The chemical reacts inside the reactor (MC; 20 cm) formed a red-coloured product. Passage of the coloured compound through the flow cell of

spectrophotometer (D; 514 nm) resulted in a transient absorbance that was recorded (R) as a peak with a height proportional to the arbutin content in the sample solution.

2.1.6 Recommended Procedure

Using the proposed FI manifold as shown in Figure 8, 300 μL of standard and/or sample of arbutin were injected into the carrier stream of 0.2 mol L^{-1} borate buffer solution, pH 9.4, with an optimum flow rate of 0.8 mL min^{-1} (P1). After loop-based injection, the arbutin zone separated with a glass tube packed with silica C18 as a mini-column (6.0 mm × 0.20 mm), and merged with 1.0×10^{-2} mol L^{-1} of 4-AP solution (R2) followed by 1.0×10^{-1} mol L^{-1} of hexacyanoferrate (III) solution (R3) with the flow rate of 1.2 mL min^{-1} (P2), respectively. The standard and/or sample and reagents reacted inside the reactor (MC; 20 cm) forming a red colour product which was then passed through the flow cell of a spectrophotometer (D; 514 nm), resulting in a transient absorbance that was recorded (R) as a peak with the height proportional to the arbutin content in the sample, and the calibration graph was constructed.

2.2 Flow Injection Analysis of Curcuminoids in Medicinal Plants and Pharmaceutical Formulations

2.2.1 Instruments and Apparatus

The instruments and apparatus used were as follows:

1. Peristaltic pump: Three channels and variable speed, Pharmacia, Sweden
2. Tygon tubing (0.508 - 1.521 mm i.d.): Cole-Parmer Instrument Company, Chicago, IL, U.S.A
3. Disposable plastic syringe: SGE, Australia.
4. Three-way connector (Y-shaped): Omnifit®, Cambridge, United Kingdom
5. Flow through cell: Cell volume 100 μL, Perkins Elmer, USA
6. UV-visible spectrophotometer (Model speckol 1200): Jena Analytic, Jena, Germany
7. Filter membrane (Whatman®, No. 41 filter paper): Whatman Company Ltd., Maidestone, United Kingdom
8. pH meter: Inolab WTW, Germany
9. Ultrasonicator: Model 889: Cole Parmer, USA
10. Analytical balance: Mettler Toledo AG 285, Switzerland
11. Linomat IV sample applicator: Camag, Wilmington, NC, USA
12. Silica gel 60 GF TLC plate (20 × 20 cm): Merck, Darmstadt, Germany
13. Rotary evaporator: Buchi, Switzerland

2.2.2 Chemicals and Reagents

Deionized water was used throughout all experiments. All chemicals used are of analytical reagent grade which are listed as follows:

1. 4-aminoantipyrine (4-AP, CH_3-$C_9H_7N_3O$-CH_3): ACROS Organic (98%); Morris Plains, New Jensey, U.S.A
2. Potassium hexacyanoferrate ($K_3Fe(CN)_6$): May & Baker Ltd., (99%); Dagenham, United Kingdom
3. Boric acid (H_3BO_3): Sigma-Aldrich, Exeter, United Kingdom
4. Potassium chloride (KCl): Merck, Darmstadt, Germany

5. Sodium hydroxide (NaOH): BDH, Poole, United Kingdom
6. Methanol: HPLC grade, E. Merck, Germany
7. Acetonitrile: HPLC grade, E. Merck, Germany

2.2.3 Flow Injection Apparatus

A schematic diagram of the flow system used for the determination of total curcuminoids content is shown in Figure 9. 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., as flow lines.

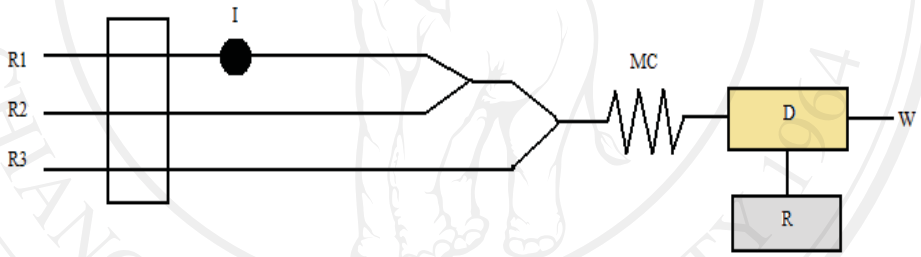


Figure 9 Schematic configuration of the FIA system: R1; carrier stream of borate buffer solution, R2; 1.0×10^{-1} mol L^{-1} 4-AP solution, R3; 1.0×10^{-3} mol L^{-1} potassium hexacyanoferrate (III) solution, P; peristaltic pump, I; injection valve, MC; reaction coil, D; detection, R; recorder, W; waste.

The standard and/or sample solution containing curcuminoids was injected by using a 20 µL disposable micro syringe into the borate buffer solution stream via the 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 in a spectrophotometer model Speckol 1200 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 was used to scan the spectra of curcuminoids.

2.2.4 Preparation of Standard, Reagents and Samples

a) Preparation of Standard Solutions

A stock solution (1000 μg mL^{-1}) of standard curcuminoids was freshly prepared 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.

b) Reagents

The 4-AP solution 1.0×10^{-1} mol L^{-1} was prepared by dissolving 10.1625 g of 4-AP in 500 mL of DI 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 of DI water and prepared daily. The borate buffer solution pH 9.4 was prepared by dissolving the calculated amount of 0.025 M of $Na_2B_4O_7 .10H_2O$ in deionized water and 0.1 mol L^{-1} hydrochloric acid solution was used to adjust the pH at 9.4.

c) Sample Pretreatments

Fresh turmeric was purchased from commercial sources in Chiang Mai Province, Thailand: sample codes; Turmeric (1) and Turmeric (2): purchased from Tonpayorm market, sample code; Turmeric (3): purchased from Warorot market and Phitsanulok Province, Thailand, sample codes; Turmeric (4) and Turmeric (5): purchased from Watyai market and sample code; Turmeric (6) and Turmeric (7): purchased from Kokmatoom market. A voucher specimen of the plant was deposited in the herbarium

of the faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand for reference. The samples were washed and dried in a hot air oven at 50 ^{o}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 ^{o}C until further use.

Fresh *Curcuma longa* L. was purchased from Chiang Mai and Phitsanulok Province, 1 kg of the turmeric root was chopped into small pieces and dried in a hot air oven at 50 ^{o}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 ^{o}C by means of a rotary evaporator. Then the residue was reconstituted in 1 mL of ethanol. This solution was used for separation by preparative TLC. A Linomat IV sample applicator was used to apply 100 µL sample solution on a silica gel 60 GF TLC plate, 20 × 20 cm. 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) as 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.

2.2.5 Chemical Reaction

It has been reported that phenols react with 4-AP in the presence of alkaline potassium hexacyanoferrate (III) as oxidizing 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 present 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.

2.2.6 Recommended Procedure

Using the fabricated FI manifold (Figure 9) the experimental conditions for determining total curcuminoids were optimized 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 solution, pH 9.5) 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.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Flow Injection Analysis of Arbutin in Medicinal Plants and Pharmaceutical Formulations

3.1.1 Preliminary Study

Initially, a three-line FI manifold was tested for determination of arbutin. The arbutin standard solution was injected into a buffer solution pH 9.4, carrier streams with a flow rate of 0.8 mL min^{-1} and separated with a glass tube packed with silica C_{18} as a microcolumn (6.0 cm × 0.2 mm). The 4-AP (1.0×10^{-4} mol L^{-1}) and hexacyanoferrate (III) (1.0×10^{-3} mol L^{-1}) solution were used as the reagent streams with a flow rate of 1.6 mL min^{-1}. The effect of sodium carbonate solution added to hexacyanoferrate (III) solutions was initially examined. Various alkaline media such as sodium carbonate and sodium hydroxide at the same condition, 1% in 1.0×10^{-3} mol L^{-1} hexacyanoferrate (III) solutions were tested as a reagent stream in the FI manifold, whereas the reagent stream was 1.0×10^{-4} mol L^{-1} in 4-AP solution. The absorbance of 1.0, 5.0, 10.0 and 15.0 μg mL^{-1} arbutin standard solutions were measured at 514 nm. It was found that when sodium carbonate was added to the hexacyanoferrate (III) solution, the sensitivity (defined as slope of calibration graph) increased steeply with increasing the concentration of sodium carbonate up to 1%. Afterwards the sensitivity decreased rather rapidly.

3.1.2 Manifold Design

The FI configuration used for the determination of arbutin was then designed and fabricated. A simple three channel FI system was employed. The first FI configuration (Figure (10a)) was used in which the arbutin standard solution was injected into the carrier stream containing 2.5×10^{-2} mol L^{-1} borate buffer solution and 1.0×10^{-4} mol L^{-1} 4-AP and 1.0×10^{-3} mol L^{-1} hexacyanoferrate (III) solution were

used as the reagent streams. Both streams were operated at the same flow rate of 1.6 mL min^{-1}. Next, the FI configuration was adopted in Figure (10b) 1.0×10^{-4} mol L^{-1} 4-AP solution was used as the carrier streams while the reagent stream were 2.5×10^{-2} mol L^{-1} borate buffer and 1.0×10^{-3} mol L^{-1} hexacyanoferrate (III) solutions with a flow rate of 1.6 mL min^{-1}.

In the position specified in the figure, a standard or sample volume (I) of 300 μL was selected by the sample loop. After standard or sample injection the solution zone was carried through separated silica C_{18} as a mini-column (C) and merged with reagent streams. The chemical reacts inside the reactor formed a red coloured product. Passage of the coloured compound through the flow cell of spectrophotometer (D) resulted in a transient absorbance that was recorded (R) as a peak with a height proportional to the arbutin content in the sample solution. Results indicated that the maximum sensitivity was obtained when the standard or sample solution was injected into a carrier stream of borate buffer solution and then mixed with the reagent streams of 4-AP and hexacyanoferrate (III) solution before delivery to the detector. Therefore this was chosen for subsequent studies.

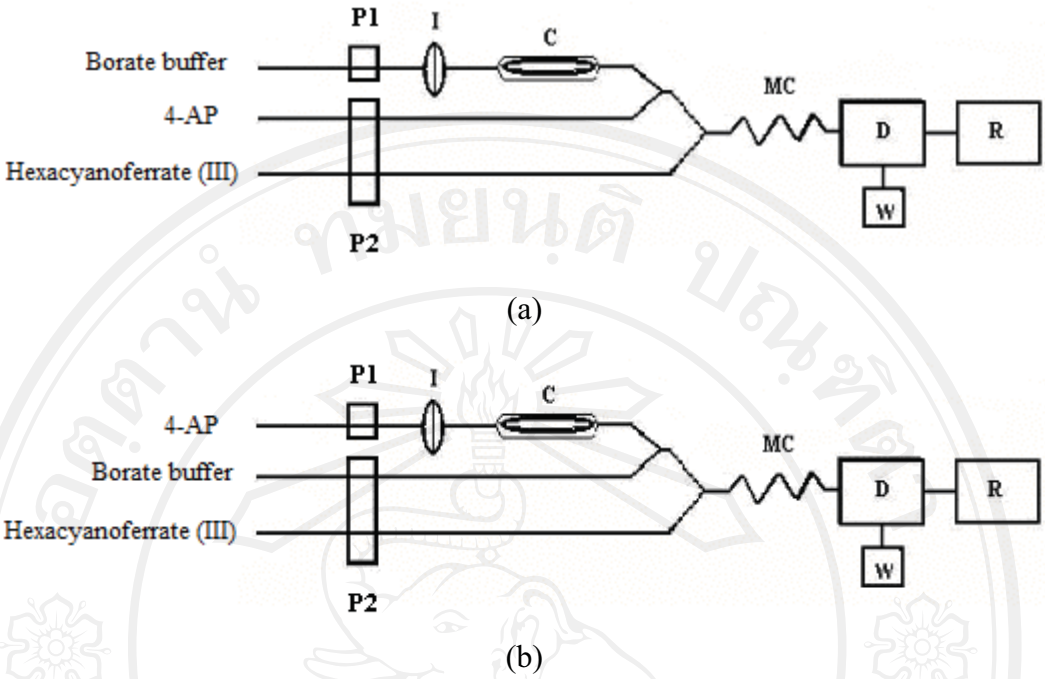
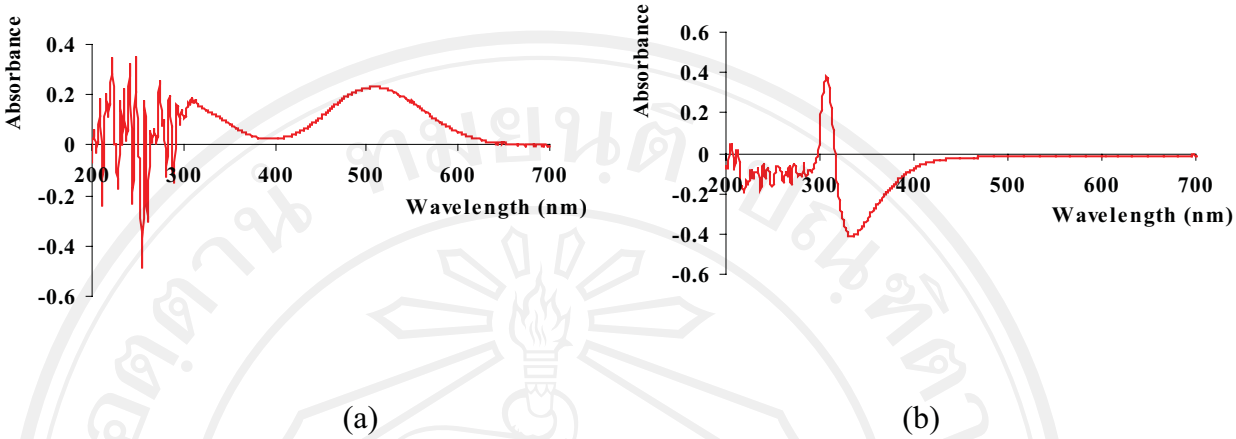


Figure 10 FI configurations tested for arbutin determination: (a) borate buffer solution was used as a carrier stream and 4-AP and hexacyanoferrate (III) solution were used as the reagent streams; (b) 4-AP solution was used as the carrier streams while the reagent stream were borate buffer and hexacyanoferrate (III) solutions; P1 and P2, pump; I, injection valve; C, a glass tube packed with silica C_{18} as a mini-column; MC, mixing coil reactor; D, detector; R, recorder; W, waste.

3.1.3 Absorption Spectra of Arbutin

Arbutin reacts with 4-AP and hexacyanoferrate (III) resulting in a red coloured product in buffer solution pH 9.4, medium. The borate buffer solution pH 9.4, was used because arbutin gives a strong and stable red colour with 4-AP in presence of hexacyanoferrate (III) at pH 9.4. The product presented an absorption maximum at 514 nm whereas the arbutin exhibited its absorption maximum at 308 nm under the same experimental conditions. The molar absorptivity at 514 nm was 3.15×10^{-3} L mol^{-1} cm^{-1} for buffer solution pH 9.4 medium. In this work, a FI spectrophotometric procedure was proposed for determining arbutin using 4-AP and hexacyanoferrate (III) reagents.

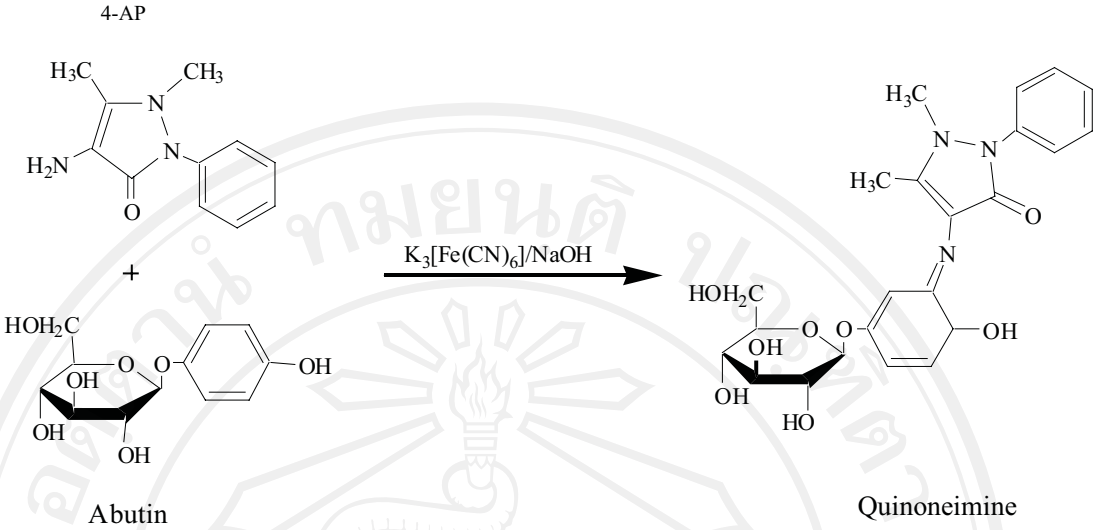


(a) (b)

Figure 11 The product presents an absorption maximum at 514 nm (a) whereas the arbutin exhibits its absorption maximum at 308 nm (b)

3.1.4 Chemical Reaction

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



Scheme 1 Assumed scheme of reaction between 4-AP and arbutin and formation of a highly colored quinineimine

3.1.5 Delivery Order of Reagent and Sample

In FIA measurements that involve multiple reagents, it is necessary to optimize the delivery order of reagents and sample. There are two possibilities to sandwich the sample; 4AP-sample-hexacyanoferrate (III) (peak height = 1.63 mV), hexacyanoferrate (III)-4AP-sample (peak height = 0.21 mV) and hexacyanoferrate (III)-sample-4AP (peak height = 0.72 mV). The order 4AP-sample-hexacyanoferrate (III) was chosen.

3.1.6 Optimization of Experimental Variables

The optimization of experimental conditions was carried out by means of a univariate method. The optimum was investigated by injecting 300 μL of 1.0, 5.0, 10.0 and 15.0 μg mL^{-1} arbutin standard solutions into the carrier stream of buffer solution with the flow rate 0.8 mL min^{-1} and reagent streams of 4-AP and hexacyanoferrate (III) with the same flow rate 1.2 mL min^{-1}, respectively. The optimized parameters included the reagent concentrations and some physical variables, including the flow rate and the sample injection volume.

3.1.6.1 Effect of Reagent Concentrations

The effect of varying concentrations of 4-AP, hexacyanoferrate (III) and sodium carbonate were studied.

3.1.6.1.1 The Effect of 4-AP Concentration

The effect of various concentrations of 4-AP solutions (1.0×10^{-6} - 5.0×10^{-1} mol L^{-1}) on the absorption of the arbutin (as peak height) at 514 nm was examined. The results obtained are shown in Table 1. The 4-AP concentrations in the studied range were sufficient for complete colour development. At higher 4-AP concentrations, the sensitivity of calibration curve increases. The 4-AP concentration which exhibited the largest sensitivity increment with reasonable sample throughput was found to be 1.0×10^{-2} mol L^{-1} as shown in Figure 12, and was considered as optimum value.

3.1.6.1.2 The Effect of Hexacyanoferrate (III) Concentration

The effect of various concentrations of hexacyanoferrate (III) solutions (1.0×10^{-4} - 1.0 mol L^{-1}) in 1% sodium carbonate on the absorption of the arbutin (as peak height) at 514 nm was examined (Table 1). The hexacyanoferrate (III) concentrations in the studied range were sufficient for complete color development. At higher hexacyanoferrate (III) concentrations, the sensitivity of calibration curve increases. The hexacyanoferrate (III) concentration which exhibited the largest sensitivity increment with reasonable sample throughput was found to be 1.0×10^{-2} mol L^{-1} as shown in Figure 12, and was considered as optimum value.

Table 1 Effect of 4-AP and hexacyanoferrate (III) concentrations on sensitivity

4-AP concentration (mol L^{-1})	Sensitivity* (mV/µg mL^{-1})	Correlation coefficient (r^2)	Hexacyanoferrate (III) concentration (mol L^{-1})	Sensitivity* (mV/µg mL^{-1})	Correlation coefficient (r^2)
1.0×10^{-6}	0.0234	0.9969	1.0×10^{-4}	0.0424	0.9973
1.0×10^{-5}	0.0263	0.9998	1.0×10^{-3}	0.1652	0.9932
1.0×10^{-4}	0.0629	0.9763	1.0×10^{-2}	0.4783	0.9949
1.0×10^{-3}	0.1579	0.9950	1.0×10^{-1}	0.2459	0.9838
1.0×10^{-2}	0.1814	0.9963	0.5	0.1721	0.9962
1.0×10^{-1}	0.1670	0.9978	1.0	0.0985	0.9982
0.5	0.1667	0.9815			

*Mean of three determinations

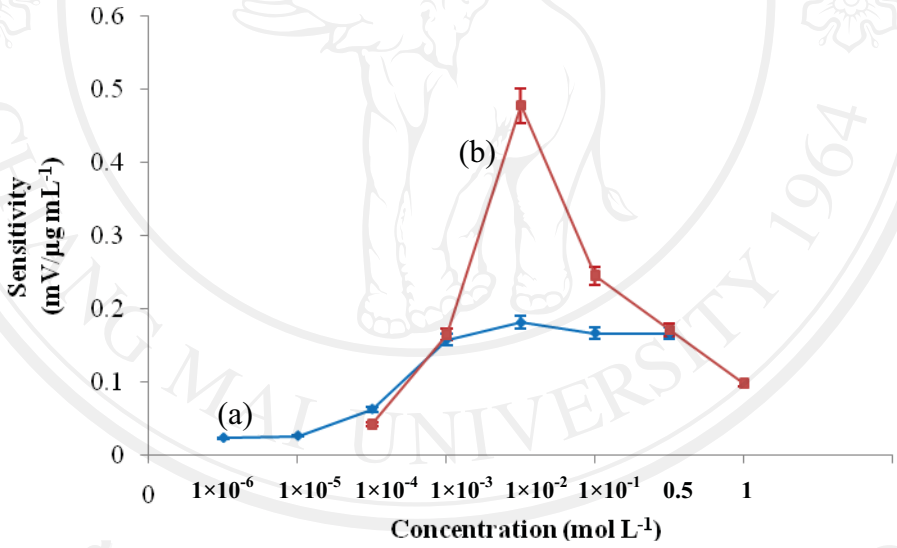


Figure 12 Effect of (a) 4-AP concentration and (b) hexacyanoferrate (III) concentration on the sensitivity of calibration curve (n = 3) of 1.0 - 15.0 µg mL^{-1} arbutin standard solutions

3.1.6.1.3 The Effect of Alkaline Media

The effect of alkaline media added to potassium hexacyanoferrate (III) solution was initially examined. Various alkaline media such as Na_2CO_3 and NaOH 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 1.0% Na_2CO_3 gave the greatest sensitivity (slope of the calibration graph).

3.1.7 Optimization of Manifold Parameters

The variables studied under the optimized reagent concentrations were the flow rate, the reaction coil length and the injection sample volume.

3.1.7.1 The Effect of Flow rate

The effect of flow rate on the sensitivity of the coloured reaction product was investigated over the range of 0.4 - 2.4 mL min^{-1} (Table 2). The results obtained showed that a flow rate of carrier steam (0.8 mL min^{-1}) and total flow rate of both reaction streams (1.2 mL min^{-1}) gave the highest sensitivity as shown in Figure 13, and was used in all subsequent experiments.

3.1.7.2 Mixing Coil Length and Internal Diameter

Mixing coil length and its internal diameter (i.d.) were essential parameters that affected the sensitivity of the colored reaction product and were investigated over the ranges of 10 - 90 cm and 0.508 - 1.521 mm i.d. respectively. The results obtained showed that a coil length with the internal diameter of 20 cm and 0.635 mm respectively, gave the highest sensitivity and was used in all subsequent experiments (Figure 14).

3.1.7.3 Injection Volume

The volume of the injected sample was varied between 100 - 500 μL using different length sample loop. The results obtained indicated that injection of 300 μL sample gave the highest sensitivity and was used in all subsequent experiments (Figure 15).

Table 2 Effect of flow rate of reaction and carrier stream on sensitivity

Flow rate of reaction (mL min^{-1})	Sensitivity* (mV/µg mL^{-1})	Correlation coefficient (r^2)	Flow rate of carrier (mL min^{-1})	Sensitivity* (mV/µg mL^{-1})	Correlation coefficient (r^2)
0.6	0.0814	0.9995	0.4	0.1014	0.9969
0.8	0.0911	0.9988	0.6	0.1016	0.9998
1.2	0.1023	0.9985	0.8	0.1018	0.9950
1.6	0.1016	0.9982	1.2	0.1000	0.9963
2.0	0.1012	0.9998	1.6	0.0970	0.9982
2.4	0.1011	0.9921			

*Mean of three determinations

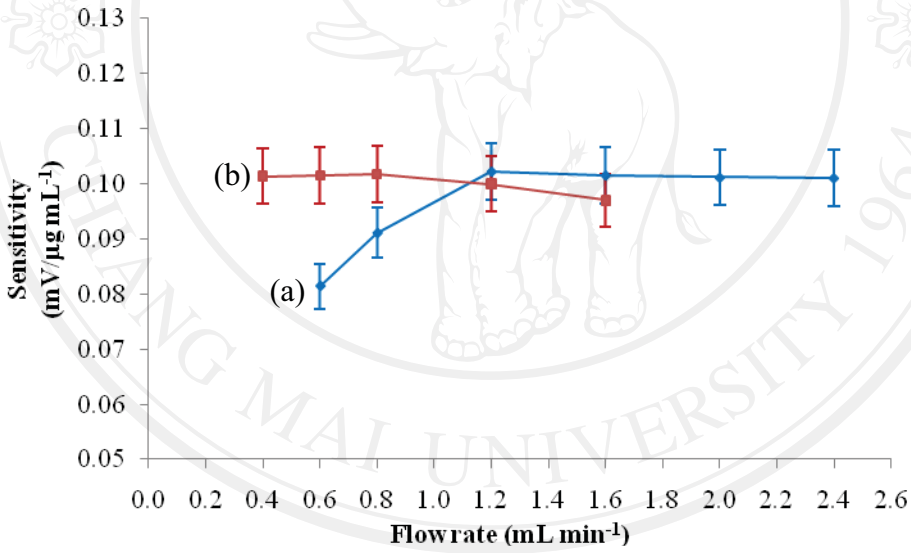


Figure 13 Effect of flow rate of (a) reaction and (b) carrier streams on the sensitivity of calibration curve (n = 3) of 1 - 15 µg mL^{-1} arbutin standard solutions

Table 3 Effect of mixing coil length and mixing coil internal diameter on sensitivity

Mixing coil length (cm)	Sensitivity* (mV/μg mL^{-1})	Correlation coefficient (r^2)	Mixing coil internal diameter (mm)	Sensitivity* (mV/μg mL^{-1})	Correlation coefficient (r^2)
10	0.0797	0.9980	0.508	0.0923	0.9982
20	0.0797	0.9975	0.635	0.1016	0.9998
30	0.0762	0.9999	1.521	0.0867	0.9957
60	0.0652	0.9991			
90	0.0498	0.9957			

*Mean of three determinations

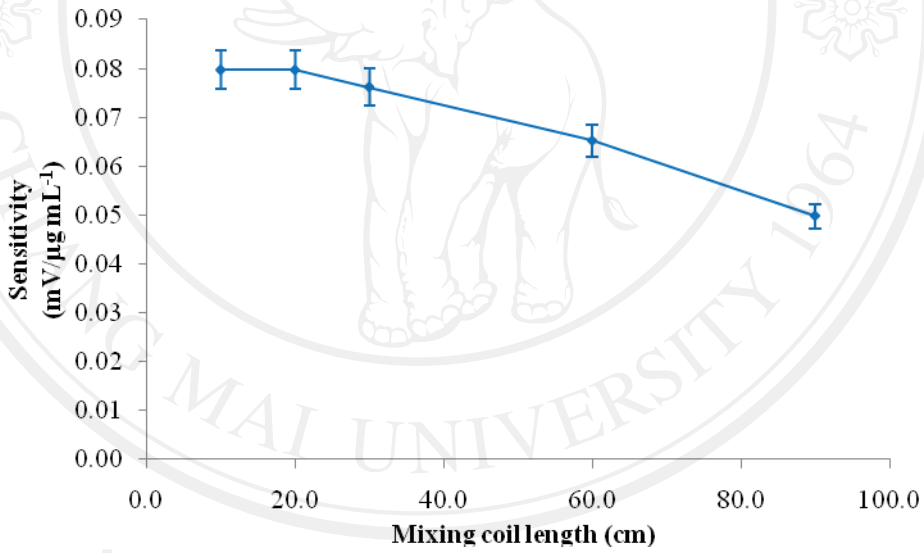


Figure 14 Effect of mixing coil on the sensitivity of calibration curve (n = 3) of 1.0 - 15.0 μg mL^{-1} arbutin standard solutions

Table 4 Effect of injection volume on sensitivity

Injection volume (μL)	Sensitivity* (mV/μg mL^{-1})	Correlation coefficient (r^2)
100	0.0752	0.9998
200	0.0961	0.9988
300	0.1110	0.9999
400	0.1047	0.9994
500	0.1001	0.9979

*Mean of three determinations

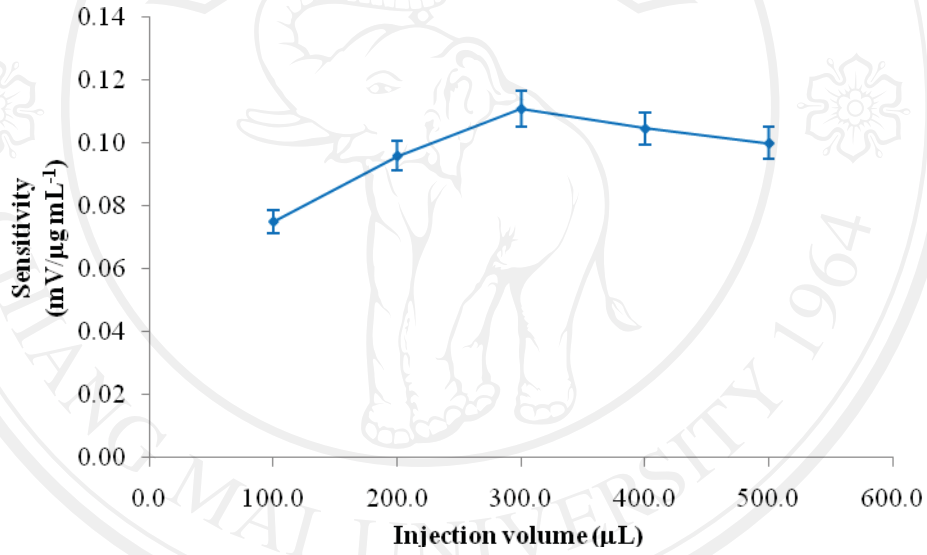


Figure 15 Effect of injection volume on the sensitivity of calibration curve (n = 3) of 1 - 15 μg mL^{-1} arbutin standard solutions

3.1.8 Summary of the Optimum Conditions

The selected optimum conditions for arbutin determination were achieved by univariate method. The performance data for the optimization is shown in Table 5.

Table 5 The optimum conditions for determination of arbutin

Parameter Studies	Range Studies	Optimum
Wavelength (nm)	200 - 700	514
4-AP concentration (mol L^{-1})	1.0×10^{-6} - 5.0×10^{-1}	1.0×10^{-2}
Hexacyanoferrate (III) concentration (mol L^{-1})	1.0×10^{-4} - 1.0	1.0×10^{-2}
Type of alkaline medium	Na_2CO_3, NaOH	Na_2CO_3
Sodium carbonate (%, w/v)	0.1 - 2.0	1.0
Flow rate of 4-AP (mL min^{-1})	0.6 - 2.4	1.2
Flow rate of hexacyanoferrate (mL min^{-1})	0.6 - 2.4	1.2
Flow rate of borate buffer (mL min^{-1})	0.4 - 1.6	0.8
Mixing coil length (cm)	10 - 90	20
Mixing coil I.D. (mm)	0.508 - 1.521	0.635
Sample injection volume (μL)	100 - 500	300

3.1.9 Analytical Characteristics for Arbutin Determination

Analytical characteristics for determination of arbutin were studied under the selected experimental conditions in Table 5.

3.1.9.1 Linearity of Calibration Curve

Using the proposed FI manifold for determination of arbutin under the optimum conditions, the linear calibration curve over the range of 1.0 - 30.0 μg mL^{-1} arbutin was established which can be expressed by the regression equation; $y = 0.2186 \pm 0.0036x + 0.1196 \pm 0.0366$ ($r^2 = 0.9981$, n = 5)

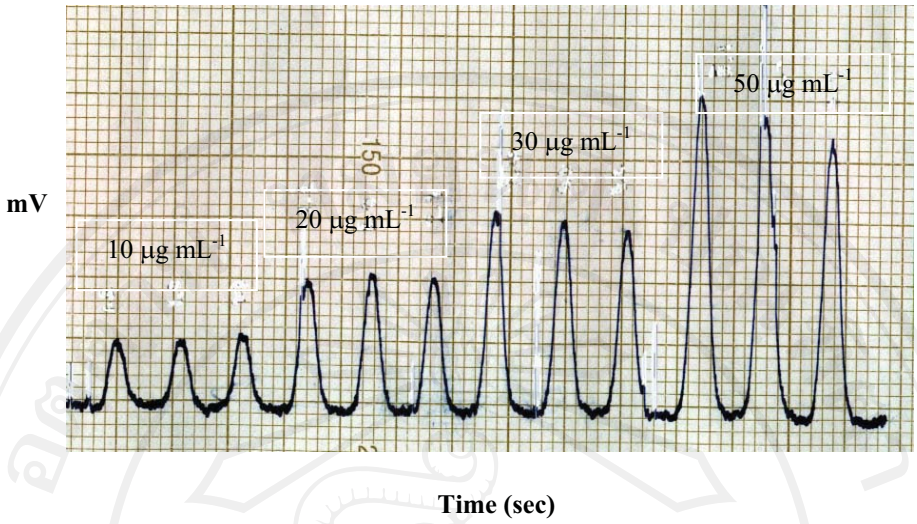


Figure 16 FI grams of arbutin standard

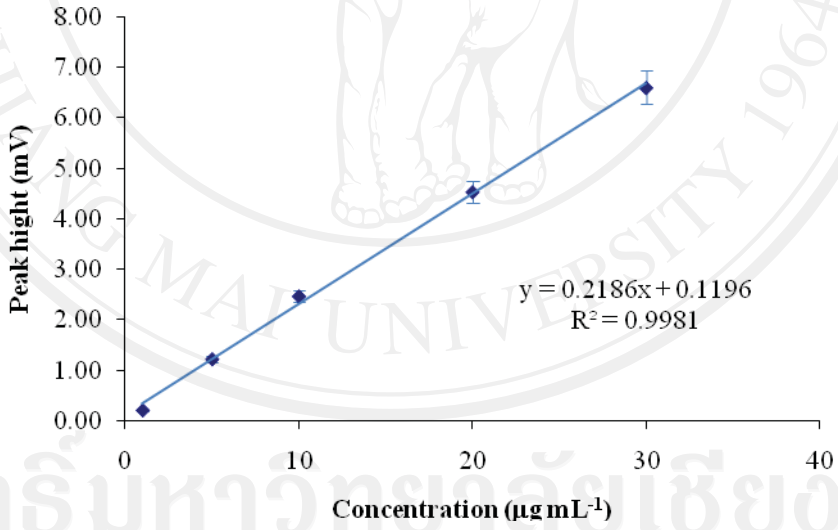


Figure 17 Calibration curve of arbutin standard

3.1.9.2 Sensitivity, Detection Limit and Quantitation Limit

The detection limit (LOD) is defined as the concentration of analyte that gives the signal that different from the blank by an amount equal to three times the standard deviation of blank signal (3σ). It was found to be 0.04 µg mL^{-1}. The quantitation limit (LOQ) is defined as the concentration of analyte that gives the signal that

different from the blank by an amount equal to ten times the standard deviation of blank signal (10σ). It was found to be 0.13 μg mL^{-1}. The molar absorption coefficient was found to be 3.15×10^3 L mol^{-1} cm^{-1}.

3.1.9.3 Interferences

The effects of some possible excipients in commercial formulation (glucose, tween 80, sorbital, sodium metabisulphite, methyl paraben and propyl paraben) were investigated for the maximum ratio of interfering species to the analyte concentration. Synthetic sample solutions containing 20.0 μg mL^{-1} arbutin and different concentrations of interferences were tested, and the peak heights obtained were recorded. Interestingly, glucose, tween 80 and sorbital had no significant effect on the determination of arbutin. The most serious interferences were from the used preservatives compounds, methyl paraben and propyl paraben (Table 6). However, these preservatives must not be used over than 1.0% in cosmetic preparations [(50)] the organic and organic salt interferences were removed in the step of the extraction. Effect of some interference in peel pear extracts (Quercetin, catechin, chlorogenic acid, caffeic acid, p-coumaric acid, and rutin) were investigated[(51)]. The maximum concentration ratio of the interfering species was analyzed. The result showed that these interferences had no significant effect on the determination of arbutin, because some interference in this studied are not soluble in cold water and were removed in the extraction step.

Table 6 The effect of interferences on the peak height (mV) of 20.0 μg mL^{-1} arbutin standard solution

Interferences	Maximum tolerable concentration ratio* (arbutin : interference)
Glucose	20:500
Tween 80	20:750
Sorbital	20:500
Sodium metabisulphite	20:500
Methyl paraben	20:20
Propyl paraben	20:20

*Maximum ratio test

3.1.9.4 Reproducibility and Accuracy

The relative standard deviation of the proposed method (peak height in mV) obtained by five replicate injections of 5.0 and 20.0 μg mL^{-1} of arbutin for intra-day and inter-day were found to be 1.24 - 1.40% and 1.70-2.65%, respectively (Table 7). The recoveries were determined with the standard addition method. Arbutin (5.0 and 20.0 μg mL^{-1}) were added and mixed with 2.00 g of a fine powder material and 0.10 g of whitening creams. The sample was extracted and analyzed using the proposed method. The percentage accuracy of the method was determined in term of recovery of the added standard arbutin. The mean percentage recoveries were found to be 92.00-99.25, respectively (Table 8), indicating an agreement between the true values and the values found.

Table 7 Intra-day and inter-day variability of the FI spectrophotometric determination of arbutin

Samples	Intra-day variability*		Inter-day variability*	
	5 μg mL^{-1}	20 μg mL^{-1}	5 μg mL^{-1}	20 μg mL^{-1}
Chinese pear	5.00	20.00	5.02	19.80
	4.95	19.80	4.83	19.80
	5.00	20.00	4.65	19.60
	4.95	19.80	5.02	19.80
	5.10	20.40	5.02	19.00
	5.00	20.00	4.83	19.60
	4.90	19.60	4.83	19.20
	5.00	20.00	5.02	20.00
	5.10	20.40	4.83	19.20
	5.00	19.60	4.83	19.80
Average	5.00	19.96	4.89	19.58
S.D	0.06	0.28	0.13	0.33
%R.S.D	1.24	1.40	2.65	1.70

*Mean of five determinations

Table 8 Comparison of accuracies for arbutin determination by FIA and HPLC methods

Samples	FIA proposed method				HPLC method[52]	*t*-values[a]
	Arbutin content (%,w/w)	Added ($\mu g\ mL^{-1}$)	Found ($\mu g\ mL^{-1}$)	%Recovery*	Arbutin content (%,w/w)	
Ya Pear	0.02	5	4.94	98.80	0.02	0.54
		20	19.85	99.25		
Fragrant Pear	0.07	5	4.76	95.20	0.07	0.73
		20	19.42	97.10		
Early-Matured pear	0.03	5	4.87	97.40	0.03	0.48
		20	19.39	96.95		
Chinese pear	0.08	5	4.89	97.80	0.08	0.56
		20	19.67	98.35		
Shiseido cream	6.86	5	4.75	95.00	7.02	1.94
		20	19.50	97.50		
Aunyamanee cream	0.30	5	4.82	96.40	0.30	0.72
		20	19.58	97.90		
Arbuwhite cream	0.30	5	4.79	95.80	0.31	1.65
		20	19.55	97.75		

* Mean of three determinations

[a] Tabulated *t*-value for $P = 0.05$ and four degrees of freedom is 2.7764.

Table 9 Comparison of the proposed FI method with selected earlier reported methods

Techniques	Samples	Linear range ($\mu g\ mL^{-1}$)	Correlation coefficient (r^2)	Limit of detection; LOD ($\mu g\ mL^{-1}$)	Limit of quantitative; LOQ ($\mu g\ mL^{-1}$)	References
High performance liquid chromatography	Skin whitening cosmetics	0.0375-0.60	0.9999	0.037	-	(52)
High performance liquid chromatography	Skin whitening cosmetics	100.0-500.0	0.9998	1.0	15.0	(53)
Capillary electrophoresis microchips	Cosmetic	-	0.990	3.0	10.0	(54)
Indirect Spectrophotometry (oxidation by periodate and complexation with ferric chloride)	Skin whitening agents	25.0-125.0	0.9778	-	25.0	(55)
Proposed FI method	Medicinal plants (pear) and commercial whitening creams	1.0-30.0	0.9990	0.04	0.13	proposed method

3.1.10 Analytical Applications

The proposed FI method was successfully applied to the determination of arbutin in whitening creams (C(1), C(2) and C(3)) and medicinal plants (Ya pear, Fragrant pear, Early-Matured pear and Chinese pear). The mean contents of arbutin in whitening creams were found to be 6.86, 0.30 and 0.31% (w/w), respectively. The mean contents of arbutin in medicinal plants were found to be 0.016, 0.068, 0.027 and 0.074% (w/w), respectively. The assay results are present in Table 8.

The assay results for arbutin content in samples obtained using the proposed FIA method and the high performance liquid chromatographic method[52] were compared by applying the paired *t*-test[56]. The calculated *t*-value for arbutin content is less than tabulated *t*-value at the 95% confidence level. Therefore there is no significant difference in a determined content of arbutin by both methods.

The sensitivity of the proposed FI method is compared with the published analytical methods. It is found that the proposed FI method is as sensitive as the HPLC method[52]. It is also more sensitive than the HPLC method[53], capillary electrophoresis[54] and indirect spectrophotometry[55] for determining skin whitening cosmetics as shown in Table 9.

3.1.11 Flow Injection Analysis of Arbutin

A novel flow injection (FI) method using the 4-aminoantipyrine (4-AP) in hexacyanoferrate (III) solution was developed. Appropriate FI conditions for the quantitative analysis of arbutin were achieved. A simple flow injection (FI) manifold with spectrophotometric detection was fabricated and tested for arbutin determination. It is based on the measurement at 514 nm of a red colored product formed by the complexation reaction between arbutin and 4-aminoantipyrine (4-AP) in the presence of potassium hexacyanoferrate (III) in an alkaline medium. The method was successfully applied to the determination of arbutin in four selected fruits and three commercial whitening creams extracts. There were no interference effects from some common excipients used in medicinal plants and commercial whitening creams. The method was simple, rapid, selective, accurate, reproducible and relatively inexpensive.

3.2 Flow Injection Analysis of Curcuminoids in Medicinal Plants and Pharmaceutical Formulations

3.2.1 Preliminary Study

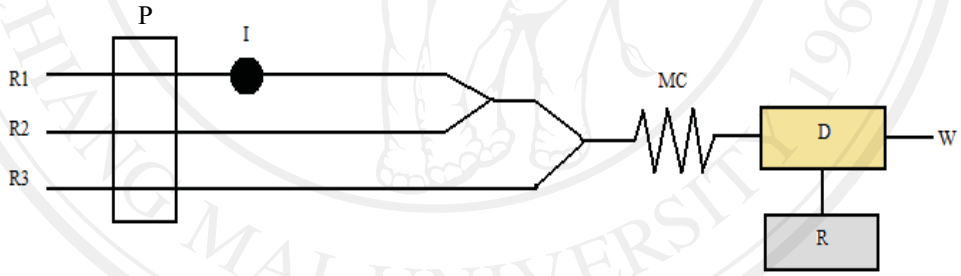
Initially, a three line FI manifold was used for testing the reactivity of curcuminoids and reagent solution. Standard solution of curcuminoids (5 μg mL^{-1}) was injected into a water carrier stream, whilst the reagent and oxidant streams were 4-AP (1.0×10^{-2} mol L^{-1}) and potassium hexacyanoferrate (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 colored 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 optimize 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 Figure 18 (c). It is preferable to inject the sample into the buffer solution before merging with the reagent streams because it gave the better sensitivity.

3.2.2 Manifold Design

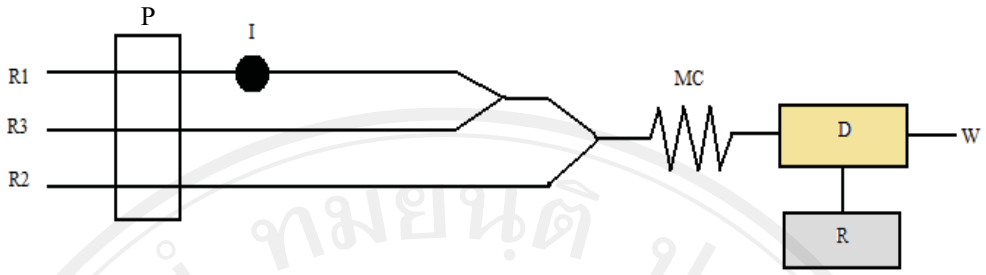
The FI configuration used for the determination of curcuminoids was then designed and fabricated. A simple three channel FI system was employed. The first FI configuration in Figure 18 (a) was used in which the curcuminoids standard solution was injected into the carrier streams containing 2.5×10^{-2} mol L^{-1} borate buffer solution and 1.0×10^{-1} mol L^{-1} 4-AP and then 1.0×10^{-3} mol L^{-1} potassium hexacyanoferrate (III) solution was used as the reagent streams. Both streams were operated at the same flow rate at a flow rate of 0.8 mL min^{-1}. Next, the FI configuration was adopted in Figure 18 (b) and was used in which the curcuminoids standard solution was injected into the carrier streams containing 2.5×10^{-2} mol L^{-1} borate buffer solution and 1.0×10^{-3} mol L^{-1} potassium hexacyanoferrate (III) solution and then 1.0×10^{-1} mol L^{-1} 4-AP was used as the reagent streams. Both streams were

operated at the same flow rate at a flow rate of 0.8 mL min^{-1}. Finally, the FI configuration was adopted in Figure 18 (c) and was used in which the curcuminoids standard solution was injected into the carrier streams containing 2.5 × 10^{-2} mol L^{-1} borate buffer solution and then 1.0 × 10^{-1} mol L^{-1} 4-AP and 1.0 × 10^{-3} mol L^{-1} potassium hexacyanoferrate (III) solution were used as the reagent streams. Both streams were operated at the same flow rate at a flow rate of 0.8 mL min^{-1}

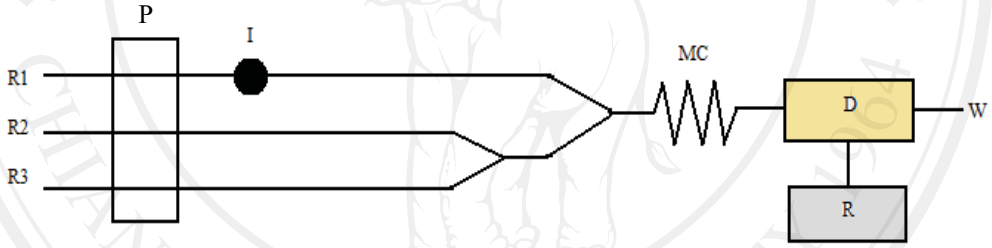
In the position specified in the figure, a standard or sample volume (I) of 100 µL was selected by the sample loop. The solutions were mixed at a coiled reactor which was made from the PTFE tubing (i.d. 0.635 mm and 60 cm in length). The chemical reacts inside the reactor formed a red coloured product. Passage of the coloured compound through the flow cell of spectrophotometer (D) resulted in a transient absorbance that was recorded (R) as a peak with a height proportional to the curcuminoids content in the sample solution.



(a) The curcuminoids standard solution was injected into the carrier streams containing 2.5 × 10^{-2} mol L^{-1} borate buffer solution and 1.0 × 10^{-4} mol L^{-1} 4-AP and then 1.0 × 10^{-3} mol L^{-1} hexacyanoferrate (III) solution was used as the reagent streams with a flow rate of 0.8 mL min^{-1}.



(b) The curcuminoids standard solution was injected into the carrier streams containing 2.5×10^{-2} mol L^{-1} borate buffer solution and 1.0×10^{-3} mol L^{-1} potassium hexacyanoferrate (III) solution and then 1.0×10^{-1} mol L^{-1} 4-AP was used as the reagent streams with a flow rate of 0.8 mL min^{-1}.



(c) The curcuminoids standard solution was injected into the carrier streams containing 2.5×10^{-2} mol L^{-1} borate buffer solution and then 1.0×10^{-1} mol L^{-1} 4-AP and 1.0×10^{-3} mol L^{-1} potassium hexacyanoferrate (III) solution were used as the reagent streams with a flow rate at a flow rate of 0.8 mL min^{-1}

Figure 18 Schematic configuration of the FIA systems: R1; carrier stream of borate buffer solution, R2; 4-AP solution, R3; potassium hexacyanoferrate (III) solution, P; peristaltic pump, I; injection valve, MC; reaction coil, D; detection, R; recorder, W; waste.

Results indicated that the maximum sensitivity was obtained when the standard or sample solution was injected into carrier streams of borate buffer solution and 4-AP solution and then mixed with the reagent streams of potassium hexacyanoferrate (III) solution before delivery to the detector. Therefore this was chosen for subsequent studies.

3.2.3 Absorption Spectra of Curcuminoids

Curcuminoids reacts with 4-AP and potassium hexacyanoferrate (III) resulting in a red-colored. The product presents absorption at 425 nm whereas the curcuminoids exhibits its absorption maximum at 456 nm under the same experimental conditions (Figure 19).

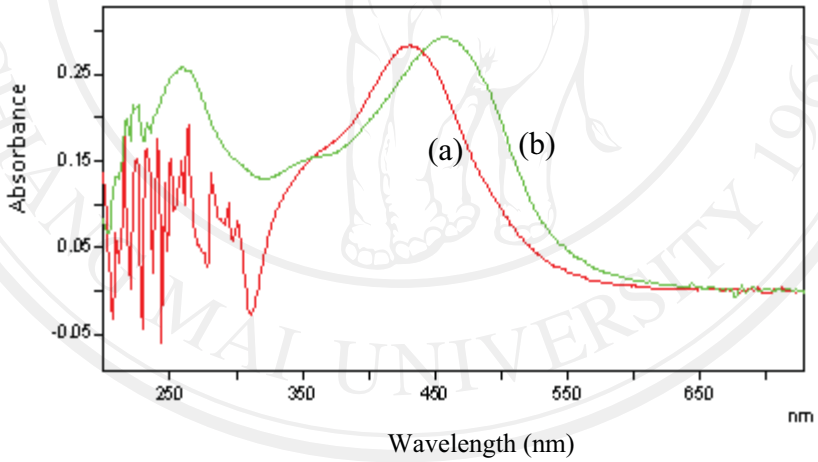
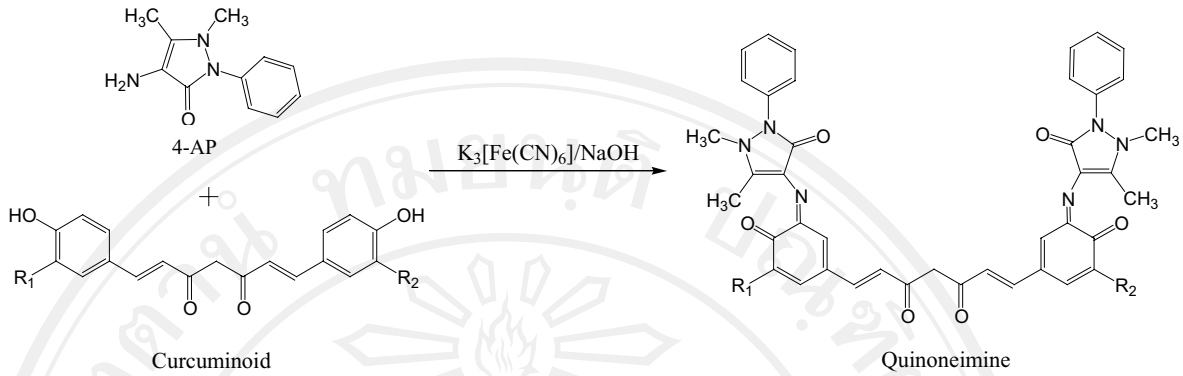


Figure 19 Absorption spectra of (a) curcuminoids (λ 425 nm) and (b) curcuminoids and 4-AP and potassium hexacyanoferrate in an alkaline solution (λ 456 nm)



Scheme 2 Assumed scheme of reaction between curcuminoids and 4-AP and formation of a highly colored quinoneimine

3.2.4 Chemical Reaction

It has been reported that phenols react with 4-AP in the presence of alkaline potassium hexacyanoferrate (III) as oxidizing agent yielding N-substituted quinoneimine, which in turn is known to spontaneously react with phenolic compounds, leading to a red-colored 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 2.

3.2.5 Delivery Order of Reagent and Sample

In FIA measurements for determination of curcuminoids that involve multiple reagents, it is necessary to optimize the delivery order of reagents and sample. There are two possibilities to sandwich the sample; sample-4-AP-potassium hexacyanoferrate (III), sample-potassium hexacyanoferrate (III)-4-AP and 4-AP-potassium hexacyanoferrate (III)-sample. Interchanging the order of the two reagents and sample zone was studied. Apparently, the order 4AP-potassium hexacyanoferrate (III)-sample was chosen (Table 10).

Table 10 Effect of delivery order of reagents and sample

Aspiration order	Sensitivity*; (mV/μg mL^{-1})
sample- 4-AP - potassium hexacyanoferrate (III)	3.0
sample - potassium hexacyanoferrate (III) - 4-AP	0.4
4AP - potassium hexacyanoferrate (III) - sample	8.1

*Mean of three determinations

3.2.6 Optimization of Experimental Variables

The optimization of experimental conditions was carried out by means of a univariate method. The optimum was investigated by injecting 100 μL of 1.0, 5.0, 10.0 and 15.0 μg mL^{-1} curcuminoids standard solutions into the carrier stream of buffer solution and 4-AP solution and then reagent stream of potassium hexacyanoferrate (III) solution with the same flow rate 0.8 mL min^{-1}, respectively. The optimized parameters included the reagent concentrations and some physical variables, including the flow rate and the sample injection volume

3.2.6.1 The Effect of Alkaline Media

The effect of alkaline media added to potassium hexacyanoferrate (III) solution was initially examined (Table 11). Various alkaline media such as Na_2CO_3, NaOH and KOH solutions at the same concentration and various concentrations of all alkaline solutions (0.5 - 1.5%) 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 (Figure 20).

3.2.6.2 The Effect of 4-AP Concentration

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 (1.0 - 15.0

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.

Table 11 Effect of alkaline media added to hexacyanoferrate (III) solution

Curcuminoids concentration (μg mL^{-1})	Peak height* (AU)		
	Na_2CO_3	NaOH	KOH
1.0	0.0002	0.0008	0.0026
5.0	0.1158	0.1347	0.1032
10.0	0.2998	0.3718	0.2446
15.0	0.4941	0.6174	0.3963
Sensitivity*; (AU/μg mL^{-1})	0.0446	0.0356	0.0282
Correlation coefficient	0.9943	0.9965	0.9985

*Mean of three determinations

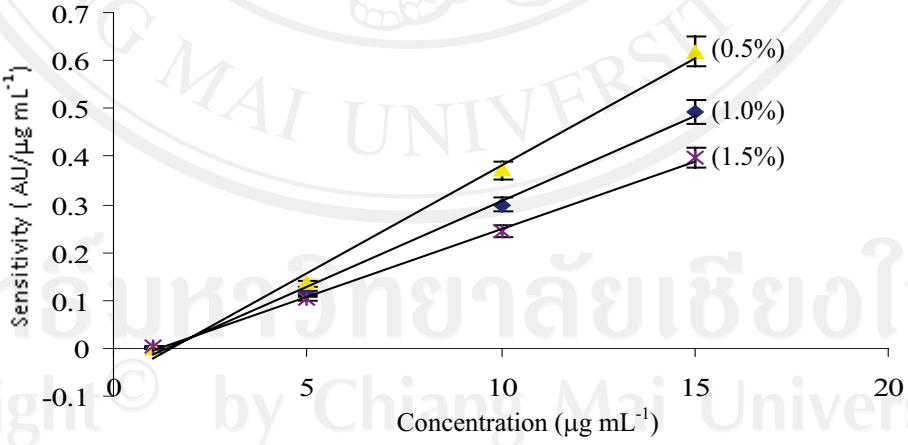


Figure 20 The effect of concentrations of alkaline media added to potassium hexacyanoferrate (III)

3.2.6.3 The Effect of Potassium Hexacyanoferrate (III) Concentration

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 (1.0 - 15.0 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.

3.2.6.4 The pH Effect of Buffer Solution

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 of pH 8-10. Curcuminoids standard solutions (1.0 - 15.0 μg mL^{-1}) were injected into a water 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 9.4. For further increments of the pH, the absorbance increases very slightly and remains almost constant. Thus, a pH of 9.4 was chosen as appropriate pH for further investigations, due to reproducibility and sensitivity.

Table 12 Effect of 4-AP and hexacyanoferrate (III) solutions

4-AP concentration (mol L^{-1})	Sensitivity*; (AU/ µg mL^{-1})	Correlation coefficient (r^2)	Hexacyanoferrate (III) concentration (mol L^{-1})	Sensitivity*; (AU/µg mL^{-1})	Correlation coefficient (r^2)
1.0×10^{-4}	0.2694	0.9899	1.0×10^{-4}	2.4003	0.9951
1.0×10^{-3}	0.1710	0.9812	1.0×10^{-3}	2.6256	0.9999
1.0×10^{-2}	2.0613	0.9778	1.0×10^{-2}	2.0197	0.9876
1.0×10^{-1}	2.5285	0.9737	1.0×10^{-1}	0.9227	0.9808
1.0	2.3390	0.9854	1.0	0.7745	0.9818

*Mean of three determinations

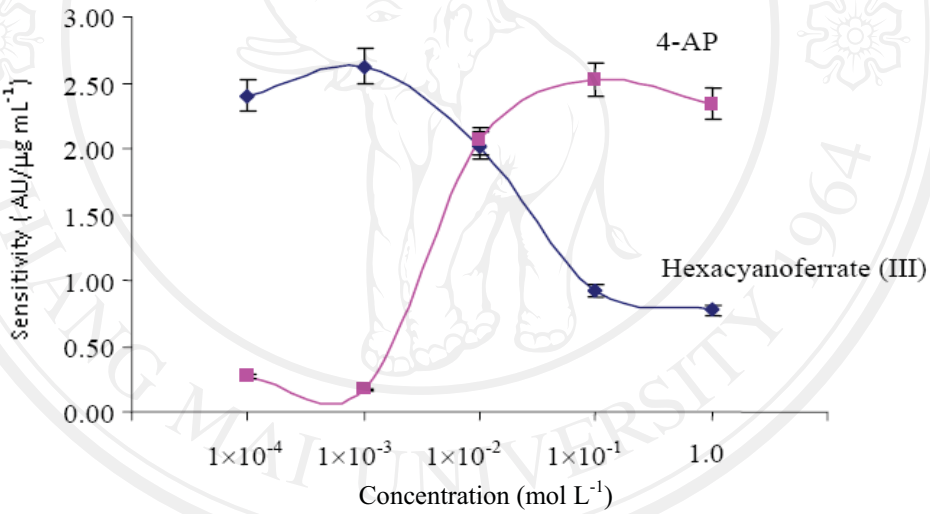


Figure 21 Effect of 4-AP concentrations and hexacyanoferrate (III) concentrations on the mean of sensitivity (slope) of 1.0 - 15.0 µg mL^{-1} curcuminoids standard solutions

Table 13 Effect of pH of borate buffer solution on sensitivity

pH of buffer solution	Sensitivity*; (AU/µg mL^{-1})	Correlation coefficient (r^2)
8.0	1.7738	0.8796
8.2	1.9277	0.9269
8.4	1.9050	0.9444
8.6	1.9288	0.9786
8.8	1.8822	0.9534
9.4	1.9673	0.9733
9.6	1.9537	0.9708
9.8	1.8500	0.9870
10.0	1.6657	0.9890

*Mean of three determinations

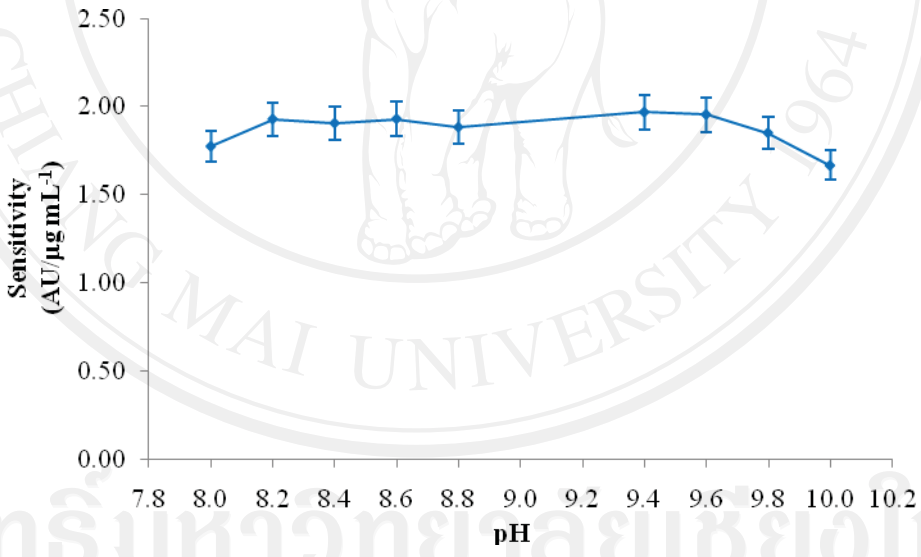


Figure 22 The effect of pH on carrier stream

3.2.7 Optimization 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.

3.2.7.1 The Effect of Mixing Coil Diameter and Length

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 20 - 90 cm. Thus, a-60 cm tubing is considered to be suitable due to its good sensitivity and precision.

Table 14 Effect of mixing coil length and internal diameter on sensitivity

Mixing coil length (cm)	Sensitivity* (AU/μg mL^{-1})	Correlation coefficient (r^2)	Mixing coil internal diameter (mm)	Sensitivity* (AU/μg mL^{-1})	Correlation coefficient (r^2)
20	1.1189	0.9972	0.508	0.1923	0.9977
30	2.0060	0.9983	0.635	0.2016	0.9984
60	2.4671	0.9988	1.521	0.1867	0.9965
90	2.1021	0.9973			

*Mean of three determinations

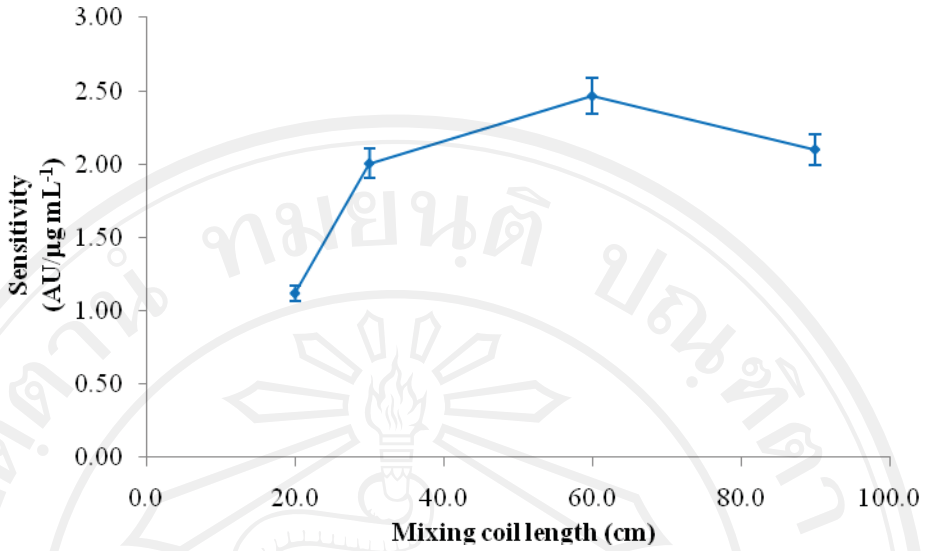


Figure 23 The influence of the tubing length for making the mixing coil

3.2.7.2 The Effect of Flow Rate

The flow rate is one of the most important parameters to be optimized 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}, respectively. 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.

Table 15 Effect of flow rate, carrier and reagent streams on sensitivity

Flow rate of carrier (mL min^{-1})	Sensitivity*; (AU/µg mL^{-1})	Correlation coefficient (r^2)	Flow rate of reagent (mL min^{-1})	Sensitivity*; (AU/µg mL^{-1})	Correlation coefficient (r^2)
0.5	1.0716	0.9978	0.5	0.8409	0.9973
0.8	1.6753	0.9971	0.8	1.8840	0.9952
1.3	1.6094	0.9954	1.3	1.6389	0.9973
1.7	1.4959	0.9945	1.7	1.4336	0.9972
2.0	1.4554	0.9825	2.0	1.3232	0.9974
2.5	1.4181	0.9820	2.5	1.2074	0.9969

*Mean of three determinations

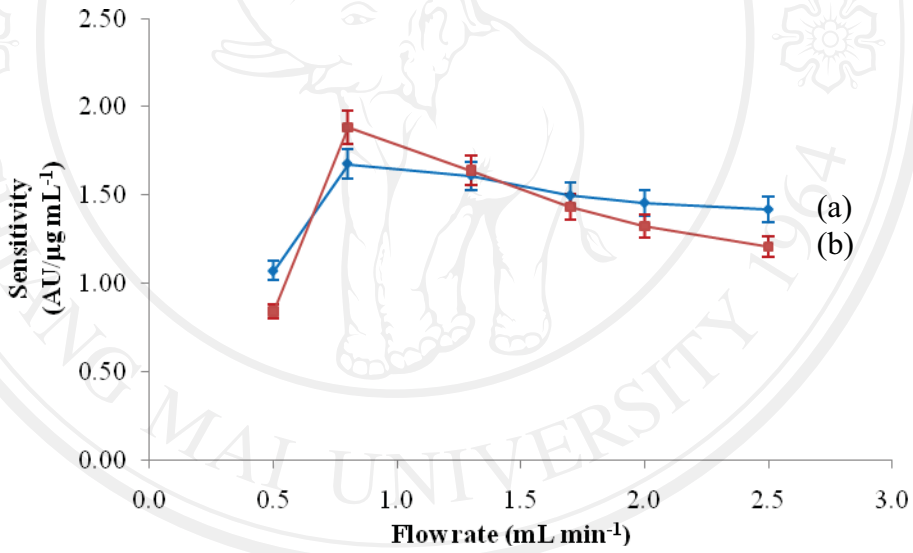


Figure 24 The effect of flow rate for carrier stream (a) and reagent stream (b)

3.2.7.3 The Effect of Injection Volume

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.

Table 16 Effect of injection volume on sensitivity

Injection volume (µL)	Sensitivity*; (AU/µg mL^{-1})	Correlation coefficient (r^2)
10	1.3378	0.9981
20	2.2524	0.9938
40	2.9782	0.9940
50	3.1189	0.9789
100	3.5346	0.9904

*Mean of three determinations

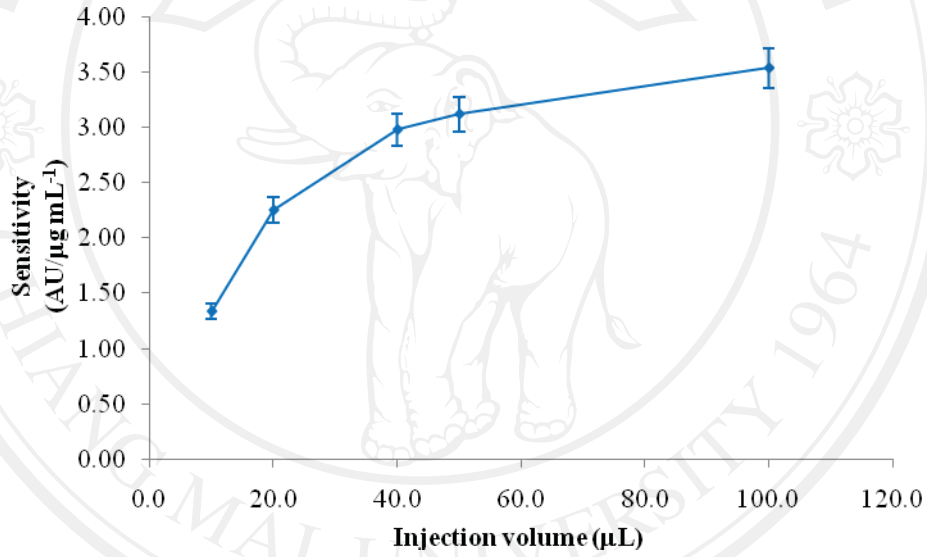


Figure 25 The influence of injection volume on absorbance sensitivity

3.2.8 Summary of the Optimum Conditions

The selected optimum conditions for curcuminoids determination were achieved by so-called univariate method. The performance data for the optimization is shown in Table 17.

3.2.9 Analytical Characteristics for Curcuminoids Determination

Under the selected experimental conditions (Table 17) and using the FI system as illustrated in Figure 18 (c), the linear calibration range, the detection limit (LOD) and the quantitation limit (LOQ) for the determination of curcuminoids were investigated.

3.2.9.1 Linearity of Calibration Curve

The linearity of calibration curve was determined using the optimal experimental parameters in Table 17. Linear calibration curve of curcuminoids over the concentration range of 5.0 - 50.0 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; A = 0.003C - 0.0053 (r^2 = 0.9997).

Table 17 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)	0.5 - 1.5	0.5
pH of Borate buffer solution	8.0 - 10.0	9.4
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

Table 18 Analytical characteristics for curcuminoids determination

Analytical characteristics	FI proposed method
Linear regression equation [a]	A = 0.003C-0.0053
Linear range (μg mL^{-1})	5.0 - 50.0
Correlation coefficient	0.9997
LOD [b] (μg mL^{-1})	0.60
LOQ [c] (μg mL^{-1})	1.80

[a] The number of data for each calibration graph correspond to eight different concentration levels, with five replicates for each level

[b] Limit of detection calculated according to the IUPAC definitions: $3S_0/S$, when S is the slope of the standard curve

[c] Limit of quantitation calculated according to the IUPAC definitions: $10S_0/S$

3.2.9.2 Sensitivity, Detection Limit and 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 5.0 - 50.0 μg mL^{-1} with the correlation coefficient of 0.9997. The detection limit (3σ) and the quantitation limit (10σ) were found to be 0.6 μg mL^{-1} and 1.8 μg mL^{-1} respectively.

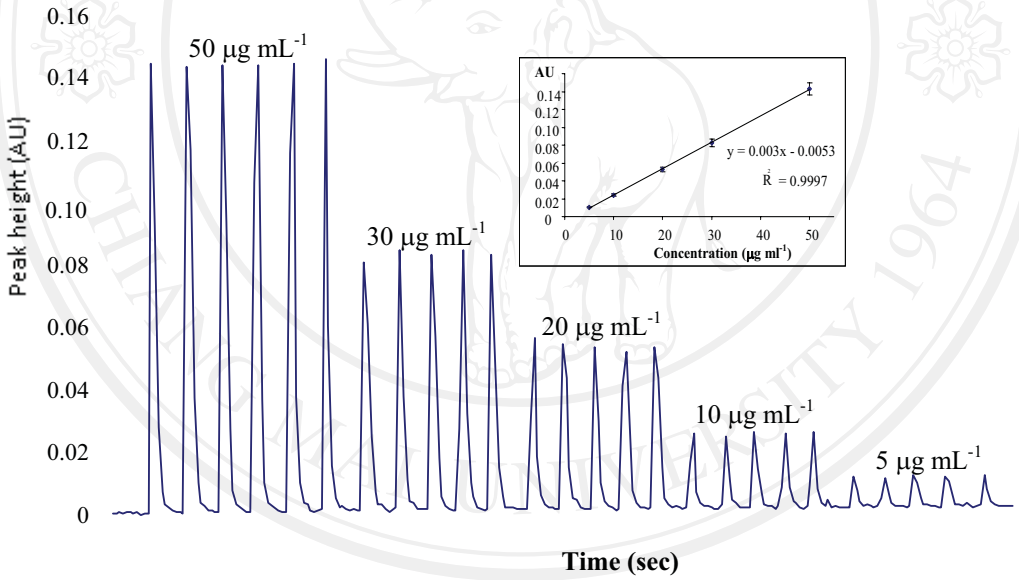


Figure 26 Calibration curve of curcuminoids in concentration range 5.0 - 50.0 μg mL^{-1}

3.2.9.3 Precision and Accuracy

The relative standard deviation of the proposed method (peak height in AU) calculated from 10 replicate injections of 5.0, 10.0 and 15.0 μg mL^{-1} of curcuminoids were found to be 2.09%, 1.08% and 0.79%, respectively. The percentage recoveries were determined with the standard addition method in herb samples. Curcuminoids (5.0, 10.0 and 15.0 μg mL^{-1}) were added and mixed with 0.5000 g of a fine powder of

Turmeric, the samples was extracted and analyzed using the proposed method. The mean percentage recovery of 5.0, 10.0 and 15.0 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.

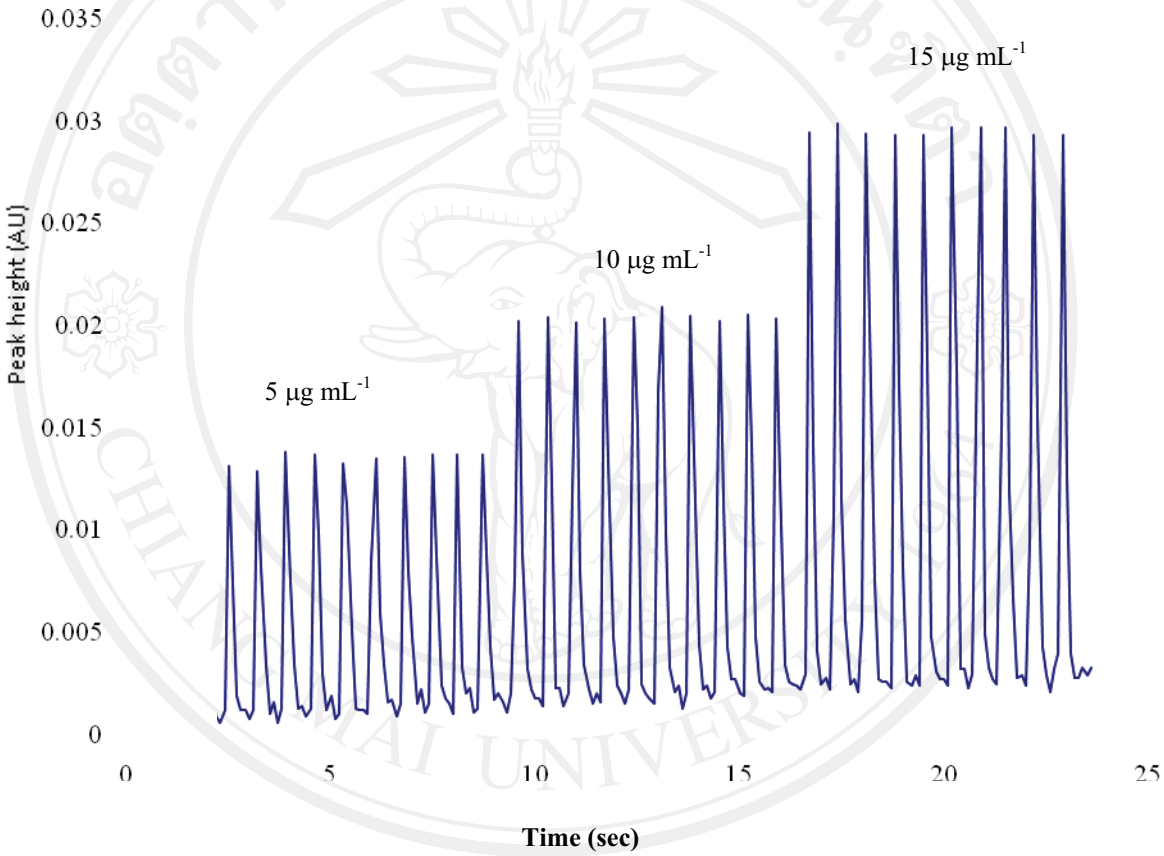


Figure 27 Precision of the proposed FI method

Table 19 Precision of the proposed FI method

Concentration of curcuminoids (μg mL^{-1})	AU mean (n = 10)	S.D	%R.S.D
5	1.33×10^{-2}	2.80×10^{-4}	2.09
10	2.03×10^{-2}	2.21×10^{-4}	1.08
15	2.94×10^{-2}	2.23×10^{-4}	0.79

3.2.10 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 as mentioned above, and the calculated contents of curcuminoids were shown in Table 21. The mean contents of curcuminoids from turmeric purchased from commercial sources in Chiang Mai province (three samples) were found to be 3.4, 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. Results were compared flavorable with the spectrophotometric method[57]. The assay results for total curcuminoids content in turmeric samples (Table 21) obtained using the proposed FIA method and the spectrophotometric method were compared by applying the paired *t*-test[56]. 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 20 Accuracy of proposed FIA method for determination of curcuminoids

Location	Samples	Code	FIA proposed method Curcuminoids content (%,w/w)	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery* (%)
Tonpayorm market, Chiang Mai	Turmeric (*Curcuma longa* L.)	T1	3.30	5	4.93	98.66
				10	9.93	99.33
				15	15.26	101.78
Tonpayorm market, Chiang Mai	Turmeric (*Curcuma longa* L.)	T2	4.14	5	4.83	96.67
				10	9.90	99.00
				15	15.10	100.67
Warorot market, Chiang Mai	Turmeric (*Curcuma longa* L.)	T3	2.67	5	4.97	99.33
				10	9.97	99.67
				15	14.77	98.44
Watyai market, Pitsanulok	Turmeric (*Curcuma longa* L.)	T4	0.92	5	4.87	97.33
				10	9.87	98.67
				15	15.27	101.78
Watyai market, Pitsanulok	Turmeric (*Curcuma longa* L.)	T5	4.34	5	4.93	98.67
				10	9.97	99.67
				15	14.93	99.56
Kokmatoom market, Pitsanulok	Turmeric (*Curcuma longa* L.)	T6	3.78	5	5.07	101.33
				10	9.43	94.33
				15	14.40	96.00
Kokmatoom market, Pitsanulok	Turmeric (*Curcuma zedoaria* (Berg) Roscoe)	T7	1.06	5	5.40	108.00
				10	9.77	97.67
				15	14.73	98.22

*Mean of three determinations

Table 21 Comparative determination of total curcuminoids using the proposed FIA method and spectrophotometric method

No.	Turmeric samples	Total curcuminoids content (%,w/w), (n=3) The proposed FIA method	Spectrophotometric method[57]	*t*-values [a]
1	Turmeric (*Curcuma longa* L.); Tonpayorm market, Chiang Mai	3.3	3.5	1.89
2	Turmeric (*Curcuma longa* L.); Tonpayorm market, Chiang Mai	4.1	4.4	2.34
3	Turmeric (*Curcuma longa* L.); Warorot market, Chiang Mai	2.7	2.7	0.57
4	Turmeric (*Curcuma longa* L.); Watyai market, Phitsanulok	0.9	0.9	0.76
5	Turmeric (*Curcuma longa* L.); Watyai market, Phitsanulok	4.3	4.4	1.24
6	Turmeric (*Curcuma longa* L.); Kokmatoom market, Phitsanulok	3.8	3.8	0.72
7	Turmeric (*Curcuma zedoaria* (Berg) Roscoe); Kokmatoom market, Phitsanulok	1.1	1.1	0.65

[a] Tabulated *t*-value for $P = 0.05$ and four degrees of freedom is 2.7764.

3.2.11 Flow Injection Analysis of Curcuminoids

A simple flow-injection analysis procedure was proposed for the determination of curcuminoids content in turmeric extracts. The method was based on the formation of a colored complex between 4-aminoantipyrine and curcuminoids, in the presence of an oxidizing 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. The proposed system was applied to the determination of curcuminoids content in turmeric. The proposed FI method is simple, economic, rapid and especially suitable for quality control in pharmaceutical plants. 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.

The experimental conditions of FI method were optimized and the final procedure allowed a successful determination of total curcuminoids.

CHAPTER IV

CONCLUSIONS

4.1 Flow Injection Analysis of Arbutin in Medicinal Plants and Pharmaceutical Formulations

A simple flow injection (FI) manifold with spectrophotometric detection was fabricated and tested for arbutin determination. It is based on the measurement at 514 nm of a red colored product formed by the complexation reaction between arbutin and 4-aminoantipyrine (4-AP) in the presence of hexacyanoferrate (III) in an alkaline medium. Injection of 300 μL standard solutions at various concentrations of arbutin into the FI system under optimum conditions, a linear calibration graph over the range of 1.0-30.0 μg mL^{-1} arbutin was established. It is expressed by the regression equation $y = 0.2188 \pm 0.0036x + 0.1019 \pm 0.0366$ ($r^2 = 0.9990$, n = 5). The detection limit (3σ) and the limit of quantitation (10σ) were 0.04 μg mL^{-1} and 0.13 μg mL^{-1} respectively. The R.S.D of intra-day and inter-day precisions were found to be 1.2-1.4% and 1.7-2.7% respectively. The method was successfully applied to the determination of arbutin in four selected fruits and three commercial whitening creams extracts with the mean recoveries of the added arbutin over the range of 96.2-99.0%. The FI method was successfully applied to the determination of arbutin in whitening creams (C(1), C(2) and C(3)) and medicinal plants (Ya pear, Fragrant pear, Early-Matured pear and Chinese pear). The mean contents of arbutin in whitening creams were found to be 6.86, 0.30 and 0.31% (w/w), respectively. The mean contents of arbutin in medicinal plants were found to be 0.016%, 0.068%, 0.027% and 0.074% (w/w), respectively. The assay results are presented in Table 8. Other common excipients (e.g., glucose, tween 80, sorbital, sodium metabisulphite, methyl paraben and propyl paraben) in formulations were tested. They were not cause serious problem on the analysis of the studied arbutin (Table 6). The method was successfully applied to the determination of arbutin in medicinal plants and pharmaceutical formulations with a sample throughput rate of 40 sample h^{-1}.

The assay results for arbutin content in samples obtained using the proposed FIA method and the high performance liquid chromatographic method[52] were compared by applying the paired *t*-test[56]. The calculated *t*-value for arbutin content is less than tabulated *t*-value at the 95% confidence level. Therefore there is no significant difference in a determined content of arbutin by both methods.

The sensitivity of the proposed FI method is compared with the published analytical methods. It is found that the proposed FI method is as sensitive as the HPLC method[52]. It is also more sensitive than the HPLC method[53], capillary electrophoresis[54] and indirect spectrophotometry[55] for determining skin whitening cosmetics as shown in Table 9.

The proposed FI spectrophotometric procedure has been successfully applied to the determination of arbutin in commercial whitening cream and medicinal plants with reasonably accepted detection limit and speed. Sample pretreatment is not necessary. This method is simple, fast, relatively inexpensive, precise, accurate, sensitive, using minimum number of reagents and reaction sequence. The speed, cost effectiveness and the precision make this method suitable for the quality control of arbutin in commercial whitening creams and in some medicinal plants and/or pharmaceuticals.

4.2 Flow Injection Analysis of Curcuminoids in Medicinal Plants and Pharmaceutical Formulations

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 colored complex between 4-aminoantipyrine and curcuminoids, in the presence of an oxidizing 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 5.0 - 50.0 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 analyzed by using the proposed method. The percentage recoveries were found to be 94.3 - 108.0. The FI 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). Results were compared flavorable with the spectrophotometric method[57]. The assay results for total curcuminoids content in turmeric samples (Table 20) obtained using the proposed FIA and the spectrophotometric methods were compared by applying the paired *t*-test[56]. 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.

In this work, the proposed FI spectrophotometric method has presented to be a simple method 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 proposed FI method is simple, economic, rapid and especially suitable for quality control in medicinal plants.