PART III

DEVELOPMENT AND VALIDATION OF MICROFLUIDIC SYSTEMS FOR DETERMINATION OF DRUG RESIDUE IN HONEY SAMPLES COUPLES WITH CHEMILUMINESCENCE DETECTOR AND LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

CHAPTER I

INTRODUCTION

The field of microfluidics combines the fabrication methods of micro and nanotechnology with knowledge about the behavior of fluids on the fundamental microscopic level to give rise to very powerful techniques in controlling and measuring chemical reactions and physical and biological processes on the micro and nanoscale.

In the lab-on-a-chip, a miniaturized fluidic system, measurements or sample manipulations that otherwise requires considerable human involvement and sizeable laboratory equipment and space is performed on a chip that would fit in the palm of a hand. The development of the lab-on-a-chip also bring about possibilities of handling very small volumes, in the pico liter range, bringing about the opportunity to analyze samples that were previously beyond our reach. In addition it has proven to have the capacity to increase both speed and sensitivity.

1.1 The Third Generation-Microfluidic Systems as Micro Total Analysis (μTAS) or Lab-on-a Chip Technique

1.1.1 Lab-On-a-Chip[124]

A lab-on-a-chip (LOC) is a device that integrates one or several laboratory functions on a single chip of only millimeters to a few square centimeters in size. LOCs deal with the handling of extremely small fluid volumes down to less than pico liters. Lab-on-a-chip devices are a subset of microelectromechanical systems (MEMS) devices and often indicated by "Micro Total Analysis Systems" (μTAS) as well. Microfludics is a broader term that describes also mechanical flow control devices like pumps and valves or sensors like flow meters and viscometers. However, strictly regarded "Lab-on-a-Chip" indicates generally the scaling of single or multiple lab processes down to chip-format, whereas "μTAS" is dedicated to the integration of the total sequence of lab processes to perform chemical analysis. The term "Lab-on-

a-Chip" was introduced later on when it turned out that μTAS technologies were more widely applicable than only for analysis purposes.

1.1.1.1 Chip Materials and Fabrication Technologies

The basis for most LOC fabrication processes is photolithography. Initially most processes were in silicon, as these well-developed technologies were directly derived from semiconductor fabrication. Because of demands for e.g. specific optical characteristics, bio- or chemical compatibility, lower production costs and faster prototyping, new processes have been developed such as glass, ceramics and metal etching, deposition and bonding, polydimethylsiloxane (PDMS) processing (e.g., sift lithography, thick-film- and stereolithography as well as fast replication methods via electroplating, injection molding and embossing. Furthermore the LOC field more and more exceeds the borders between lithography-based microsystem technology, nano technology and precision engineering.

1.1.1.2 Advantages of LOCs

LOCs may provide advantages, which are specific to their application. Typical advantages are:

- Low fluid volumes consumption (less waste, lower reagents costs and less required sample volumes for diagnostics)
- Faster analysis and response times due to short diffusion distances, fast heating, high surface to volume ratios, small heat capacities.
- Better process control because of a faster response of the system (e.g. thermal control for exothermic chemical reactions).
- Compactness of the systems due to integration of much functionality and small volumes.
- Massive parallelization due to compactness, which allows high-throughput analysis.
- Lower fabrication costs, allowing cost-effective disposable chips, fabricated in mass production.

- Safer platform for chemical, radioactive or biological studies because of integration of functionality, smaller fluid volumes and stored energies.

1.1.1.3 Disadvantages of LOCs

- Novel technology and therefore not yet fully developed.
- Physical and chemical effects that become more dominant on small-scale sometimes make processes in LOCs behave more complex than in conventional lab equipment (like capillary forces, surface roughness, chemical interactions of construction materials on reaction processes).
- Detection principles may not always scale down in a positive way, leading to low signal-to-noise ratio.
- Although the absolute geometric accuracies and precision in microfabrication are high, they are often rather poor in a relative way, compared to precision engineering for instance.

1.1.2 Molecularly Imprinted Polymers (MIPs)

During the last decade, research interest in molecular imprinted polymers (MIPs) has strongly increased with potential applications ranging from solid phase extraction materials[125-130] to receptors in sensors[131-134] and immuno-like assays[135-137]. Due to their highly selective recognition properties, MIPs are often referred to as artificial antibodies. Moreover, MIPs possess several advantages over their biological counterparts such as low cost, ease of production and good physical and chemical stability.

Molecular imprints have been demonstrated against many classes of compounds. These include drugs, hormones, pesticides, proteins, amino acids, peptides, carbohydrates, coenzyme, nucleotides, nucleotide bases, steroids and dyes[138].

1.1.2.1 Theory of Molecularly Imprinted Polymers

Molecular imprinting is a technique for preparation of polymeric materials that have tailor-made selectivity for the templates molecule. The recognition properties of polymers are resulted from functional group interaction between template molecule

and functional monomer. The type of interaction can be hydrogen bonds, ion paring, π-π interaction or driven by the hydrophobic effect. The affinity increases with the number of interacting groups. However, each individual interaction can be strongly dependent on the properties of the solvent such as protic or aprotic, dielectric constants, presence of complex forming agents, etc.

The selectivity of MIPs in comparison to non-imprinted polymers (NIPs)-same MIPs procedure without using template molecule- can only occur when the analyte and/or the matrix compounds of the sample have an increased number of interaction points. Therefore, single and part of the dual point interactions that take place between the template molecule and the polymer will affect the selectivity.

1.1.2.2 Molecular Imprinting Strategies and Procedures

The procedures for synthesis of MIPs have been described in details which generally use the following strategies. During the molecular imprinting process, highly cross-linked co-polymers are formed around analyte molecules acting as cavity-creating templates. The template molecules are then removed, providing binding sites ideally complementary in size, shape and functionality to the templated molecule. Upon reintroduction of the template preferential rebinding within the cavity should occur.

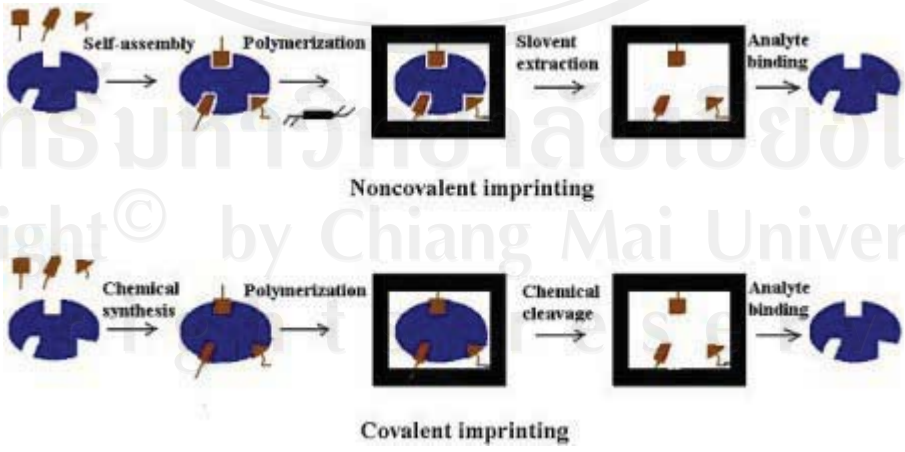


Figure 41 Principle of molecular imprinting

In the pre-polymerization mixture, the dissolved target analyte interacts by covalent, non-covalent or metal coordination interactions which responsible for localizing the chemically active moieties of the target molecules during copolymerization. Consequently, molecular imprinting is classified into two types according to the type of interaction between functional monomer and target molecules in the pre-polymerization as mixture covalent and non-covalent imprinting (Figure 41).

1.1.2.3 Parameters of MIP Syntheses[128, 139]

In the processes of MIP synthesis various parameters such as template molecule, function monomers, cross-linkers, porogens and initiators have to be considered in order to create the selective site to the analyte.

1.1.2.3.1 Template

In the molecular imprinting processes, the template molecule is of central importance that it directs the organization of the functional groups pendent to the functional monomers. Unfortunately, not all templates are directly amenable to templating. In terms of compatibility with free radical polymerization, templates should have the following properties:

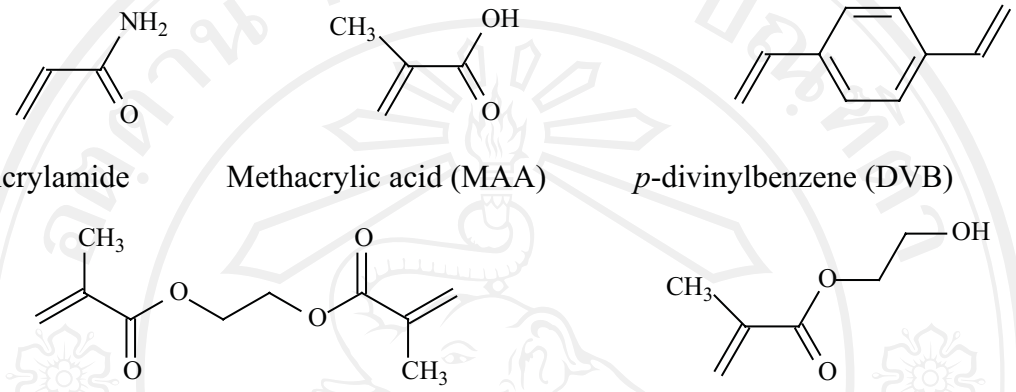
- should be chemically inert under the polymerization conditions
- do not contain any polymerizable groups
- have no functionality that could potentially inhibit a free radical polymerization
- should be stable at initiate temperatures of initiator

A wide variety of template molecules have been used in various imprinting protocols.

1.1.2.3.2 Functional Monomers

In an imprinted polymer the functional monomers are responsible for the binding interactions with the template molecule for pre-polymerization that this creates the binding sites in the imprinted polymer before the assembled complex is trapped with a

cross-linker to form a rigid polymer network. Several functional monomers have been used in molecular imprinting protocols that the most are acrylate-based, acrylamide-based and styrene-based systems. The chemical structures of a selection of the more important functional monomers are shown in Figure 42.



Acrylamide Methacrylic acid (MAA) *p*-divinylbenzene (DVB)

Ethylene glycol dimethacrylate (EGDMA) 2-hydroxyethyl methacrylate (2-HEMA)

Figure 42 The function monomers used in the non-covalent approach

Ethylene glycol dimethacrylate (EGDMA) *p*-divinylbenzene (DVB)

Tetramethylene dimethacrylate (TDMA) 1,4-phenylene diacrylamide

Figure 43 The cross-linkers used for molecular imprinting

1.1.2.3.3 Cross-Linkers

Generally, the high degrees of cross-linker (70-90%) are preferred. The cross-linker has three major functions:

- The cross-linker is important in controlling the morphology of the polymer matrix (gel-type, macroporous or a microgel poeder).
- It serves to stabilize the imprinted binding site.
- It imparts mechanical stability to the polymer matrix.

Originally, isomers of divinylbenzene were used for cross-linker of styrene and other functional monomers into polystyrenes. Recently, acrylate or methacrylate-based systems (*di*-, *tri*- or *tetra*-unsaturated) are commonly employed in several systems. The chemical structures of several cross-linkers are shown in Figure 43.

1.1.2.3.4 Solvents (Porogens)

The solvent plays an important role in the outcome of molecular imprinted processes. The main responsibilities of solvent are as follows:

- It brings all the components in the polymerization, such as template, functional monomer, cross-linker and initiator into one phase.
- It creates the pores in macroporous polymers. For this reason it is quite common to refer to the solvent as the "porogen". When macroporous polymers are being prepared, the nature and the level of the porogen can be used to control the morphology and the total pore volume.
- It is particularly pronounced in self-assembly systems. As porogen in polymerization, the solvent govern the strength of non-covalent interactions.

1.1.2.3.5 Initiators

Polymerization of monomers can be done by various types of mechanisms. In MIPs, free radical chemistry is widely used for polymerization. However, in the polymerization reactions, the templates are included in the prepolymerize mixture. If the template was unstable under photochemical or thermal reaction, the condition

should be triggered thermally and/or photochemically. The chemical structures of selected polymerization initiators are shown in Figure 44.

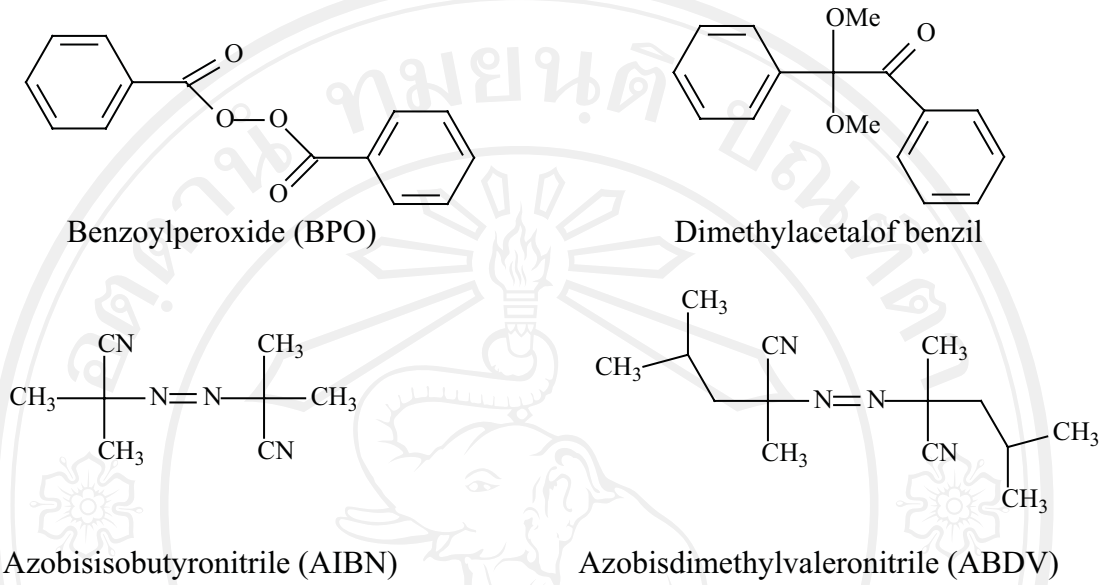


Figure 44 Chemical structures of selected chemical initiators

1.1.3 Chemiluminescence

1.1.3.1 Theory of Chemiluminescence

Chemiluminescence is defined as the emission of electromagnetic radiation by a chemical reaction. Chemiluminescence reactions usually produce a product in an electronic excited state, which produces light on returning to the ground state (S_0)[140]. The route of light emission in chemiluminescence is the same as in photoluminescence (fluorescence, phosphorescence) except for the production of the excited state. In fluorescence and phosphorescence an excited state is produced as a result of the absorption of ultraviolet or visible light. In fluorescence, emission is from a singlet electronically excited state (S_1) and phosphorescence emission is from a triplet electronically excited state (T_1) (Figure 45)[140].

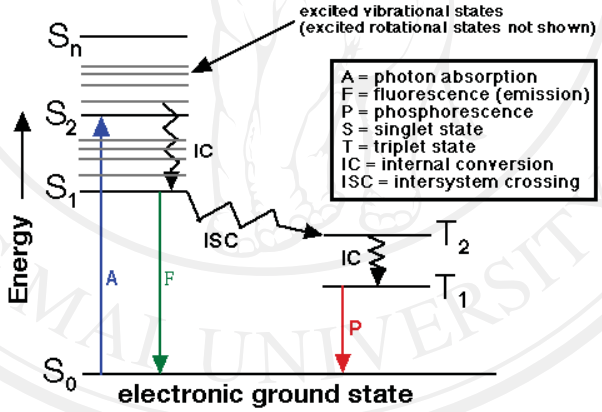


Figure 45 Jablonski diagram to show electronic states of a molecule absorbing and emitting light. Where S_0: ground singlet state, S_1, S_2: excited singlet states, T_1, T_2: excited triplet state, IC: internal conversion, ISC: intersystem crossing, F: fluorescence, P: phosphorescence

Generally, a chemiluminescence reaction can be generated by two mechanisms, direct or indirect (sensitized or energy transfer) chemiluminescence (Figure 46). Direct chemiluminescence involves two regents, a substrate, usually a chemiluminescence precursor (a reductant) and an oxidant in the presence of cofactors to give a product (or intermediate) in an electronically excited state, which returns to the ground state emitting a photon. Indirect chemiluminescence is based on the initial interaction between an oxidant and reductant to generate an intermediate excited species, followed by a transfer of energy from this excited species to a fluorphore, which itself becomes excited and releases a photon upon returning to the ground state.

For a chemical reaction to produce light, certain criteria must be met. An exothermic reaction is required to generate enough energy to produce an electronically excited state (40-70 kcal mol^{-1})[141]. The reaction pathway has to be such that it favours the energy to be channeled into producing an electronically excited state. The deactivation process of the excited state must favour photon emission over other non-radiative processes such as molecular dissociation, reaction with other species, intramolecular energy transfer (indirect chemiluminescence), isomerisation and physical quenching. Chemiluminescence reagents are usually reduced species that can be easily oxidized, such as molecules containing amino and hydroxy groups and polycyclic aromatic ring systems. Strongly alkaline arylamino groups deprotonate making them more susceptible to oxidation.

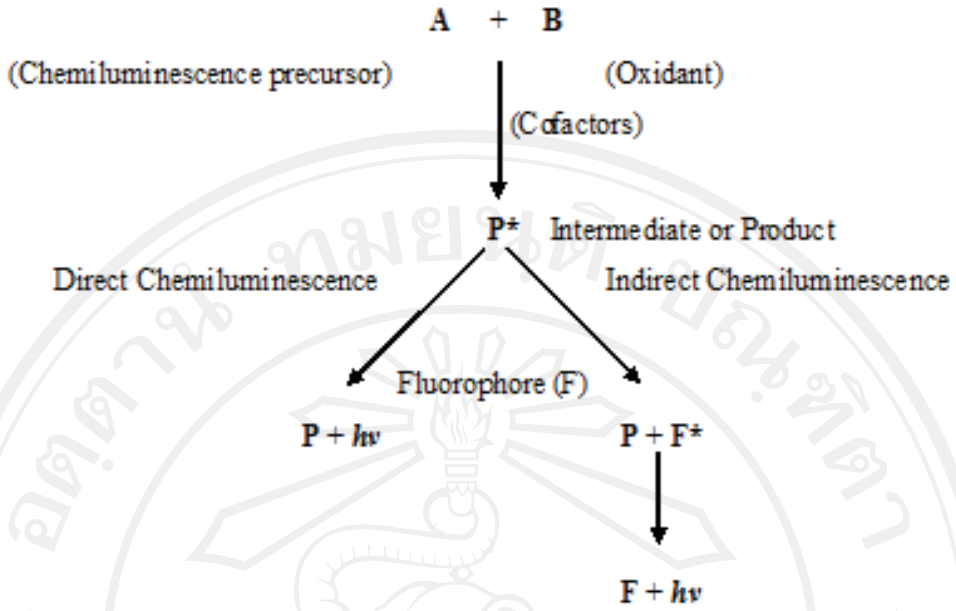


Figure 46 Schematic showing the different chemiluminescence reaction mechanisms (direct and indirect chemiluminescence)[36].

The intensity of the light emitted is dependent on the efficiency of producing electronically excited molecules, characterized by the quantum efficiency (quantum yield) and the rate of the reaction, see equation 3.

$$I_{CL} = \Phi_{CL} - dA/dt \qquad (3)$$

Where, I_{CL} = *emission intensity (photons/second)*

Φ_{CL} = *quantum yield (efficiency of chemiluminescence reaction (ratio of number of photons emitted to the number of reactant molecules reacting))*

$-dA/dt$ = *rate at which chemiluminescence precursor A is consumed*

For direct chemiluminescence, Φ_{CL} is defined in equation 4.

$$\Phi_{CL} = \Phi_C \times \Phi_E \times \Phi_F \qquad (4)$$

Where, Φ_C = *chemical yield (ratio of number of molecules that react via the reaction pathway to the total number of molecules reacted)*

Φ_E = *excitation yield (ratio of the number of molecules that form an electronically excited product to the number of molecules that react via the chemiluminescence pathway)*

Φ_F = *quantum yield of fluorescence of the light emitting species*

For indirect chemiluminescence, Φ_{CL} is defined in equation 5.

$$\Phi_{CL} = \Phi_C \times \Phi_E \times \Phi_F \times \Phi_{ET} \qquad (5)$$

Where, Φ_C, Φ_E *and* Φ_F *are defined above and* Φ_{ET} = *efficiency of energy transfer between the initial excited species and the secondary product*

Under defined experimental conditions Φ_C is the constant of proportionality between the observed intensity of chemiluminescence (I_{CL}) and the rate of consumption of the initial chemiluminescence precursor (A) (equation 5). Therefore, on rapid mixing a direct chemiluminescence reaction will produce an emission with intensity I_{CL}, which can be measured as a function of time. If the reaction is first order with respect to analyte A with rate constant k_r, then equation 5 can be rearranged to give equation 6. So the measurement of the maximum intensity can be related to the concentration.

$$I_{CL} = \Phi_{CL} k_r [A] \qquad (6)$$

Several factors affect the chemiluminescence intensity; these include the chemical structure of the chemiluminescence precursor, the nature and concentration of other species that may affect the chemiluminescence pathway, the presence of a catalyst, temperature, pH and ionic strength, hydrophobicity of the solvent and solution composition and the presence of energy transfer acceptors.

As it has been demonstrated, chemiluminescence can be utilized for quantitative analysis because the reaction rate is a function of the chemical concentration. The advantages of using chemiluminescence as a detection method are:

1. No external light source is required unlike other photoluminescence and spectrophotometric techniques, which gives chemiluminescence the advantage of having a decreased background signal and a reduction in scattering, producing high sensitivity with wide dynamic ranges.

2. The instrument required for chemiluminescence detection is relatively simple and consists of a reaction cell, light-tight housing, a method of introducing reagent and sample (static or flowing stream), a light detector and a method of collecting the data.

3. The technique is applicable to a wide variety of species that can participate in chemiluminescence reactions such as chemiluminescence substrates, chemiluminescence precursors, oxidants and reagents needed for the reaction, as well as species that affect the rate of the chemiluminescence reaction including activator, catalysts and inhibitors.

Chemiluminescence detection can be also used as a detector for chromatography, capillary electrophoresis and immunoassays. Problems associated with chemiluminescence detection include (i) the need of good mixing of the sample and reagents (ii) the fact that different reactions have different kinetics, which leads to the problem of where the light is being emitted and (iii) light scattering.

1.2 Development and Validation of Microfluidic Systems for Determination of Drug Residue in Honey Samples Coupled with Chemiluminescence Detector and Liquid Chromatography-Mass Spectrometry

1.2.1 Drug Residue (Chloramphenicol)

Antibiotics are widely used in animals for the treatment of diseases and also as animal growth promoters. The use of antibiotics may lead to drug residues present in animal-derived foods; the side effects of which would threaten public health. To minimize possible exposure to antibiotics, an allowable level of antibiotics in food has been established by the U.S. Food and Drug Administration and European Union.

Chloramphenicol (CAP) is a broad spectrum antibiotic produced by *Streptomyces venezuelae*[142-143]. It is freely soluble in alcohol, acetonitrile, acetone, ethyl acetate and propylene glycol, but is only slightly soluble in water. The molecular weight of CAP is 323.1. It sublimes in high vacuum and is sensitive to light. This drug inhibits protein synthesis in bacteria by binding to peptidyl transferase enzyme. The structure of CAP is shown in Figure 47.

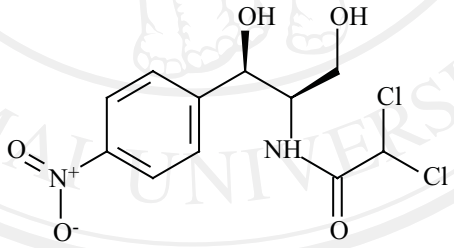


Figure 47 Chemical structure of chloramphenicol

CAP is widely used in human and veterinary medicine. In human, it has been used in the treatment of various bacteria eye infections, microbial infections such as typhoid, paratyphoid and meningitis, and also used as a “back up” drug for various veneral diseases. In veterinary, it is used as antibacterial agent for systematic salmonellosis infections in cattle, respiratory infections in calves and control disease in aquaculture species. Furthermore, CAP may be contaminated in animal-derived foods such as egg, milk, meat and shrimp.

This drug is harmful to humans because it can cause aplastic anemia leading to leukemia. The possible mechanism of action involves the biotransformation of CAP by intestinal bacteria to dehydrochloramphenicol and subsequent nitroreduction. The metabolite formed can cause singlestrand breaks of DNA to bone marrow and induction of faulty chromatid exchange. For these reasons, the International Agency for Research on Cancer (IARC) in 1990 considered CAP as "probably carcinogenic to humans". In view of highly toxic effects of CAP to humans, it has been banned as veterinary drug within the United States and the European Union (EU). However, this drug is still used in Asian countries. Therefore, the European Union severely restricted the import of food contaminated with CAP. The European Union established minimum required performance limit (MRPL) for CAP detection in food products as low as 0.3 ng g^{-1}. Therefore, it is necessary to develop the specific and sensitive analytical method to monitor the presence of CAP residues in shrimp and milk powder.

1.2.2 Previous Analytical Methods for Chloramphenicol Residue Determination

Various methods have been developed for determining chloramphenicol residues, including immunoassay[144], microbiological methods[145], sensors[146] and chromatographic methods using GC-MS[147] and LC-MS/MS[148]. The use of molecularly imprinted polymers for the determination of chloramphenicol has been previously described in milk, serum, honey and shrimp[149-152].

The technique of molecular imprinting polymerization (MIP) was introduced in 1972. The pre-organized approach, mainly developed by Wulff[153] where the aggregates in solution prior to polymerization is maintained by covalent bonds and the self-assembly approach, mainly developed by Mosbach and Ramstrom[154] where the pre-aggregates between the print molecule and the functional monomers are formed by non-covalent or coordination interactions. Molecularly imprinted polymers are extensively cross-linked polymers containing specific recognition sites with a predetermined selectivity for analyses of interest. The technique involves complexation in a solution of target molecules (template) with functional monomers through either covalent or non-covalent bonds, followed by a polymerization reaction with an excess of cross-linkers. Removal of the templates leaves behind specific

recognition sites that are complementary to the template in terms of its shape, size and functionality in the polymer network. These recognition sites enable imprinted polymers to be used as the mimics of enzymes, receptors and antibodies for screening various kinds of compounds from a mixture with abundant interferences. Up to now, there have been many reviews summarizing the development of MIP[155-157], which have covered many aspects from the sorbents for sample preconcentration and stationary phase for separation to bioassays, biosensors and mimics for enzymes, receptors and catalysts.

The molecular imprinting technique which combines the advantages of tailor-made sorbents and physical durability is one way to solve this problem. This technique has been extensively used in the production of molecularly imprinted polymers (MIPs) with specific binding sites for a wide variety of molecules[158]. Molecular imprinting technology is a synthetic approach to imitate natural molecular recognition. The printing process is performed by co-polymerizing functional and cross-linking monomers in the presence of a template molecule. The subsequent removal of the imprint molecule reveals binding sites in the polymer network, which are complementary to the template in size and shape. That allows the highly specific rebinding of the template. Furthermore, usually MIPs are reusable, need low cost of preparation, exhibit a high mechanical and chemical stability. A relatively new development in the area was used MIPs for sample clean-up and sensor determination.

In recent years, Chemiluminescence (CL) technique is becoming a powerful analytical tool for analysis. CL is well known for its high sensitivity and low detection limit that can be obtained. Chemiluminescence reactions have been used widely for sensitive and selective detection in flow injection and chromatographic analysis[159]. One of the most interesting series of chemiluminescence reactions are those involving the oxidation of tris(2,2'-bipyridyl) ruthenium(II), $Ru(bipy)_3^{2+}$ to (2,2'-bipyridyl) ruthenium(III), $Ru(bipy)_3^{3+}$, which is then followed by reduction with an analyte species with subsequence emission of light. There has been a variety of method employed to obtain the active oxidized reagent $Ru(bipy)_3^{3+}$, including chemical, photochemical, electrochemical oxidation and in situ electrogenerated chemiluminescence. Chemical generation of $Ru(bipy)_3^{3+}$ has been achieved by a

range of reagents such as cerium(IV) sulphate[160], lead dioxide[161] and potassium permanganate[162-163].

The CL reactions between primary, secondary or tertiary amine and $Ru(bipy)_3^{2+}$ are very sensitive and have been widely applied to the determination of chloramphenicol as containing an amine functionality. The chemical of electrogenerated chemiluminescence activity of tertiary amines with $Ru(bipy)_3^{2+}$ and their CL reaction mechanism have been reviewed by Knight and Greenway[164]. The chemical of electrogenerated chemiluminescence activity of chloramphenicol with $Ru(bipy)_3^{2+}$ and their CL reaction mechanism have been reported by Lindino[165].

It is well known that in the CL system containing $Ru(bipy)_3^{2+}$, the CL light emission is due to the reaction of $Ru(bipy)_3^{3+}$ with reductants ([the radical intermediates]*) to give $[Ru(bipy)_3^{2+}]^*$ as follows:

$$Ru(bipy)_3^{2+} + Ce(IV) \longrightarrow Ru(bipy)_3^{3+} + Ce(III) \quad (7)$$

$$Ru(bipy)_3^{3+} + [\text{the radical intermediates}]^* \longrightarrow [Ru(bipy)_3^{2+}]^* + \text{products} \quad (8)$$

$$[Ru(bipy)_3^{2+}]^* \longrightarrow Ru(bipy)_3^{2+} + h\nu \quad (9)$$

In the recent years, the use of the microsensor on a chip for its inherent advantages of portability, low reagents consumption and the reduction of analysis time have noticeable attention in a variety of applications.

This research describes the development of the microsensor on a chip for determination of chloramphenicol in honey samples. The firstly studied chemiluminescence reaction of $Ru(bipy)_3^{2+}$ and Ce(IV) in sulfuric acid enhanced by chloramphenicol, and on line enrichment chloramphenicol using molecularly imprinted polymer as molecule recognition element in the flow CL microsensor. The chloramphenicol imprinted polymers was synthesized with diethylaminoethyl methacrylate (DAM) as functional monomer, glycol dimethacrylate (EGDMA) as cross-linker and 2, 2'-dimethoxy-2-phenylacetophenone (DMPA) as initiator in the presence of chloramphenicol template. The chloramphenicol imprinted polymer was prepared with photo polymerization on microchip. The $Ru(bipy)_3^{2+}$ and Ce(IV) in sulfuric acid flowed through the micro sensor and reacted with on line adsorbed chloramphenicol and produced strong CL. The proposed method was proved to be

simple, rapid, selective and sensitive for the quantitative analysis. The method has been successfully applied to the determination of chloramphenicol in honey samples.

1.3 Research Aims

In this research, the development of microfluidic system will be undertaken for the determination of the drug residue in honey samples. The aim of this research can be summarized as follows:

1.3.1 To develop and validate microfluidic systems known as micro total analysis (μTAS) or lab-on-a chip technique for determining drug residue in honey samples coupled with chemiluminescence detector and liquid chromatography-mass spectrometry.

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 Microflow Chemiluminescence Sensor for Determination of Chloramphenicol in Honey Based On Molecularly Imprinted Polymer

2.1.1 Instruments and Apparatus

The instruments and apparatus used are as follows:

1. Superwhite crown borosilicate glass plate 50 × 40 mm: Alignrite, Bridgend, United Kingdom
2. Miniaturised photomultiplier tube: H5784, Hamamatsu Photonics, Enfield, United Kingdom
3. Microcomputer power supply: ITE power supply, ARTESYN, China.
4. UV light: Handheld UV lamp, UVL-56, Ultra-violet Products Ltd. Cambridge, United Kingdom
5. Centrifuged: Gilson, ANACHEM,
6. Micro syringe pump: Fusion 100, CHEMYX Inc., USA.
7. Tygon tubing (0.508 - 1.521 mm i.d.): Cole-Parmer Instrument Company, Chicago, IL, U.S.A
8. Yellow tubing: PTFE capillary 100 mm i.d./360 mm o.d., Sigma-Aldrich, United Kingdom
9. Disposable plastic syringe: SGE, Australia
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
14. Cabinet oven: Build-in, The University of Hull, United Kingdom

2.1.2 Chemicals and Reagents

Deionized water was used throughout all experiment. All chemicals used are analytical reagent grade, listed as follows:

1. Chloramphenicol: 99% pure, Sigma-Aldrich, United Kingdom.
2. Hydrofluoric acid: Sigma-Aldrich, United Kingdom.
3. Ammonium fluoride: Sigma-Aldrich, United Kingdom.
4. Tris(2,2'-bipyridyl) ruthenium (II) chloride hexahydrate: Sigma-Aldrich (Sigma-Aldrich Inc. SL Louis, MO, USA.
5. Cerium (IV) sulphate 4-hydrate: BHD Limited, Poole, United Kingdom.
6. Diethylaminoethyl methacrylate (DAM): Sigma-Aldrich, United Kingdom.
7. Methacrylic acid (MAA): Sigma-Aldrich, United Kingdom.
8. Ethylene glycol dimethacrylate (EGDMA): Sigma-Aldrich, United Kingdom.
9. 2, 2'-dimethoxy-2-phenylacetophenone (DMPA): Sigma-Aldrich, United Kingdom.
10. Acetonitrile and methanol: HPLC grade, E. Merck, Germany.
11. Sulfuric acid: E. Merck, Germany.
12. Toluene: E. Merck, Germany.
13. Dodecanol: E. Merck, Germany.
14. 3-(trimethoxysilyl) propyl methacrylate: Sigma-Aldrich, United Kingdom.

2.1.3 Microfluidic Device Design and Instrumentation

The MIPs on microchip was fabricated in house[166] using photolithography and wet etching techniques. The channel network was etched into a Superwhite Crown

borosilicate glass plate 50 × 40 mm. The glass was obtained precoated with chromium and photo resist layers, which were first, patterned using photolithography.

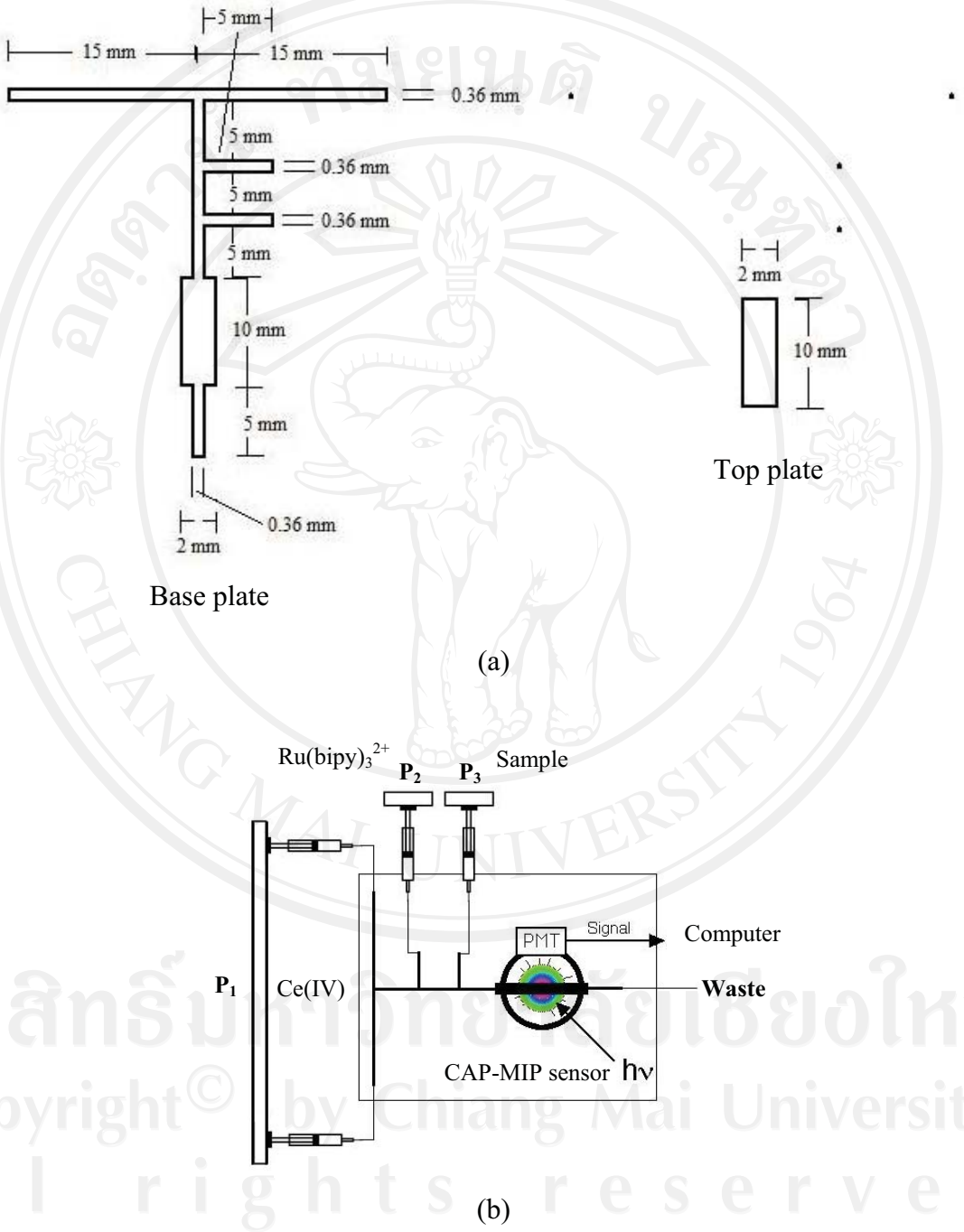


Figure 48 (a) Schematic diagram of dimension on the micro-channel on chip. (b) Schematic diagram of micro flow sensor on chip for determination of chloramphenicol (P_1, P_2, P_3: Fusion 100 micro-syringe pump)

The channels were then etched using aqueous 1% hydrofluoric acid solution buffered with 5% ammonium fluoride for 1 h at 70 ^{o}C. This produced the channels network consisting of four channels, intersecting at 90^{o}, 360 μm wide and 100 μm deep in base plate. The MIPs micro flow sensor cell was 10 mm long, 2 mm wide and 150 μm deep. The chip was then completed by thermally bonding a 3 mm top plate and base plate of Superwhite Crown borosilicate glass plate. A diagram of the chip can be seen in Figure 48.

The instrument was contained in a custom built, light tight box. A miniaturised photomultiplier tube (H5784, Hamamatsu Photonics, Enfield, UK) with a microcomputer power supply (ITE power supply, ARTESYN, China) 12 V. Solutions were pump through the flow system using syringe pumps (Fusion 100, CHEMYX Inc.US). UV light (Handheld UV lamp, UVL-56, Ultra-violet Products Ltd. Cambridge, UK) for the polymer preparations.

2.1.4 Standard, Reagents and Sample Preparations

a) Preparation of Standard Solutions

The stock standard solution of CAP was freshly prepared as a 3.09 μmol L^{-1} (1.00 mg L^{-1}) in aqueous solution by dissolving of 10 mg of CAP (accurately weighed) and diluting to 10 mL with 20 mmol L^{-1} phosphate buffer, pH 8.0 (stored at 4 ^{o}C in the dark). This standard solution was further diluted with 20 mmol L^{-1} phosphate buffer, pH 8.0 to give an appropriate concentration for the working standard solutions and was then exposed under ultraviolet light (365 nm) for 12 h.

b) Reagents

The solution of phosphate buffer (20.0×10^{-3} mol L^{-1}) pH 4.5 was prepared by dissolving the calculated amount of NaH_2PO_4 .H_2O and Na_2HPO_4 .$7H_2O$ in deionized water and 0.1 mol L^{-1} HCl solution was used to adjust the pH at 4.5

An aqueous solution of 5.0×10^{-3} mol L^{-1} $Ru(bipy)_3^{2+}$ was prepared by dissolving 0.2500 g of tris(2, 2'-bipyridyl) ruthenium (II) chloride hexahydrate in appropriate amount of 1.0×10^{-1} mol L^{-1} H_2SO_4 solution, then diluting to 100 mL in a volumetic flask. The solution was protected from light by using amber glass bottles.

An oxidant solution of 1.5×10^{-2} mol L^{-1} Ce(IV) was prepared by dissolving 3.9424 g of cerium(IV) sulphate 4-hydrate and making up to 100 mL with 1.0×10^{-1} mol L^{-1} H_2SO_4 solution.

c) Sample Pretreatments

The honey samples were purchased from local retail markets in United Kingdom. These samples were stored at 4 ^{o}C before used; 1.00 g of honey sample was accurately weighed. The honey sample was then spiked with known variable amounts of chloramphenicol ranging from 3.09×10^{-3} to 3.09×10^{-2} μmol L^{-1}. These samples were diluted with 10 mL of 20 mmol L^{-1} phosphate solution at pH 8.0. Each sample solution was mixed with a vortex mixer and centrifuged (Gilson, ANACHEM) for 10 min. Then the supernatant was filtered through a 0.45 μm filter membrane. The sample blank was prepared in the same way but without the compound-spiking step.

2.1.5 Procedures

a) Polymer Preparation

The internal wall surface of the glass microchip was first silanized to enable covalent attachment of the monolith to the walls using the method previously described in Reference[167]. Briefly, the microchip was rinsed with acetone and water, activated with sodium hydroxide (0.2 mol L^{-1}) for 30 min, and finally rinsed with ethanol. A 20% solution of 3-(trimethoxysilyl)propyl methacrylate in 95% ethanol, its pH was adjusted to 5 with acetic acid and then pumped through the capillary using syringe pump, and left for 5 h., followed by washing with ethanol and dried in a stream of nitrogen.

A prepolymerisation solution consisting of 0.75×10^{-3} mol L^{-1} (0.25 g) CAP, 5 mmol function monomer (1.00 mL DAM, or 0.42 mL MAA), 24.5×10^{-3} mol L^{-1} (4.62 mL) EGDMA, 0.18×10^{-3} mol L^{-1} (45 mg) DMPA and 1.10 mL toluene and 8.60 mL dodecanol were prepared in a screw-capped glass vial. The molar ratio of the template for the prepared MIPs was 1:2. The solution was sonicated for 20 min, and then purged with a stream of nitrogen for 10 min. A microchip was filled to 1 cm with the polymerized mixture. The microchip was placed in UV block by photo-

initiated with a UV light, which emitted light at 365 nm and irradiated for 30 min. The microchip was then flushed with acetonitrile at flow rate 2 μL min^{-1}, followed by 50% of methanol in water using the same flow rate by means of a micro syringe pump in order to remove the template and residues of nonreactive species. Non-imprinted polymers (NIP) were prepared simultaneously under the same conditions without the addition of the template.

b) On Line Pre-Concentration and Detection of Chloramphenicol

A schematic diagram of the instrument set up is shown in Figure 48 (b). The procedure for analyte enrichment and determination could be summarized in six steps.

- *Step 1,* Pump 1 was used to deliver the $Ru(bipy)_3^{2+}$ and Ce(IV) solutions into the microfluidic device at a flow rate 10 μL min^{-1} for 1 min. The CL signal obtained was used as the blank signal.
- *Step 2*, Pump 1 was stopped pump 2 was then used to remove any remaining $Ru(bipy)_3^{2+}$ and Ce(IV) solutions for 2 min before introducing pH 8.0 buffer solution at the same flow rate for pre-conditioning the MIP.
- *Step 3*, Pumps 1 and 2 were stopped and pump 3 was used to introduce the sample into MIP channel at a flow rate 5 μL min^{-1} for 6 min, during which time the chloramphenicol was trapped on to the MIPs.
- *Step 4*, Pump 2 was used to pump water into the MIP channel at 5 μL min^{-1} for 2 min, to wash the surface of the MIP.
- *Step 5*, $Ru(bipy)_3^{2+}$ and Ce(IV) solutions were then introduced by pump 1 at flow rate 10 μL min^{-1} for 1.5 min. The CL intensity was measured and then the concentration of chloramphenicol was quantified by the peak height corresponding to CL intensity.
- *Step 6*, Pump 2 was then started and the system was cleaned by methanol. The process was continued again.

2.2 LC-MS/MS on Microfluidic Device for Confirmatory of Chloramphenicol Determination in Honey Samples Based On Molecular Imprinted Polymers

2.2.1 Chromatographic Evaluation of MIPs Chloramphenicol

Polymer particles of the correct particle size (25 μm) were slurry packed into a stainless steel column (150 × 4.6 mm i.d.) using a pump (Fusion 100, CHEMYX Inc.). Molecular imprinted polymers were prepared by packing the imprinted polymer (2.50 g) in a stainless steel column. Acetonitrile/water (80:20, v/v) was used as the pushing solvent. A number of mixed mobile phases were investigated for selecting suitable chromatographic eluents including acetonitrile/water and methanol/water. The mixture ratios were between 100:0 and 10:90 (v/v). Analyses were performed at a flow rate of 1.5 mL min^{-1} in an isocratic mode and a detection wavelength of 275 nm. A sample and/or standard volume of 20 μL were injected. The retention factor; $k = (t_r-t_0)/t_0$ when t_r = retention time of a given analyte, t_0 = retention time of the internal standard as marker and the imprinted factor IF = k_{MIPs}/k_{NIP} when k_{MIPs} = retention factor of the MIPs, and k_{NIP} = retention factor of the NIP were calculated.

2.2.2 LC-MS/MS Instrumentation

An isocratic liquid chromatograph was connected with Hamilton syringe pump and then was connected to the injector (2 μL loop, Valco) from which the chip was interfaced to an electrospray ionization mass spectrometer (ESI-MS).

ESI-MS/MS was performed on a Finnigan LCQ classic quadrupole ion trap mass spectrometer equipped with standard electrospray ion source (Thermo Finnigan LOC, San Jose, USA) in negative-ion mode. An electrospray voltage of 4.0 kV and nitrogen sheath flow rate of 60 arbitrary units was employed. The temperature of the heated capillary was set to 200°C. Mass spectra were recorded on a personal computer with Xcalibur software version 1.2. The monitored ion was *m/z* 321 and the product ions for quantification were *m/z* 257, 194 and 152.

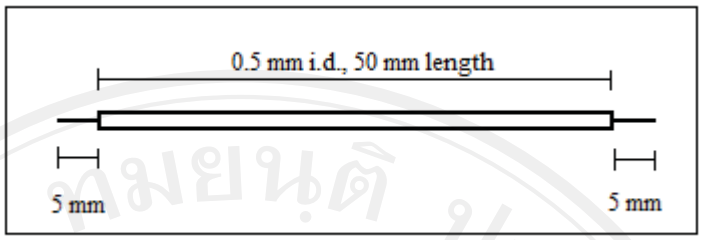


Figure 49 Schematic diagram of dimension on chip

CHAPTER III

RESULTS AND DISCUSSION

3.1 Microflow Chemiluminescence Sensor for Determination of Chloramphenicol in Honey Samples Based on Molecularly Imprinted Polymer

Chloramphenicol (CAP) is a broad-spectrum antibiotic that was first isolated in 1974 from culture of *Streptomyces venezuelae*[142-143] (Figure 50). It exhibits activities against both aerobic and anaerobic microorganisms. However, CAP has been shown to possess several harmful side effects in human, such as Grey syndrome, bone marrow suppression and fatal aplastic anaemia[144]. CAP is still illegally used in animal farming because of its easy access and low cost. Further, in the EU, application of CAP to food production has been prohibited since 1994[145]. Moreover, the EU has defined a minimum required performance limit (MRPL) of 0.3 $\mu g\ kg^{-1}$ for CAP in food of animal origin[146], because a safe level of CAP dosage has yet to be identified. With growing concerns over food safety and the need to increase sample-throughput in analytical testing laboratories, there is a constant requirement for accurate, simpler, faster and improved analytical methods. The complexity of food matrices and the presence of much potential interferences, require specific and selective methods for analysis.

Molecularly imprinted polymers are extensively cross-linked polymers containing specific recognition sites with a predetermined selectivity for analyses of interest. The printing process is performed by co-polymerizing functional and cross-linking monomers in the presence of a template molecule. The subsequent removal of the imprint molecule reveals binding sites in the polymer network, which are complementary to the template in size and shape. That allows the highly specific rebinding of the template.

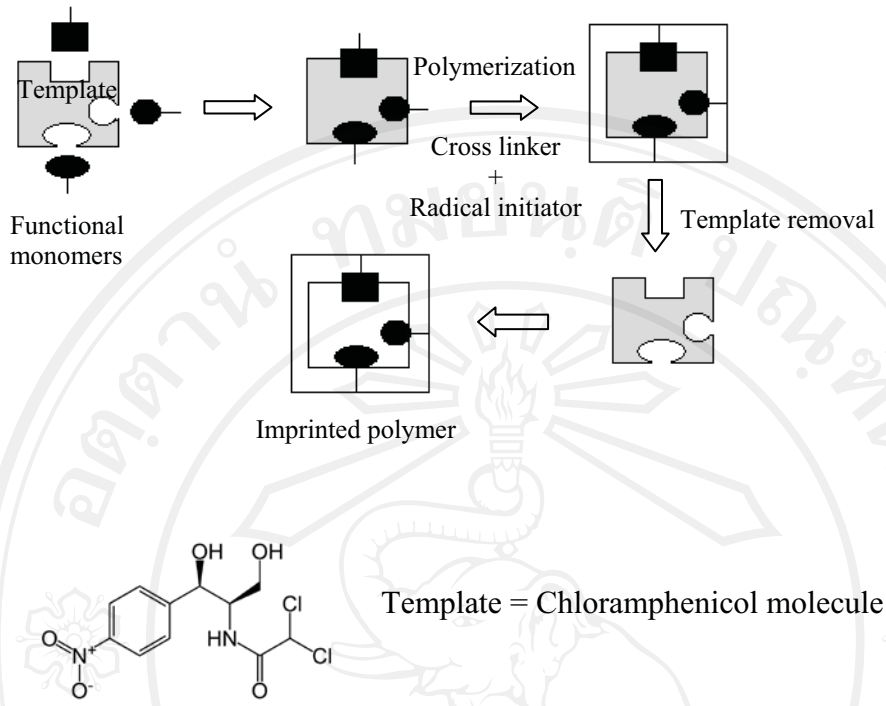


Figure 50 Structure of chloramphenicol used in this work and molecularly imprinted polymer procedure

The photochemical reaction and chemiluminescence detection have been applied to the determination of aromatic amines[168], photo-induction of glucose[169], determination of citrate[170], nitrate[171-172] and generation of singlet molecular oxygen[173-174]. Chloramphenicol in aqueous solution at room temperature (29-30 ^{o}C) degrades gradually after exposure to sunlight, UV light (365 nm) and red light[175]. The photochemical reaction-chemiluminescence detection for determination of chloramphenicol with a luminal-Co(II) system and potassium permanganate in sulfuric acid medium have been reported by David *et. al*.[176] and Catala Icardo *et. al*.[177] respectively.

In recent years, the miniaturisation of analytical systems has been shown to be advantageous due to the inherent advantages of portability, low reagents consumption and the reduction of analysis time. This research work describes the development of a microfluidic device for the determination of chloramphenicol in honey samples. The device incorporated a selective online enrichment channel with an MIP synthesized with diethylaminoethyl methacrylate (DAM) as the functional monomer,

glycol dimethacrylate (EGDMA) as cross-linker and 2, 2'-dimethoxy-2-phenylacetophenone (DMPA) as initiator in the presence of chloramphenicol. The method has been successfully applied to the determination of chloramphenicol in spiked honey samples.

3.1.1 Preliminary Study

3.1.1.1 Evaluation of the MIP

The method for the preparation of the MIP was adapted from that described by Schirmer *et al*.[152]. The ability of the MIP to trap the chloramphenicol was initially evaluated in bulk using CL detection where the DAM and MAA were compared as the functional monomers. Figure 51 shows the results obtained in comparison to the polymer obtained without the incorporation of the template (blank). It can be seen from Figure 51 that much better enrichment is seen with DAM. Figure 52 shows the scanning electron micrographs for the two different monoliths, these show a porous surface with pore diameter distributions from 2-5 mm.

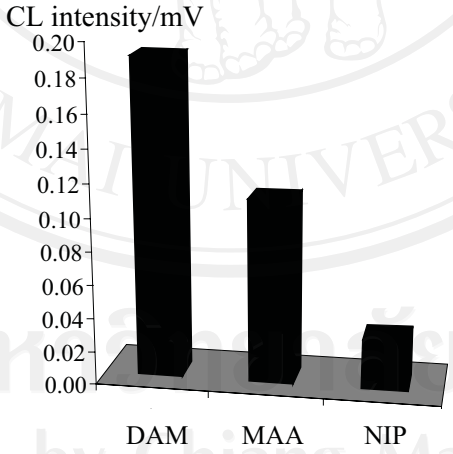
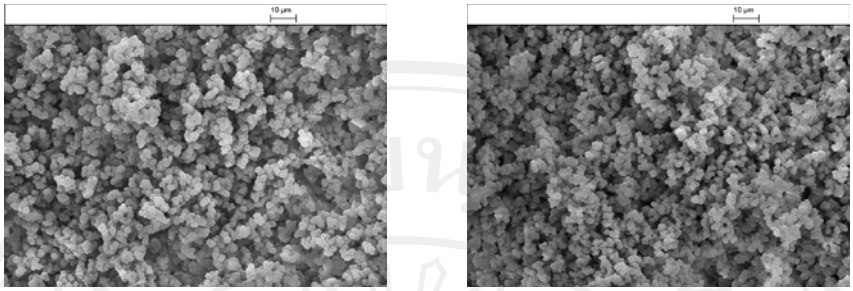


Figure 51 The CL intensity of sample with effect of adsorption comparison between DAM and MAA monomers synthesis for micro flow sensor on chip for determination of chloramphenicol (3.09×10^{-3} μmol L^{-1} of chloramphenicol).



poly(DAM-EGDMA) monolith poly(MAA-EGDMA) monolith

(a) (b)

Figure 52 Scanning electron micrographs of the MIPs by photo-polymerization method (a) DAM and (b) MAA monolith

3.1.1.2 Investigation of Parameters for CL Detection

As a starting point for this study the initial reagent concentrations were selected to be 5×10^{-3} mol L^{-1} for $Ru(bipy)_3^{2+}$, 1.5×10^{-2} mol L^{-1} for Ce(IV) in H_2SO_4 and 3.09×10^{-3} μmol L^{-1} for the chloramphenicol standard were studied. The averages of three measurements are reported for all results.

3.1.1.2.1 Effect of H_2SO_4 Concentration

The CL reaction of $Ru(bipy)_3^{2+}$ takes place in acidic medium, for the studied CL system optimum reaction media was tested at first with the concentration of 5×10^{-3} mol L^{-1} $Ru(bipy)_3^{2+}$, 1.5×10^{-2} mol L^{-1} Ce(IV) and 3.09×10^{-3} μmol L^{-1} of chloramphenicol standard. The effect of H_2SO_4 concentration was investigated in the range of 5.0×10^{-2} mol L^{-1} to 1 mol L^{-1}. The result showed that the strongest CL intensity was obtained in the concentration 1.0×10^{-1} mol $L^{-1}$of H_2SO_4 and was selected as the optimum concentration for the CL system (Table 32).

Table 32 Effect of H_2SO_4 concentration

H_2SO_4 concentration (mol L^{-1})	CL intensity* (mV)
0.05	1.07
0.1	1.34
0.5	1.18
1.0	0.68

*Mean of three determinations

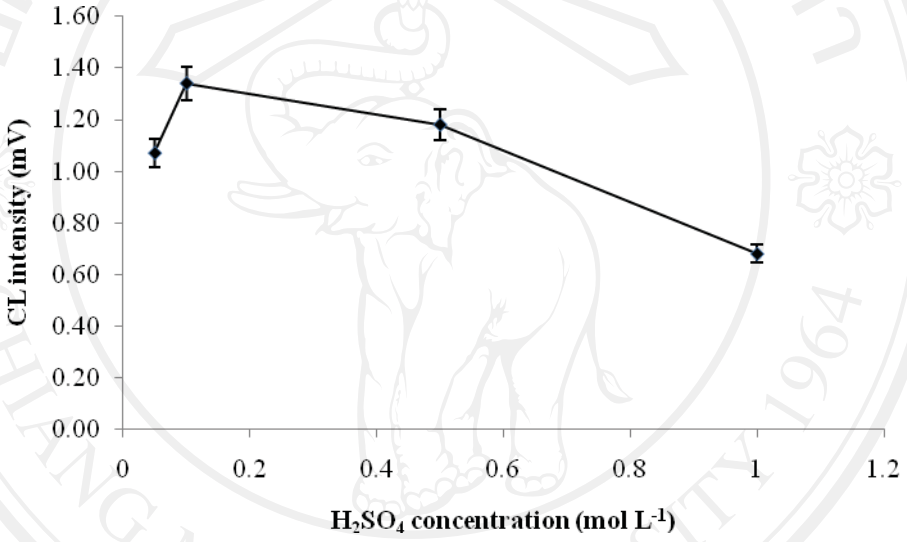


Figure 53 Effect of H_2SO_4 concentration

3.1.1.2.2 The pH Effect of Phosphate Buffer Solution on the CL Intensity

The pH effect of phosphate buffer solution on the CL intensity was subsequently optimized in the pH range of 3 - 10. It was found that the pH 8.0 of phosphate buffer solution gave maximum CL intensity. This might be due to the fact that at pH 8.0 effective CL reactions were favorered. Thus, the pH 8.0 of phosphate buffer solution was considered as the optimum.

3.1.1.2.3 Effect of $Ru(bipy)_3^{2+}$ Concentration

The effect of the concentration of $Ru(bipy)_3^{2+}$ on the CL intensity was investigated in the range of 1.0×10^{-3} mol L^{-1} to 5.0×10^{-3} mol L^{-1}. It was found that the CL intensity increased with the increase of $Ru(bipy)_3^{2+}$ concentration up to 2.0×10^{-3} mol L^{-1} as shown in Figure 54 a. The 2.0×10^{-3} mol L^{-1} $Ru(bipy)_3^{2+}$ concentration showed a good signal and then the 2.0×10^{-3} mol L^{-1} $Ru(bipy)_3^{2+}$ concentration was selected as the optimum concentration for the CL system.

3.1.1.2.4 Effect of Oxidant Concentration

The response of the CL system was also greatly affected by Ce(IV) the oxidant of the CL reaction (Figure 54 b). The influence of Ce(IV) concentration on the CL system was examined in the range of 5.0×10^{-3} mol L^{-1} to 3.0×10^{-2} mol L^{-1}. It was found that the 2.0×10^{-2} mol L^{-1} Ce(IV) concentration gave maximum CL intensity and this was used for further work.

Table 33 Effect of $Ru(bipy)_3^{2+}$ and Ce(IV) concentrations

$Ru(bipy)_3^{2+}$ concentration (mol L^{-1})	CL intensity* (mV)	Ce(IV) concentration (mol L^{-1})	CL intensity* (mV)
0.5	0.30	5.0	0.31
1.0	0.41	10.0	0.53
2.0	0.65	15.0	0.65
3.0	0.55	20.0	1.07
4.0	0.50	25.0	0.74
5.0	0.31	30.0	0.66

*Mean of three determinations

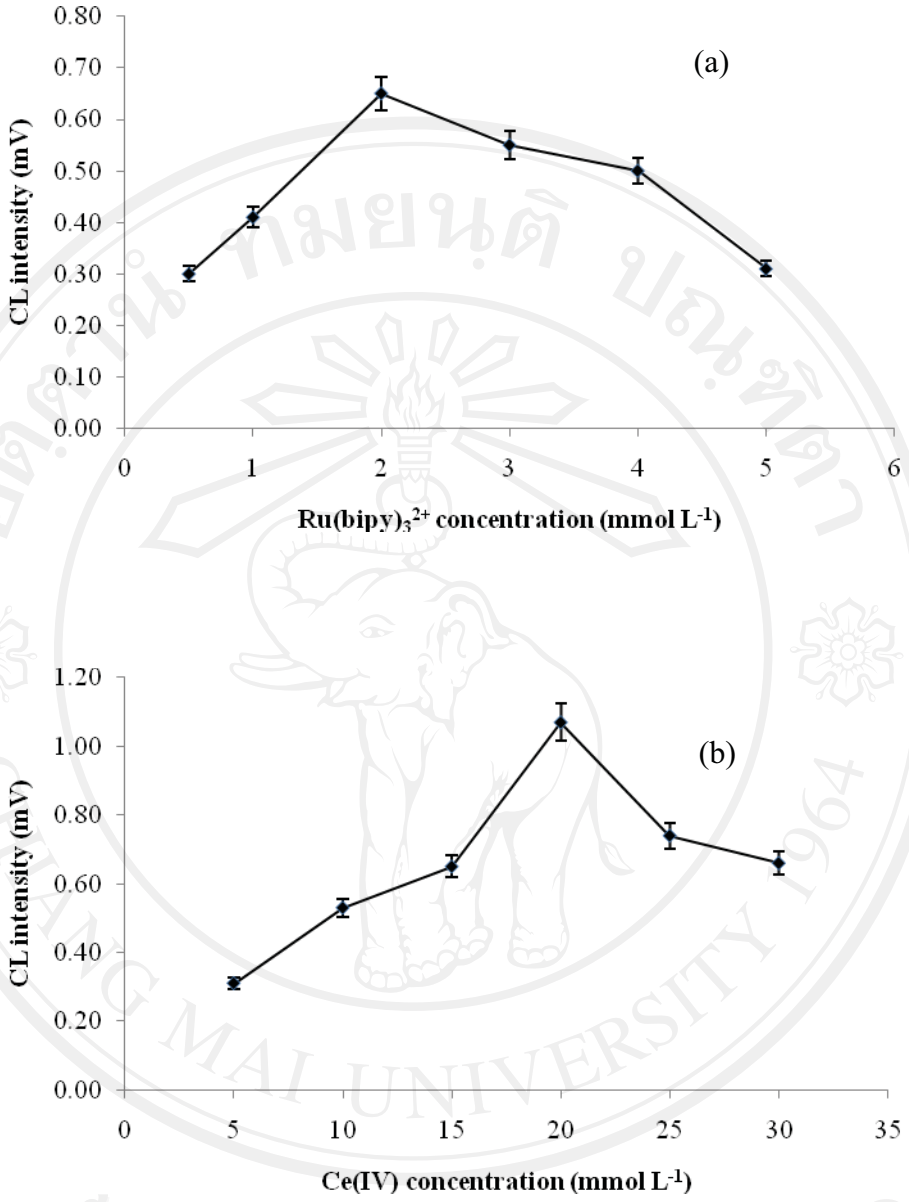


Figure 54 The effects of reagent concentration (a) $Ru(bipy)_3^{2+}$ and (b) Ce(IV) in 1.0×10^{-1} mol L^{-1} H_2SO_4

3.1.1.2.5 Effect of Flow Rates and Sample Volume

The flow rate is an important factor influencing the response of the CL system, including the relative CL intensity, sampling frequency and lifetime of the microfluidic device. The inclusion of the MIP into the device caused backpressures within the system. If the flow rate was too high, the high backpressure made it difficult to transfer the reagents and cause leaks, however, if the flow rate was too

low, the analysis time would increase. It was found that excellent reproducibility, stability and good CL response can be achieved when the flow rate of $Ru(bipy)_3^{2+}$ and Ce(IV) solution lines was fixed at the range of 2-10 μL min^{-1} (Table 34). As well as causing back pressure problem if the flow rate of the sample introduction was too high there would be insufficient time for chloramphenicol to be trapped on the MIP. The maximum CL intensity was achieved when the sample flow rate was fixed at 8 μL min^{-1} (Figure 55).

Table 34 Effect of flow rate

Flow rate (μL min^{-1})	CL intensity* (mV)
2.0	0.30
4.0	0.30
6.0	0.30
8.0	0.33
10.0	0.23

* Mean of three determinations

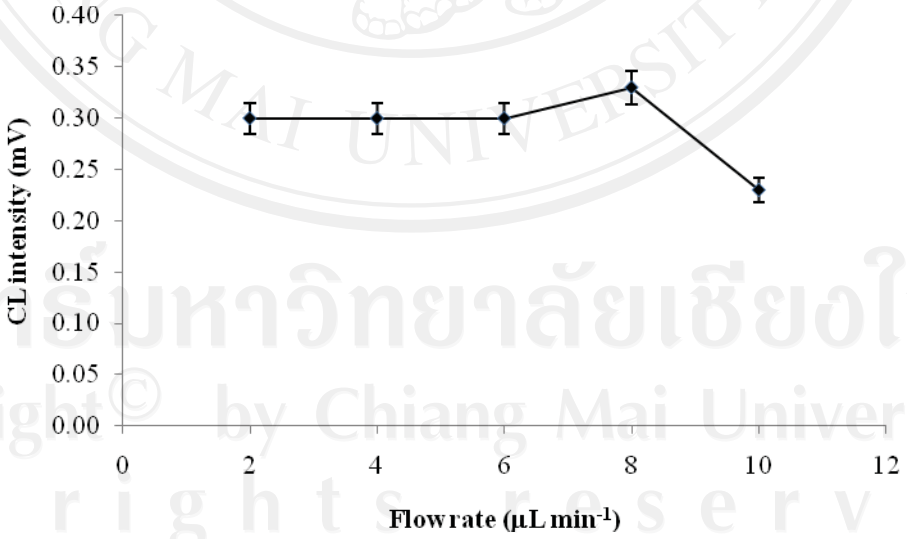


Figure 55 The effects of flow rate

This flow rate also gave sufficient cleaning time for the enrichment step. The effect of the volume of sample was then investigated at different volumes from 5 to 60 µL (Table 35). As would be expected the experimental results showed that the CL intensity increased as volume of sample increased. The 30 µL volume of sample was selected for subsequence analysis because it gave high CL intensity, short analysis time and the MIPs become saturated with high adsorbed chloramphenicol at this point as shown in Figure 56.

Table 35 Effect of sample volume

Sample volume (µL)	CL intensity* (mV)
5.0	0.52
10.0	0.60
20.0	0.73
30.0	0.77
40.0	0.78
50.0	0.77
60.0	0.78

*Mean of three determinations

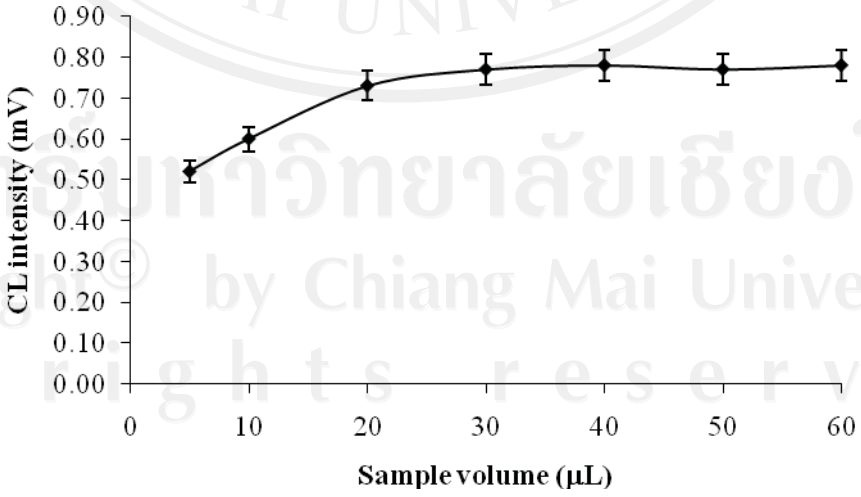


Figure 56 The effects of sample volume

3.1.1.2.6 Effect of CL Reagents Volume

The CL reagent volume, including $Ru(bipy)_3^{2+}$ and Ce(IV) sulphate were filled into the MIP microchip together by the microsyringe pump, using the flow rate at 5 μL min^{-1}. The influence of the volume of CL reagents on CL intensities were examined in the range 5 - 25 μL for $Ru(bipy)_3^{2+}$ and Ce(IV) sulphate (Table 36) respectively. Then the reagents were injected into the microchip. The results showed that the CL intensity increased with the increasing volume of the CL reagents. The CL intensity reached the maximum when the volume of the CL reagents increased up to 15 μL, after that it decreased gradually and then remained constant as shown in Figure 57.

Table 36 Effect of reagent volume

Reagents volume (μL)	CL intensity* (mV)
5.0	0.04
10.0	0.11
15.0	0.32
20.0	0.28
25.0	0.26

*Mean of three determinations

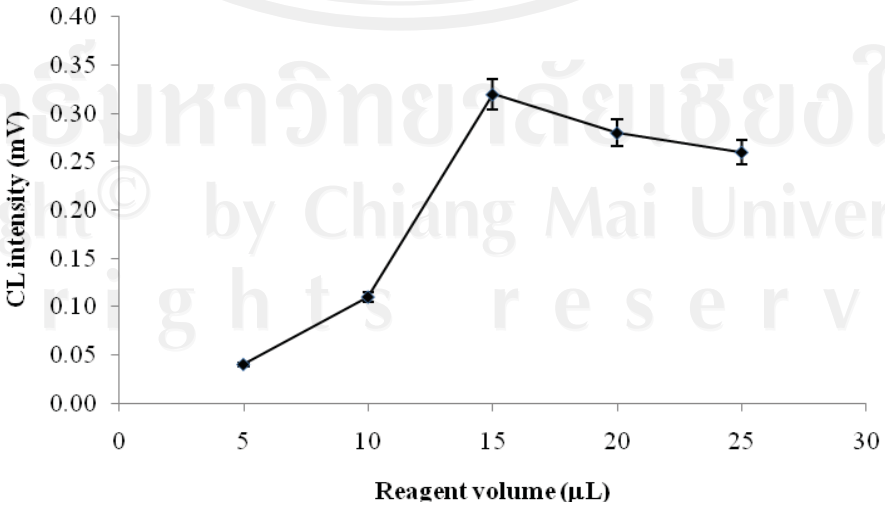


Figure 57 The effects of reagent volume

3.1.1.2.7 Effect of Binding Characteristic of MIPs Micro Flow Sensor

The binding characteristic of the MIP for chloramphenicol in the microfluidic device was investigated by comparing the results using the MIP and the polymer without the incorporation of the template (blank). When the solutions containing chloramphenicol in the concentration range of 3.09×10^{-3} - 7.55×10^{-3} μmol L^{-1} flowed through the MIP the chloramphenicol was selectively adsorbed on the MIP and the CL intensity increased. The results can be seen in terms of CL intensity in Table 37. Selectivity studies were investigated as followed by procedure steps. The optimized parameters including the reagent concentrations, the flow rate, desorption time and sample injection volume were studied.

Consequently, all these factors influencing the response of the CL system on the chip system were investigated and similar results were obtained. Therefore, these conditions were selected as optimum for the determination of chloramphenicol in the MIP micro flow sensor chip system.

Table 37 The binding characteristics of the MIP in the microfluidic device

Chloramphenical concentration (μmol L^{-1})	CL intensity (mV) MIP [a]	CL intensity (mV) NIP [b]	R.S.D. (%)
Reagent blank	0.030	0.025	5.26
3.09×10^{-3}	0.190	0.030	4.76
1.55×10^{-3}	0.110	0.028	9.09
7.55×10^{-4}	0.060	0.032	4.11

[a] MIP average of three net CL intensity signals
[b] NIP average of three net CL intensity signals

3.1.2 Summary of the Optimum Conditions

The selected optimum conditions for chloramphenicol residue determination were achieved by the so-called univariate method. The performance data for the optimization is shown in Table 38.

Table 38 The optimum conditions for the determination of chloramphenicol residue

Parameter Studies	Range Studies	Optimum
$Ru(bipy)_3^{2+}$ concentration (mol L^{-1})	1.0×10^{-3} - 5.0×10^{-3}	2.0×10^{-3}
Ce(IV) sulphate concentration (mol L^{-1})	5.0×10^{-3} - 3.0×10^{-2}	2.0×10^{-2}
H_2SO_4 concentration (mol L^{-1})	5.0×10^{-2} - 1.0	1.0×10^{-1}
pH of phosphate buffer solution	3.0 - 10.0	8.0
Flow rate of $Ru(bipy)_3^{2+}$ (μL min^{-1})	2.0 - 10.0	8.0
Flow rate of Ce(IV) sulphate (μL min^{-1})	2.0 - 10.0	8.0
Flow rate of sample (μL min^{-1})	2.0 - 10.0	8.0
Sample injection volume (μL)	5.0 - 30.0	30.0
CL reagent volume (μL)	5.0 - 25.0	15.0

3.1.3 Analytical Characteristics of the Microfluidic Device for Chloramphenicol Determination

Using the selected conditions, the linear calibration range, the detection limit (LOD) and the quantitation limit (LOQ) for the determination of chloramphenicol were investigated.

3.1.3.1 Linearity Range

A linear calibration curve was obtained for chloramphenicol over the concentration range of 1.55×10^{-4} to 3.10×10^{-3} μmol L^{-1} which covers the EU MRPL. The equation of the line was $y = 53.753x + 0.0216$ where y is relative CL emission in mV and x is chloramphenicol concentration in μmoles with a correlation coefficient of 0.9919.

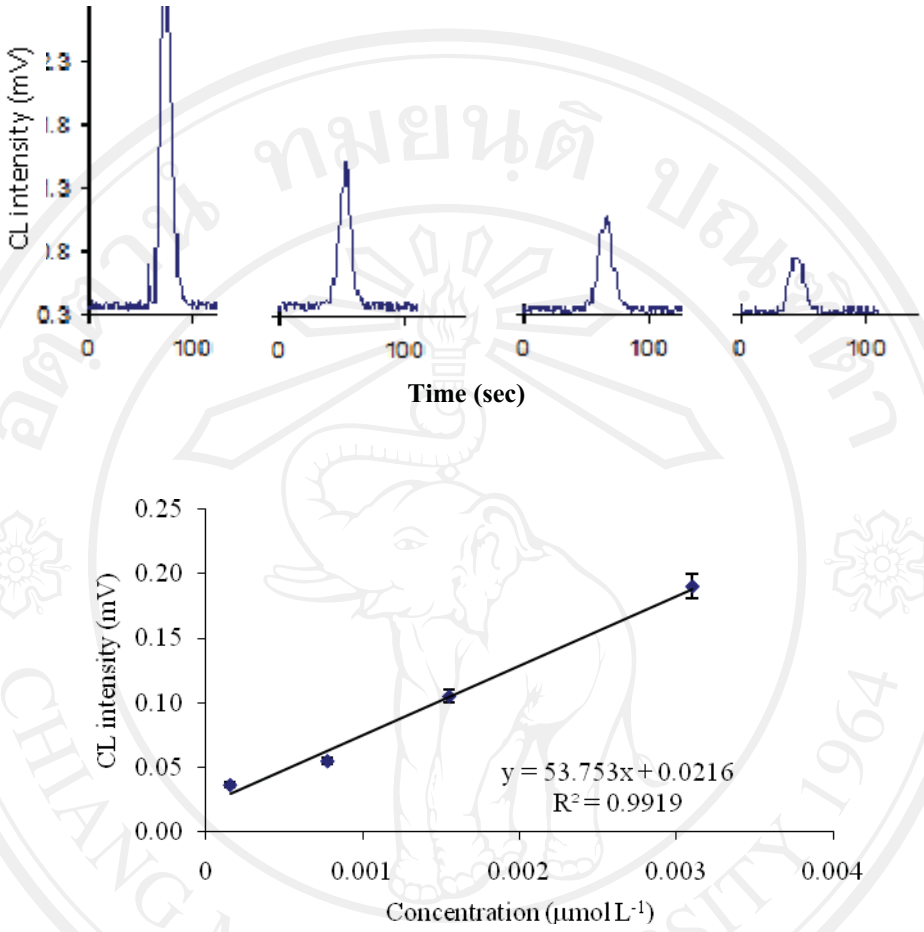


Figure 58 Calibration curve of chloramphenicol in the concentration range 1.55×10^{-4} to 3.10×10^{-3} µmol L^{-1}

3.1.3.2 Sensitivity, Detection Limit and Quantitation Limit

The sensitivity value of this method, defined as the slope of calibration curve was found to be 53.75 mV μmol L^{-1} s^{-1}. The detection limit (3σ) and the quantitation limit (10σ) were found to be 7.46 × 10^{-6} μmol L^{-1} and 2.48 × 10^{-5} μmol L^{-1} respectively.

3.1.3.3 Precision

The precision of the method was determined by measuring the repeatability (intraday precision) and the intermediate precision (inter day precision), both expressed as relative standard deviation (R.S.D) of the proposed method (peak height in mV). The precision was evaluated by assaying five replicate injections of 5.00 × 10^{-3} μmol L^{-1} and 1.55 × 10^{-2} μmol L^{-1} of chloramphenicol. The repeatability of each sample was evaluated on the same day under the same experimental conditions. The intraday precisions were found to be 4.86% and 7.81% respectively. The intermediate precision was evaluated by assaying each sample on three different days. The intermediate precisions were found to be 5.69% and 6.26% respectively (Table 39).

3.1.3.4 Accuracy

The percentage recoveries were also determined by the standard addition method. A triplicate determination of each concentration was conducted, along with statistical evaluation showing the standard deviations at difference values. The mean percentage recoveries are presented in Table 40. These results indicated that the proposed method provided highly accurate results.

3.1.4 Analytical Applications

The proposed MIP-CL method was successfully applied to the determination of chloramphenicol in honey samples using the standard addition method. Known concentrations of standard chloramphenicol (1.55 × 10^{-4}, 7.75 × 10^{-4} and 1.55 × 10^{-3} μmol L^{-1}) were added to 1.00 mL of honey sample, respectively and mixed, then analyzed using the proposed method. The standard addition curve was established by

plotting the relative CL emission intensity of chloramphenicol in mV versus the added concentration of chloramphenicol. The amounts of chloramphenicol residue could then be calculated from the standard addition curve. It was found that there is no chloramphenicol residue present in the honey samples that had been purchased.

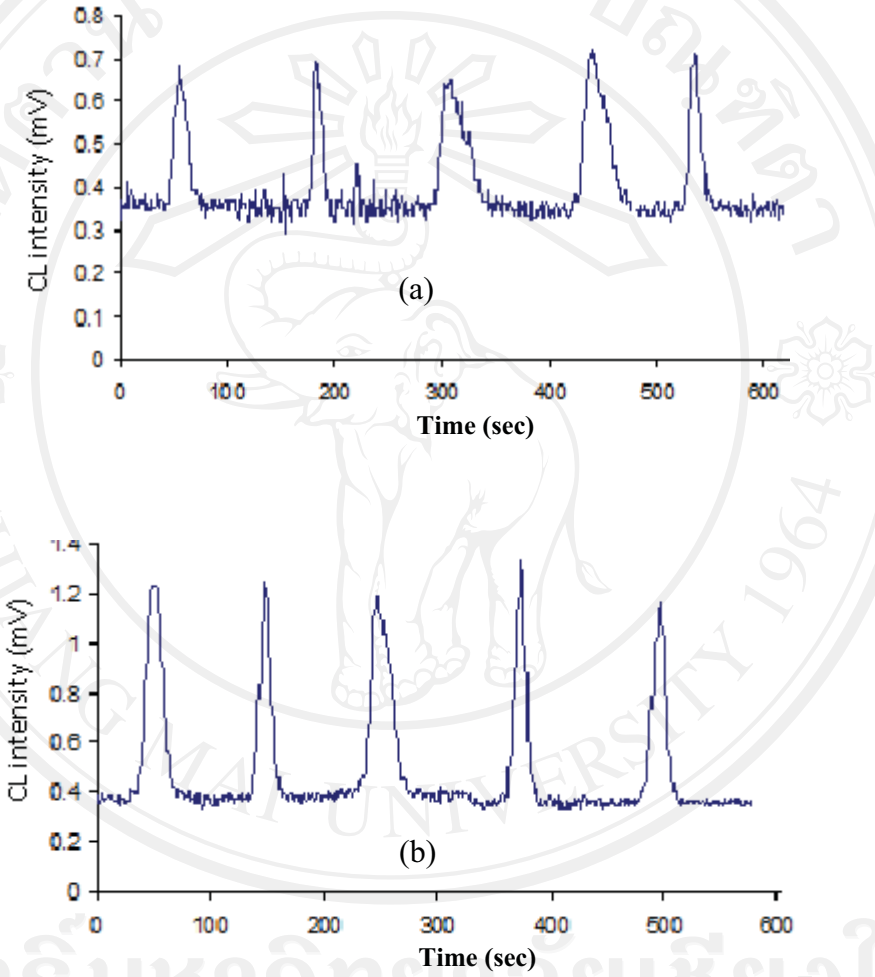


Figure 59 Precision of the proposed method in the concentration range of (a) 1.55×10^{-4} µmol L^{-1} and (b) 1.55×10^{-3} µmol L^{-1} chloramphenicol

Table 39 Precision study for chloramphenicol

Chloramphenicol ($\mu mol\ L^{-1}$)	Intra-day precision*		Inter-day precision*	
	Concentration ($\mu mol\ L^{-1}$)	% R.S.D	Concentration ($\mu mol\ L^{-1}$)	% R.S.D
1.55×10^{-4}	1.58×10^{-4}	4.86	1.54×10^{-4}	5.69
1.55×10^{-3}	1.57×10^{-3}	7.81	1.59×10^{-3}	6.26

* Each value is the average of three determinations

Table 40 Analytical recovery of chloramphenicol added to honey sample solution

Honey sample	Chloramphenicol concentration*		Recovery* (%)
	Added ($\mu mol\ L^{-1}$)	Found ($\mu mol\ L^{-1}$)	
Sample No. 1	7.75×10^{-4}	7.41×10^{-4}	97.00
	1.55×10^{-3}	1.52×10^{-3}	98.12
	3.10×10^{-3}	2.91×10^{-3}	94.08
Mean±S.D.			96.40±2.08
Sample No. 2	7.75×10^{-4}	7.44×10^{-4}	78.07
	1.55×10^{-3}	1.33×10^{-3}	86.11
	3.10×10^{-3}	2.41×10^{-3}	96.20
Mean±S.D.			86.79±9.08
Sample No. 3	7.75×10^{-4}	8.06×10^{-4}	89.08
	1.55×10^{-3}	1.46×10^{-3}	94.12
	3.10×10^{-3}	2.76×10^{-3}	104.20
Mean±S.D.			95.80±7.69

*Results from three determinations

3.2 LC-MS/MS on Microfluidic Device for Confirmatory of Chloramphenicol Determination in Honey Samples Based On Molecular Imprinted Polymers

3.2.1 Preliminary Investigation

The parameters affecting the extraction efficiency such as pH of buffer solution, extraction equilibrium profiles, extraction flow rate, desorption flow rate, desorption solvent ratio, volume of sample and type of monolith were investigated.

3.2.1.1 Optimization of the SPME Conditions

After the optimization of pH, the extraction efficiency of CAP in aqueous solution was studied in a wide pH range of 2.5 - 9.5. It was found that pH 4.5 was selected in this work because it gave high sensitivity.

The extraction equilibrium profile was increasing with the volume of the extraction sample from 100 to 1000 μL at the same extraction flow rate. The results are shown in Figure 60. The amount of CAP was increased with increasing the volume of sample. The sample volume of 600 μL was selected for subsequence analysis because high sensitivity and short analysis time were obtained.

The flow rate of extraction solution was optimized in the range of 20 to 100 μL min^{-1} by injecting 600 μL of sample solution. A flow rate of 60 μL min^{-1} gave high sensitivity, shorter extraction time and the acceptable back-pressure of the monolith capillary column. Results are shown in Figure 61.

The flow rate of desorption solution was optimized in the range of 20 to 100 μL min^{-1}. A flow rate of 40 μL min^{-1} gave high sensitivity, shorter desorption time and the acceptable back-pressure of the monolith capillary column. The results are presented in Figure 61.

Under the selected MIP-SPME conditions (Table 41) and using the HPLC and LC-MS on chip system for analysis, the linear calibration range, the detection limit (LOD) and the quantitation limit (LOQ) for the determination of chloramphenicol were investigated.

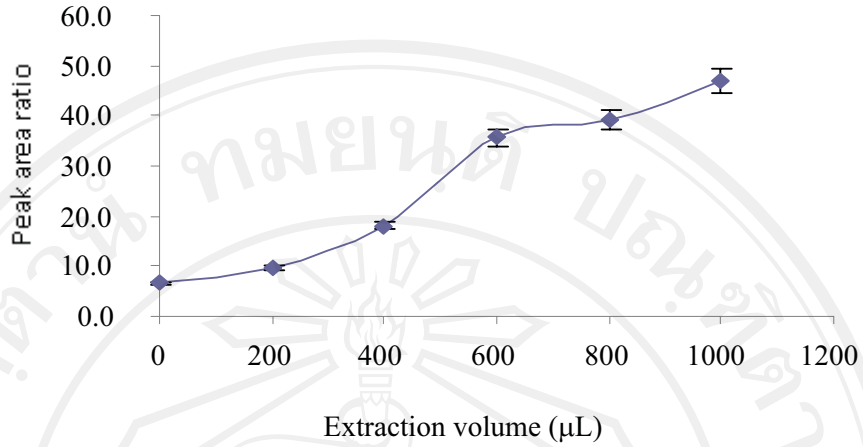


Figure 60 Extracted sample equilibrium profile of CAP for SPME

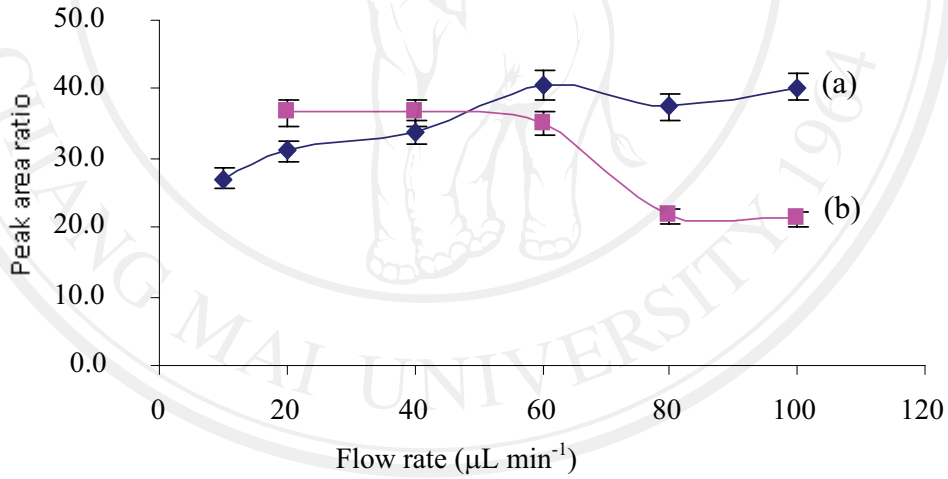


Figure 61 Extraction (a) and desorption (b) flow rate profile of CAP for SPME

Table 41 MIP-SPME conditions of CAP from (3.10×10^{-2} μmol L^{-1}) and THAP (2.80×10^{-2} μmol L^{-1}) using the MIP poly(DAM-EGDMA) as sorbent

MIP-SPME parameter*	Range of studies	Optimization value
pH of phosphate solution	2.5-9.5	4.5
Volume of extraction (μL)	200-1000	600
Flow rate of extraction (μL min^{-1})	20-100	60
Flow rate of desorption (μL min^{-1})	20-100	40
Volume of desorption (μL)	100-500	100

* The HPLC conditions are described in apparatus and procedures

3.2.2 Chromatographic Evaluation for CAP-MIP

Several bulk polymers for the separation of CAP were synthesized using DAM and MAA as functional monomers. HPLC analyses were performed to evaluate the imprinting effect of the MIPs and to identify the influence of the mobile phase composition on the retention time of CAP using THAP as internal standard. CAP and THAP retention factor on the MIPs columns relative to the NIP columns using acetonitrile/water as the mobile phase was observed. It was found that the highest retention factor was achieved with a polar solvent. Addition of protic solvent such as water to acetonitrile on the recognition properties of CAP on a CAP-imprinted bulk polymer was studied. The analyte was retained on the MIPs under polar elution conditions with THAP as a marker. Thus, in accordance with the literature[173] the analyte was retained on the MIPs under nonpolar elution conditions using acetone as a marker.

In water-rich mobile phases the retention of the target analyte was investigated by the water content. Better separation between CAP and THAP will be obtained when the water concentration at above 60% of water in acetonitrile. On replacing acetonitrile with methanol, the retention factor of CAP continuously increased with water concentration. The mixture containing methanol gave higher retention factor than mixtures containing acetonitrile (Figure 62). Thus, CAP was not eluted from the columns within 30 min when the eluent contained more than 90% of water in mixtures with acetonitrile, or more than 80% of water in the mixtures with methanol.

The imprinting factor of CAP exhibited values below acetonitrile or methanol in aqueous elution conditions, there are differences between the retention factor on the MIPs and NIP columns (Figure 63). The increased of the imprinting factor obtained with the mixtures containing methanol was much greater than that with the mixtures containing acetonitrile.

The functional monomers used for the synthesis of the corresponding polymers on the resulting recognition properties when acetonitrile was used as mobile phase. The MIPs synthesis from DAM showed higher retention factor and imprinting factor of CAP than the MIPs synthesis from MAA was relatively low.

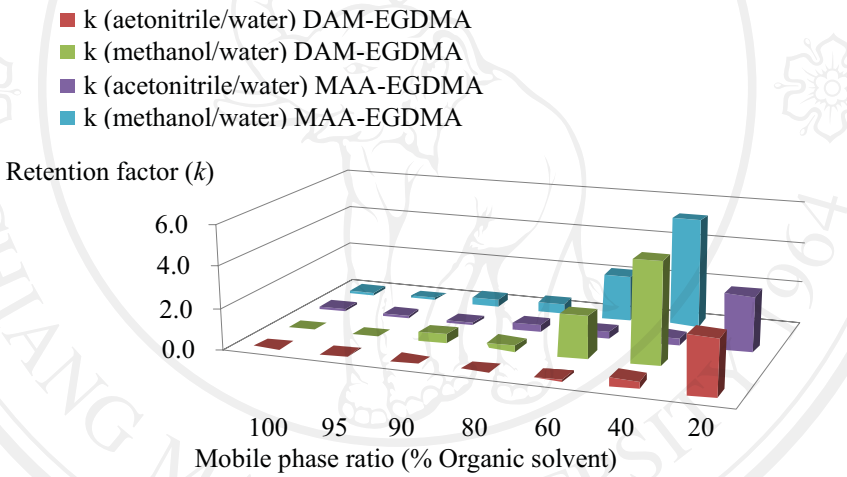


Figure 62 Retention factors (k) of CAP (3.10×10^{-2} μmol L^{-1}) and THAP (2.80×10^{-2} μmol L^{-1}) as marker, on the different MIPs columns using mixture of acetonitrile/water or methanol/water as mobile phase

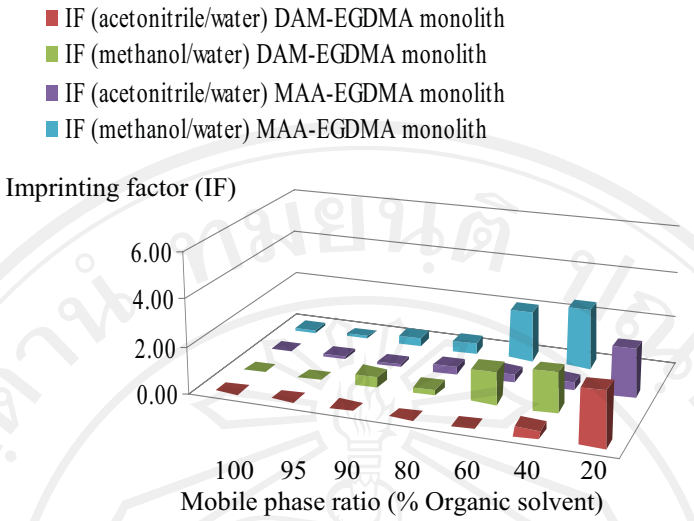


Figure 63 Imprinting factors (IF) of CAP (3.10×10^{-2} μmol L^{-1}) and THAP (2.80×10^{-2} μmol L^{-1}) as marker, on the different MIPs columns using mixture of acetonitrile/water or methanol/water as mobile phase

3.2.3 Chromatography and Mass Spectrometry

The sample was analyzed by a mass spectrometer coupled with a high performance liquid chromatograph equipped with CAP-MIP monolith on chip. A chip temperature was maintained at room temperature. The sample (5 μL) was injected into the LC-MS/MS system at a flow rate of 10 μL min^{-1}, using acetonitrile-water, 20:80 (v/v), as mobile phase. Electrospray ionization (ESI) was used in negative-ion mode and the mass spectrometer was operated in single reaction monitoring (SRM) mode. A typical mass spectrum is shown in Figure 65.

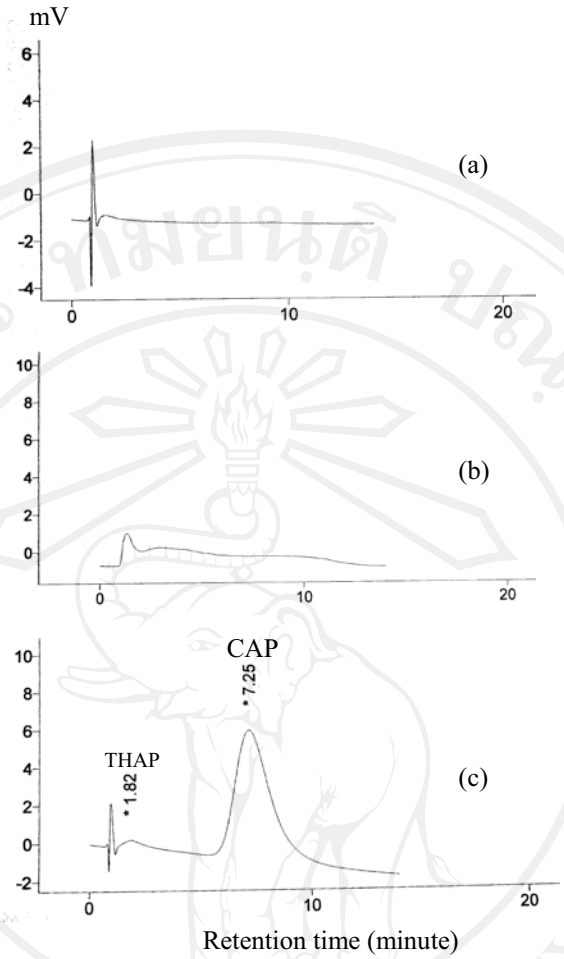


Figure 64 Chromatograms of CAP and THAP in honey sample (a) without spiked standard and passed SPME, (b) with spiked standard, and (c) with spiked standard passed SPME. Concentration of CAP (3.10×10^{-2} μmol L^{-1}) and THAP (2.80×10^{-2} μmol L^{-1}) were added.

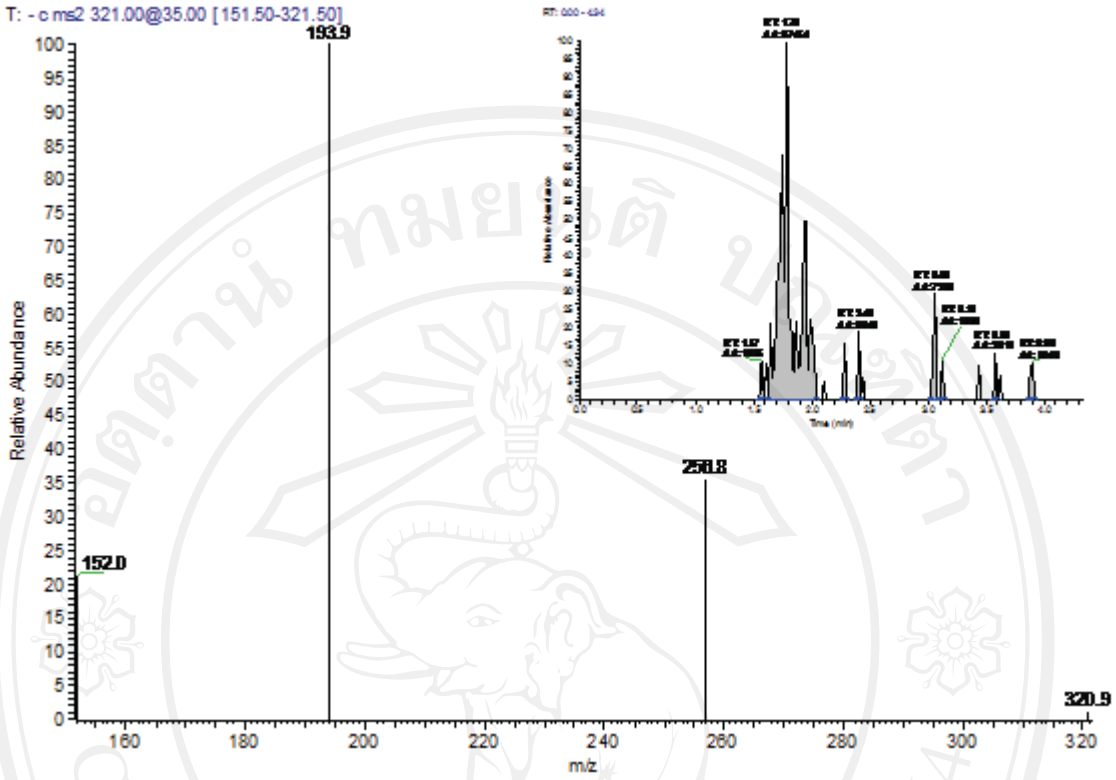


Figure 65 Full scan ESI mass spectrum of CAP residue from honey sample (MS/MS)

3.2.4 Analytical Characteristics

Using the selected conditions, the analytical characteristics of the proposed method were evaluated by examining the linear range, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and sampling frequency.

3.2.4.1 Linearity of Calibration Curve

3.2.4.1.1 High Performance Liquid Chromatography (CAP-MIP Column)

The linearity of calibration curve was determined using the selected MIP-SPME conditions (Table 41) and using the CAP-DAM-EGDMA imprinted polymer monolith column with HPLC system for the analysis (Figure 64). Linear calibration curve of CAP over the concentration range of 3.72×10^{-4} - 7.44×10^{-3} μmol L^{-1} was established. Over this concentration range, linear regression analysis of the CAP and

thiamphenicol as internal standard (peak area ratio) versus CAP concentration (x) yielded the following equation: $y = 4432.9x - 1.5043$ ($r^2 = 0.9954$)

3.2.4.1.2 LC-MS/MS (CAP-MIP on Chip)

The linearity of calibration curve was determined using the CAP-DAM-EGDMA imprinted polymer monolith on chip with LC-MS^2 system for the analysis. Linear calibration curve of CAP over the concentration range of 7.75×10^{-4} - 6.18×10^{-3} μmol L^{-1} was established. Over this concentration range, linear regression analysis of the CAP standard (peak area) versus CAP concentration (x) yielded the following equation: $y = 150512x + 16859$ ($r^2 = 0.9900$)

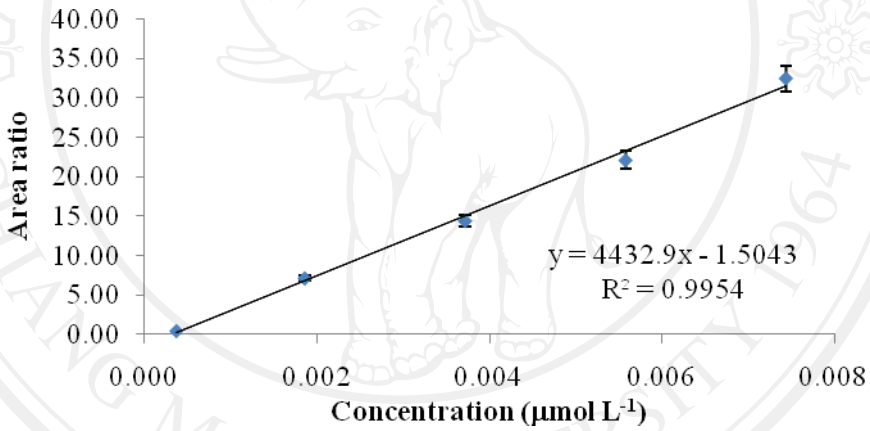


Figure 66 Linear calibration graph obtained under the optimal conditions for determination of chloramphenicol over the range 3.72×10^{-4} - 7.44×10^{-3} μmol L^{-1} using CAP-MIP column

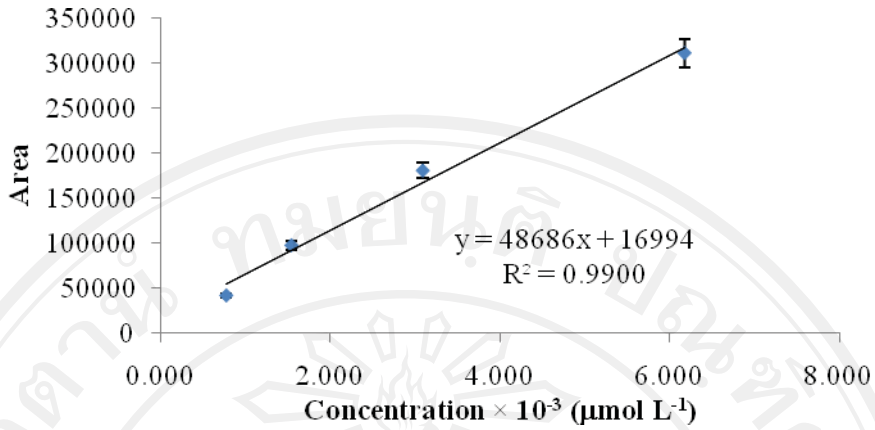


Figure 67 Linear calibration graph obtained under the optimal conditions for determination of chloramphenicol over the range 7.75×10^{-4} - 6.20×10^{-3} µmol L^{-1} using CAP-MIP on chip

3.2.4.2 Sensitivity, Detection Limit, Quantification Limit

3.2.4.2.1 High Performance Liquid Chromatography (CAP-MIP Column)

The sensitivity value of the proposed chloramphenicol molecular imprinted monolith coupled HPLC method, defined as the slope of calibration curve was found to be 533.67. Linear calibration curve was obtained over the concentration range of 3.72×10^{-4} - 7.44×10^{-3} µmol L^{-1} with the correlation coefficient of 0.9955. The detection limit (3σ) and the quantitation limit (10σ) were found to be 1.45×10^{-4} µmol L^{-1} and 4.84×10^{-4} µmol L^{-1} respectively.

3.2.4.2.2 LC-MS/MS (CAP-MIP on Chip)

The sensitivity value of the proposed chloramphenicol molecular imprinted monolith coupled LC-MS/MS method, defined as the slope of calibration curve was found to be 150512. Linear calibration curve was obtained over the concentration range of 7.75×10^{-4} - 6.18×10^{-3} µmol L^{-1} with the correlation coefficient of 0.9900. The detection limit (3σ) and the quantitation limit (10σ) were found to be 2.10×10^{-4} µmol L^{-1} and 6.79×10^{-4} µmol L^{-1} respectively (Table 42).

3.2.4.3 Precision and Accuracy

3.2.4.3.1 High Performance Liquid Chromatography (CAP-MIP Column)

The relative standard deviation of the proposed method (peak area ratio) calculated from 10 replicate injections of 1.55×10^{-2}, 3.09×10^{-2} and 4.63×10^{-2} μmol L^{-1} of CAP were found to be 0.12, 0.37 and 0.53% respectively. The percentage recoveries were determined using the standard addition method in honey samples. CAP (1.55×10^{-2}, 3.09×10^{-2} and 4.63×10^{-2} μmol L^{-1}) were added and mixed with 1.0 mL of milk sample, the sample was extracted and analyzed using the proposed method. The mean percentage recoveries of CAP were found to be in the ranges of 84.97 - 109.96, indicating that the proposed method could provide good acceptable extraction efficiency and recovery.

3.2.4.3.2 LC-MS/MS (CAP-MIP on Chip)

The relative standard deviation of the proposed method (peak area ratio) calculated from 10 replicate injections of 1.55×10^{-3}, 3.09×10^{-3} and 6.20×10^{-3} μmol L^{-1} of CAP were found to be 0.21, 2.71 and 1.34% respectively. The percentage recoveries were determined using the standard addition method. CAP (1.55×10^{-3}, 3.09×10^{-3} and 6.20×10^{-3} μmol L^{-1}) were added and mixed with 1.0 mL of honey sample, then each sample was extracted and analyzed using the proposed method. The mean percentage recoveries of CAP were found to be in the ranges of 95.18 - 96.85, indicating that the proposed method could provide good acceptable extraction efficiency and recovery.

3.2.5 Analytical Applications

The amounts of chloramphenicol residue could then be calculated from the standard addition curve. It was found that there is no chloramphenicol residue present in the honey samples.

Table 42 Comparison of the molecularly imprinted polymer analytical figures of merit of the proposed method with monolith C_{18} column HPLC method

Techniques	Linear range (μmol L^{-1})	Correlation coefficient; r^2	Limit of detection; LOD (μmol L^{-1})	Limit of quantitation; LOQ (μmol L^{-1})
HPLC (monolith C_{18} column)	3.09×10^{-3} - 6.18×10^{-2}	0.9972	2.07×10^{-4}	6.90×10^{-4}
HPLC (CAP-MIP column)	3.72×10^{-4} - 7.44×10^{-3}	0.9954	1.45×10^{-4}	4.84×10^{-4}
LC-MS/MS (CAP-MIP on chip)	7.75×10^{-4} - 6.18×10^{-3}	0.9900	2.16×10^{-4}	6.79×10^{-4}

CHAPTER IV

CONCLUSIONS

Novel microflow chemiluminescence sensor detection and LC-MS/MS techniques based on molecularly imprinted polymer have been developed for determining chloramphenicol. Both methods were successfully applied to the determination of chloramphenicol in honey samples. The research and development of both methods are carried out as follows:

4.1 Microflow Chemiluminescence Sensor for Determination of Chloramphenicol in Honey Samples Based on Molecularly Imprinted Polymer

The flow chemiluminescence sensor system describes the development of a simple FICL method for the determination of chloramphenicol. The method is based on the CL produced by the reduction of $Ru(bipy)_3^{3+}$ which is obtained by oxidation of $Ru(bipy)_3^{2+}$ with Ce(IV). One of the most widely used set of chemiluminescence reactions are those involving the oxidation of tris(2, 2'-bipyridyl) ruthenium(II), $Ru(bipy)_3^{2+}$ to (2, 2'-bipyridyl) ruthenium(III), $Ru(bipy)_3^{3+}$, which is then followed by reduction with an analyte species with subsequence emission of light. The CL reaction between tertiary amine and $Ru(bipy)_3^{2+}$ is very sensitive and has been widely applied. Chloramphenicol in aqueous solution at room temperature (29-30 ^{o}C) degrades gradually after exposure to sunlight, UV light (365 nm) and red light. The photochemical reaction-chemiluminescence detection was used for determination of chloramphenicol with a $Ru(bipy)_3^{3+}$-Ce(IV) system in sulfuric acid medium.

A schematic diagram of the instrument set up is shown in Figure 48. The procedure for analyte enrichment and determination could be summarized in six steps (see in section 2.1.5).

The parameters were investigated include reagent concentrations and some parameter for MIP-CL detection. The optimum conditions are shown in Table 38. Under the optimum conditions, linear calibration graph, the detection limit and the

quantitation limit for the studied chloramphenicol were studied. The calibration curve is linear over the range 1.55×10^{-4} to 3.09×10^{-3} μmol L^{-1} which would covers a range to below the EU MRLP if 1 g of honey was diluted to 10 mL for analysis (MRLP 9.28×10^{-4} μmol kg^{-1}). The equation of the line was $y = 53.753x + 0.0216$ where y is relative CL emission intensity in mV and x is CAP concentration in μmol L^{-1} with a correlation coefficient of 0.9919. The sensitivity value of this method, defined as the slope of calibration curve was found to be 53.75 mV μmol^{-1} L. The detection limit (3σ) and the quantitation limit (10σ) were found to be 7.46×10^{-6} μmol L^{-1} and 2.48×10^{-5} μmol L^{-1}, respectively. These limits are well below the level required to measure the MRLP and are achieved due to the high sensitivity of the CL detection. The precision of the system was evaluated by injecting chloramphenical solution at 1.55×10^{-3} μmol L^{-1} and 1.55×10^{-4} μmol L^{-1}, the percentage relative standard deviation (%R.S.D) were found to be 7.81% and 4.86% (n=5) respectively. The accuracy of the method for chloramphenicol assay was studied using the standard addition method. The mean percentage recoveries are presented in Table 40. These results indicated that the proposed method provided highly accurate results. The proposed MIP-CL method was successfully applied to the determination of chloramphenicol in honey samples using the standard addition method. Known concentrations of standard chloramphenicol (1.55×10^{-4}, 7.75×10^{-4} and 1.55×10^{-3} μmol L^{-1}) were added to accurately weighed 1.00 g of honey sample, respectively (using the procedure mentioned in section 2.1.4) and then analyzed using the proposed method. The standard addition curve was established by plotting the relative CL emission intensity of chloramphenicol in mV versus the added concentration of chloramphenicol in μmol L^{-1}. The amounts of chloramphenicol residue could then be calculated from the standard addition curve. It was found that there is no chloramphenicol residue present in the honey samples.

In this work a simple, selective and highly sensitive system has been developed for the analysis of chloramphenicol using a microfluidic device incorporating with a chloramphenicol-imprinted polymer and CL detection system. The simple instrumentation, low flow rates and reagent usage means the system could easily be developed into a portable system with computer controlled pumping. The high

sensitivity means that the system could be used for screening chloramphenicol residue in sample with the detection limit as the EU MRPL.

4.2 LC-MS/MS on Microfluidic Device for Confirmatory of Chloramphenicol Determination in Honey Samples Based On Molecular Imprinted Polymers

A molecularly imprinted polymer on microchip LC-MS/MS method is reported for determination of CAP residue in honey sample. It relies on dispersive CAP-MIP solid-phase micro extraction for clean-up of the honey sample, and HPLC on a CAP-MIP monolith on chip with ESI detection for analysis

ESI-MS/MS was performed on a Finnigan LCQ classic quadrupole ion trap mass spectrometer equipped with standard electrospray ion source in negative-ion mode. The monitored ion was *m/z* 321 and the product ions for quantification were *m/z* 257, 194 and 152. The amounts of chloramphenicol residue could then be calculated from the standard addition curve. It was found that there is no chloramphenicol residue present in the honey samples that had been purchased.

A new LC-MS/MS on chip method is reported for determination of CAP residue in honey sample. It relies on dispersive CAP-MIP solid-phase micro extraction for clean-up of the honey sample, and HPLC on a CAP-MIP monolith on chip with ESI detection for analysis. The accuracy and precision are well within the range generally regarded as acceptable.

SUGGESTION FOR FURTHER WORK

The home-made flow injection analyzer developed in this research (Part I). It has been successfully applied to the determination of bioactive compounds from medicinal plant extracts. 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. The FI method is not only a valid analytical method but will continue to provide inexpensive and reliable analytical instruments in the foreseeable future.

The SIA-LAV system was developed for simple on-line liquid-liquid semi-extraction (Part II). The proposed method has been successfully applied to the determination of solasodine in various *Solanum* species fruits. The technique is simple and economic which become an alternative cost effect systems for on-line automated extraction. Less consumption of the sample, reagent and organic solvent was achieved and compared to the conventional batch method. The proposed method should be applied to various samples, but further optimization, sample pretreatment and/or dilution step would be necessary. Although The SIA-LAV for on-line liquid-liquid semi-extraction developed in this study provided some advantages, further development to reducing the volume of organic solvent should still be studied.

The development of the microsensor on a chip for determination of chloramphenicol in honey samples (Part III). The firstly studied chemiluminescence reaction of $Ru(bipy)_3^{2+}$ and Ce(IV) in sulfuric acid enhanced by chloramphenicol, and on line enrichment chloramphenicol using molecularly imprinted polymer as molecule recognition element in the flow CL microsensor. The chloramphenicol imprinted polymer was prepared with photo polymerization on microchip. The proposed method was proved to be simple, rapid, selective and sensitive for the quantitative analysis. The method has been successfully applied to the determination of chloramphenicol in honey samples. The simple instrumentation, low flow rates and reagent usage means the system could easily be developed into a portable system with computer controlled pumping.