PART II

SEQUENTIAL INJECTION ANALYSIS WITH LAB-AT-VALVE FOR THE DETERMINATION OF SOLASODINE

CHAPTER I

INTRODUCTION

Sequential 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 Second Generation-Sequential Injection Analysis (SIA)

FIA methods, so widely accepted for many years in analytical laboratories by research people and analysts in routine laboratories, have an important functional advantage. They usually require a small volume of the sample and they do not consume large volumes of reagents. This is an advantage compared to conventional wet analytical procedures or titration methods. This is, however, not sufficiently advantageous compared to numerous modern discrete analyzer used mainly in clinical analysis[58-59].

In order to compete with these techniques the newest methodology of flow injection measurements, so-call *sequential injection analysis* (SIA), has been developed. This term is already widely accepted in the literature, although perhaps more appropriate and informative would be sequential injection analysis, as the most important feature of this method is a flow of the sample solution through the detector during detection. Continuous delivery of carrier solution and solution of reagents in typical systems with segmented flow or FIA has been replaced by a technique employed in numerous discrete analyzers. Using mostly syringe pumps and a multiport rotary selection valve, the sample and reagents are introduced to the tubing of the measuring system in an appropriate sequence segment. After changing the direction of the flow and switching one or more selecting valves, solutions are delivered to the detector. On their way segments of the solutions overlap and mix,

and then a steady-state equilibrium signal is measured in the detector. Sequential injection analysis is the new generation of flow measurement techniques in chemical analysis[61], and it quickly gains numerous applications in various fields of chemical analysis[62].

1.1.1 Principle of Measurement and Basic Instrumentation of SIA

The principle of sequential injection flow measurements invented by Ruzicka and Marshall[60] is that an analytical signal is measured in the course of the flow of series of liquid segments of sample and reagents through a detector. Therefore it is a typical flow measurement with essentially reduced consumption of reagents compared to other flow techniques, including FIA, even in the very economical version of merging zones. In the latter, there are continuously delivered at least two carrier solutions, whereas in SIA there is only one.

SIA depends on the principle of controlled partial dispersion, in combination with forward and reversed flow through a multiposition valve, which facilitates the use of different chemistry without reconfiguration of the manifold. A schematic representation of a simple SIA instrument is presented in Figure 28. Generally, the heart of an SIA instrument is a multiposition selection valve similar to that used by Ruzicka and Marshall[60] and operated in synchronisation with a pump. The multiposition valve can be connected to various sample and reagent reservoirs, typically *via* Teflon tubing. As shown in Figure 29, when the pump is operated in reverse, aliquots are sequentially drawn into a holding coil and upon forward propulsion the resultant stack of sample and reagents are dispersed into a zone of detectable product. The instrumental configuration illustrated in Figure 29 has the potential for multi-reagent chemistry by sandwiching the sample between a variety of different reagents and/or carriers and the ability to manipulate the sample volume by altering the pump aspiration time. Interestingly, Ruzicka and Marshall[60] stated that: *"The pump together with the valve serves as a precision volumetric transport device and should have zero inertia and zero elasticity, requirements that preclude the use of peristaltic pumps"* and thus *"Any computer controllable piston pump capable of forward and reversed movement, would be suitable*". In 1992, Baron *et al.*[61] replaced the sinusoidal pump with a linear syringe pump and one year later Ivaska and

Ruzicka[63] demonstrated that peristaltic pumps could indeed serve as propulsion devices for SIA. The main advantage of the latter is higher analytical frequency as there is no need to aspirate wash solution, in contrast to syringe pumps, which require priming before use and have a limited reservoir capacity. Recently GlobalFIA[64] developed the MilliGAT pump, a positive displacement piston array pump designed specifically for use in FIA/SIA. This fully programmable self-priming pump has the advantages of bi-directional pulseless flow at μL min^{-1} to mL min^{-1} rates without syringes, check valves and pulse dampers. Nevertheless the majority of researchers have employed either syringe or a peristaltic pump, the choice of which is application dependent and will be discussed in the following sections. Detection for SIA simply requires that the dimensions of the flow through cell be such that they allow solution propulsion with low-pressure pumps. Detection methods have included UV–visible, infrared, luminescence and atomic spectroscopy along with electrochemical and turbidimetric measurements.

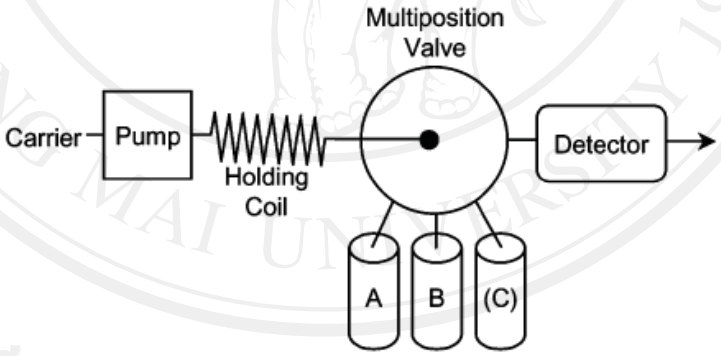

Figure 28 Schematic diagram showing a simple SIA manifold, where A, B and C represent analyte and reagent reservoirs

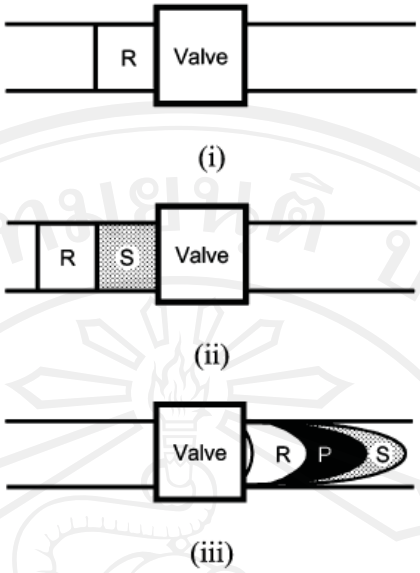

Figure 29 (i) and (ii) schematic diagram showing the pump operating in reverse to aspirate a zone of reagent followed by sample
(iii) Formation of a zone of product upon reversal of flow and propulsion toward the detector

1.1.2 Dispersion in the SIA

Ruzicka and Marshall[60] referred to the random walk model, postulating that efficient mixing in a flow system could be achieved by moving a stack of reagents back and forth, without actually travelling any net distance. As a consequence, controlled partial dispersion and reagent sequencing are the key parameters in SIA[65]. Unlike FIA, in which the sample is completely surrounded by reagent, in SIA the sample and reagent zones are sequentially stacked and, as they move through the instrument, the zones penetrate each other and become inter-dispersed[66]. This degree of penetration has been measured and terms have been defined to aid the understanding of zone dispersion. These include: D (dispersion); $S_{1/2}$ (the volume required to reach a dispersion of 2 at the peak maximum); P (degree of zone penetration). In a comprehensive study on the factors affecting dispersion in SIA, Gübeli *et al.*[65] observed that increasing the sample volume up to $S_{1/2}$ enhanced the sensitivity; however, beyond $S_{1/2}$ there was no improvement. These workers[65] also demonstrated that for single reagent chemistry the best results were achieved using at

least twice as much reagent as sample, whilst keeping the total volume less than or equal to half that of $S_{1/2}$. For two reagent chemistry, they concluded that the sample volume should be less than $S_{1/2}$ and that the reagent concentration must be sufficiently high. Not surprisingly, they observed that more than one flow reversal resulted in an increased dispersion and broadening of the signal. Marshall and van Staden[67] evaluated the precision and zone penetration to determine the effect of tubing diameter, reactor geometry and pump speed, concluding that tubing with internal diameters of either 0.8 mm or 1.5 mm gave improved precision without excessive decrease in zone penetration compared with that attained with 0.5 mm. They also found that knitted reactors were inferior to straight tubing with respect to axial dispersion, whilst the study of the effect of pump speed yielded little as the flow rate changed due to its sinusoidal nature. These workers were the first to report the importance of aspiration order, with the highest sensitivity being achieved when the reagent was introduced first[67]. The terms defined above for zone penetration (D, $S_{1/2}$, P) have provided some limited understanding of the physical dispersion process; however they do not take into account the reaction chemistry being employed and as such a rigorous optimization of all parameters would be essential for a real analytical method.

1.1.3 Software

While Ruzicka and Marshall[60] made only a brief mention of the computer-controlled instrumentation they utilised, Marshall[66] stated in his Ph.D. thesis that: *"While early FIA analysers were often manually operated and data acquisition and display was typically achieved by means of a chart recorder, this is clearly not adequate for SIA"* and *"Volumes in SIA are frequently determined by the time that a particular stream is selected or on the number of strokes a pump executes. Control of such parameters is best achieved under micro processor control"*. Hence, the requirement for accurate and precise synchronization of pumps, valves and other hardware to obtain reproducible flow patterns, together with electronic data acquisition and manipulation, necessitated the development of specialized software for overall instrument control. In most cases specific "in-house" programs have been developed to be compatible with the instrumentation available, indeed Guzman and

Compton[68], noted that the lack of commercially available software was the most limiting factor in the development of SIA. Although several researchers have developed their own programs with software written in Turbo C++, Visual Basic, Basic, the Windows 95 environment, and Lab-VIEW, there are only three papers that directly focus on the development of computer control and data acquisition for SIA.

In 1992, Marshall and van Staden[67] addressed the need for flexible computer control in publishing their novel software for the automation of flow-based analysis systems. Our group has successfully employed software written within LabVIEW for instrument control and data acquisition and recently reported the design and implementation of a user-friendly program with a graphical user interface for the chemiluminescence determination of morphine. Recently, the need to design purpose built software has been lessened, as commercially available software has become more widely available in combination with instrumentation.

1.2 Sequential Injection Analysis with Lab-at-Valve (SI-LAV) for the Determination of Solasodine

1.2.1 Steroidal Glycoalkaloids (Solasodine)

Glycoalkaloids, a class of nitrogen-containing steroidal glycosides are naturally occuring secondary metabolites commonly found in the Solanaceae family which includes many significant agricultural plants, such as tomato, potato, eggplant, pepper, nightshade, thorn apple, and capsicum. For example, solasodine has been found in about 200 *Solanum* species[69]. Glycoalkaloids are generally found in all plant organs, with the highest concentrations occuring in flowers, sprouts, unripe berries, young leaves or shoots (metabolically active parts). They are regarded as defensive allelochemicals against a number of pathogens and predators including fungi, viruses, bacteria, insects, and worms[70]. Due to defensive character, development of new cultivars of tomato and potato with high foliar steroidal glycoalkaloid levels is underway. The types of steroidal glycoalkaloids produced by solanaceous plants differ from species to species. The differences can be manifested as a presence or absence of a C-C double bond, variety of functional groups (e.g., hydroxyl, acetyl) and sugar groups, as well as in the sterochemistry of these functional groups[71].

1.2.2 Chemical Structure of Glycoalkaloids

Steroidal alkaloids are characterized by the presence of an intact or modified steroid skeleton with nitrogen. Since nitrogen is inserted into a non-amino acid residue these compounds belong to a subgroup of pseudo-alkaloids (or isoprenoid alkaloids)[72]. Structural variation in the family of plant steroidal glycoalkaloids is limited to two main groups, based on the skeletal type of the aglycone, examples of which are represented in Figure 30. One is the spirosolan type, similar to spirostan, but with nitrogen in place of the oxygen in ring F (forming a tetrahydrofuran and piperidine spiro-linked bicyclic system) (as in solasodine, Figure 30). Second are the solanidane type, where N connects spirostan rings E and F rings (as in solanidine, Figure 30). All types can contain double bonds and hydroxyls in various positions.

At least 90 structurally different steroidal alkaloids have been found in over 350 *Solanum* species[73]. Nitrogen can be attached as a primary NH_2 group in position 3 or 20 (free or methylated), forming simple steroidal bases (e.g., conessine), ring-closed to skeletal or side-chain carbon (as a secondary NH) or annelated in two rings as a tertiary N (e.g., solanidine). This often influences the chemical character of the compound[69]. Plants often contain alkaloids in glycosidic form as glycoalkaloids.

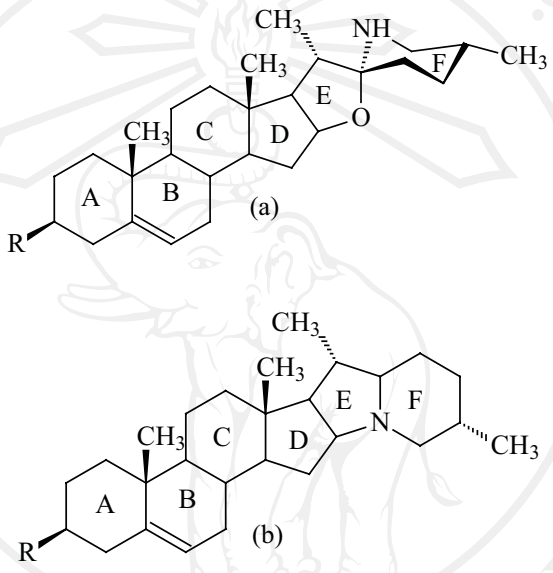

Figure 30 (a) Solasodine and (b) Solanidine

Thus, steroidal glycoalkaloids contain three portions: a non-polar steroid unit and a basic portion with either a so called indolizidine or oxa-azaspirodecane structure which together form the aglycone part; a polar, water-soluble sugar moiety with three or four monosaccharides attached to the 3-OH group of the first ring of the aglycone. The common glycoalkaloid aglycones in eggplant and potato tubers are presented in Figure 30.

1.2.3 Beneficial Effects of Glycoalkaloids

Although glycoalkaloids are toxic compounds at certain levels, they have some proposed beneficial effects. In recent years, a medicinal uses of glycoalkaloids has been a focus of scientific and pharmacological attention. For example, solamargine and solasodine exhibit potent cytotoxicity to human hepatoma cells (Hep3B) by apoptosis which is the major process responsible for cell death in various physiological events[72]. Solasodine, solamargine, and solasonine from *Solanum incanum* L. showed liver protective effects against CCl_4-induced liver damage[73]. Furthermore, α-chaconine, α-solanine, α–solamargine, α-solasonine, α-tomatine (being the most effective), and some of their hydrolysis products inhibit the growth of human colon (HT29) and liver carcinoma (HepG2) cells[74]. Plasma low-density lipoprotein cholesterol and triglycerides in hamsters is lowered by α-tomatine. The immune response is enhanced by α-tomatine inducing cytokines in immunized animals[75]. Furthermore, solasodine present in Solanaceae plants has gained significant importance globally. It can be converted to 16-dehydropregnenolone, a key intermediate in the synthesis of steroid drugs[76].

1.3 Previous Analytical Methods for Solasodine Determination

Solasodine, a spiroketal alkaloid sapogenin with a hetorocyclic nitrogen atom is one of the major starting materials for the commercial synthesis of steroid drugs. Solasodine is present in a number of *Solanum* species (Familly: Solanaceae) such as *S. khasianum*, *S. xanthocarpum*, *S. nigrum*, *S. gracile*, *S. laciniatum*, ect.[77]. A number of traditional herbs containing solasodine have been used in the Indian System of Medicine[78-81]. Solasodine exhibits anticancer[81], insecticidal[82], antiaccelerator cardiac[83], and antioxidant activities[84]. A number of analytical methods like high performance thin layer chromatography[82, 85-86], high performance liquid chromatography[87-96], capillary electrophoresis[97-99], gas chromatography[100-103] and colorimetric method[104-111] are available for determination of solasodine. Solasodine does not have a conjugated double bond in it structure. The nitrogen is protonated and forms complexes that are extractable into organic solvent like chloroform.

Liquid-liquid extraction is one of the most versatile techniques for sample matrix separation. It has been applied to various analytical fields. However, manual extractions present a series of drawbacks such as high consumption of sample and toxic organic solvent, low sampling frequency and loss of analyte through manipulation. Among them, the successful techniques are probably the on-line liquid-liquid extraction using flow systems[112-114]. A variety of flow-base systems have been reported for the on-line liquid-liquid extraction. Flow injection liquid-liquid extraction was proposed by Karlberg and Thelander[115] and Bergamin[116]. Consumption of reagent and organic solvent are lower than those in manual procedures, consequently, reducing the waste generated.

Sequential injection analysis (SIA) has also been employed for on-line liquid-liquid extraction by sequential aspiration of a small volume of organic solvent and aqueous sample into contact before either resolve them using phase separator[117-118].

In recent years, special attention was given for the development of automation for analytical procedures. A lot of new instrumental methods were described; sequential injection analysis was one of them. SIA has been introduced in 2000 by Ruzicka[119] with lab-on-valve (LOV) module. Sequential injection analysis with a simple approach called lab-at-valve (LAV), introduced by Rodjana *et al*.[120-121]. 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 SIA-LAV for on-line extraction system has been demonstrated for the determination of solasodine in *Solanum* species fruits.

In the present work, the SIA-LAV system was developed for simple on-line liquid-liquid micro-extraction. A desired component; a separation chamber was attached at one port of a multi-position selection valve. The sample solution, reagent and organic solvent were sequentially aspirated into an extraction coil. After that, the aqueous and organic phases were separated in a conical separating chamber. The organic phase containing solasodine complex was measured spectrophotometrically. The proposed method has been successfully applied to the determination of solasodine in various *Solanum* species fruits.

1.4 Research Aims

The aim of this research can be summarized as follows:

1.4.1 A novel Sequential Injection Analysis (SIA) technique will be developed.

1.4.2 Solasodione contents in various Solanum species will be determined using the proposed SIA method.

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 Sequential Injection Analysis with Lab-at-Valve (SI-LAV) for the Determination of Solasodine

2.1.1 Instruments and Apparatus

The instruments and apparatus used were as follows:

1. FIAlab® 3000 system: FIAlab® Instruments, USA
2. Six-port selection valve (Valco Instrument Co., USA
3. Tygon tubing (0.508-1.521 mm i.d.): Cole-Parmer Instrument Company, Chicago, IL, U.S.A
4. Disposable plastic syringe: SGE, Australia
5. Flow through cell: model QS1.000 Hellma flow cell (10-mm path length, 120 μL inner volume), Perkins Elmer, USA
6. Spectrophotometer (Jenway 6300, Dunmow, Essex, United Kingdom
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
14. Pipette tip 1.0 mL: Eppendoft, Germany
15. Water bath: Memmert, Germany.

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. Solasodine reference standard of 99% purity: Sigma (No. 204-774-2, Sigma Chemical Co., USA
2. Chloroform: E. Merck, Germany
3. Methyl orange: Sigma-Aldrich, United Kingdom
4. Hydrochloric acid: Sigma-Aldrich, United Kingdom
5. Sodium hydroxide (NaOH): BDH, Poole, United Kingdom
6. Methanol: Analytical grade, E. Merck, Germany
7. Ethanol: Analytical grade, E. Merck, Germany

2.1.3 Sequential Injection Apparatus

The developed SIA manifold (Figure 31) was arranged using the following equipment: a FIAlab® 3000 system consists of a syringe pump (syringe reservoir 2.5 mL) and a six-port selection valve, which is connected to a four-port switching box.

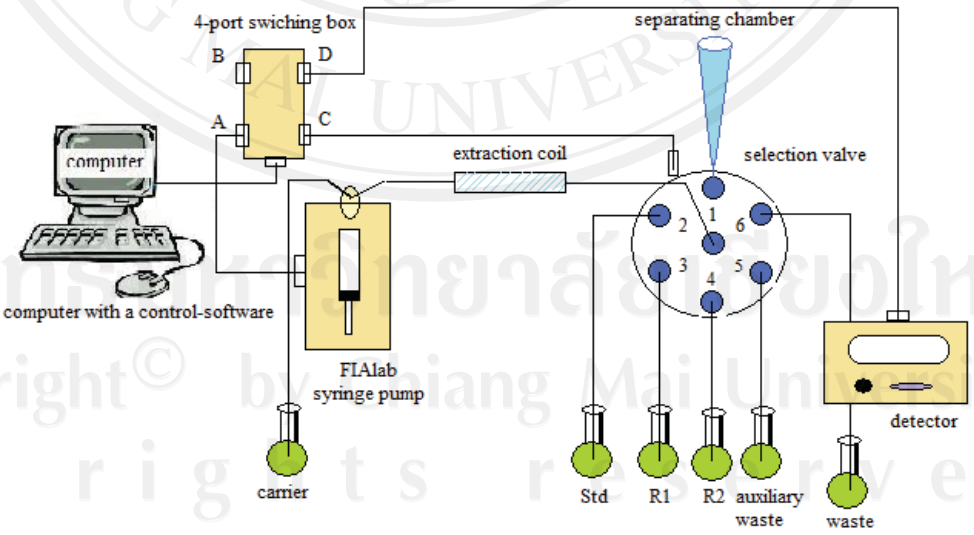

Figure 31 Schematic diagram of SIA system with LAV

The four ports undergo the following functions:

Port A is connected to a syringe control (CAVRO XL 3000 stepper motor-driven syringe pump).

Port B is available for other instruments.

Port C is connected to a valve control unit.

Port D is connected to Jenway 6300 Spectrophotometer.

A Jenway 6300 Spectrophotometer equipped with a model QS1.000 Hellma flow cell over the wavelength range 320-1000 nm. The syringe pump was connected to the center of the selection valve via the extraction coil (0.635 mm i.d.×150 cm) PTFE tubing around the small test tube) and a separating chamber type conical shape [122] (8 mm i.d. of the wider end×7 cm long) modified from a 1.0 mL pipette tip was placed at port-1 of the selection valve. The absorbance signal of the colored complex was passed through a flow-through cell in a spectrophotometer. An absorbance signal can be retrieved directly from a Jenway spectrophotometer via the RS232 interface. The absorbance signal was measured at 420 nm through a 10 mm path length flow cell. All electrical devices of the manifold were connected to computer controlled by means of a home-made program written in Microsoft Visual Basic 6.0.

2.1.4 Preparation of Standard, Reagents and Samples

a) Preparation of Standard Solutions

Stock standard solutions (1000 μg mL^{-1}) of solasodine was prepared by weighing exactly 50 mg of pure solasodine into a 50 mL volumetric flask and dissolved using 20% acetic acid and then diluted to the mark with 20% acetic acid and stored in capped plastic vial in the freezer. Working standard solutions were freshly prepared by diluting the stock solution with 20% acetic acid to obtain appropriate serial concentrations.

b) Reagents

Stock solution of methyl orange (0.04%, w/v) was prepared by dissolving 0.04 g of methyl orange in 100 mL of deionized water.

c) Sample Pretreatments

About 1.0 gram of each dried fruit was accurately weighed and transferred into a clean mortar. The sample was ground and 20 mL of 95% ethanol was added and mixed thoroughly, then transferred into a-250 mL beaker. The solution was heated in a water bath at 70 ^{o}C for 30 min., and then filtered through Whatman No. 1 filter paper into a-50 mL volumetric flask, followed by washing with several portions of 95% ethanol. Each solution was adjusted to 50 mL with 95% ethanol.

Aliquot of 5.0 ml of ethanolic extract from each sample was transferred into a-20 mL test tube and completely removed ethanol by holding tubes at 70 ^{o}C while gently blowing an air current into each tube. To each tube, 3.0 ml 1.0 mol L^{-1} hydrochloric acid was added and the temperature of water bath was increased to 100 ^{o}C and then was heated the tube at 100 ^{o}C for 2 hours. The acidic solution was neutralized by adding 3.0 mL 1.0 mol L^{-1} sodium hydroxide followed by addition of 2.0 mL glacial acetic acid into each tube. Each solution was transferred into a-25 mL volumetric flask and then diluted to the mark with distilled water. The sample solution containing aglycone (hydrolysis product of solasodine) was complexed with methyl orange and the coloured complex was extracted into chloroform and then determined by sequential injection analysis with lab-at-valve (SI-LAV).

2.1.5 Sequential Injection Analysis Method

A four-port RS232 switching box received an activation command from the PC through master port. When the system was initialized, it activated port A (Figure 31) the piston of the syringe was moved to zero position. It also activated port C to actuate with the valve at position 6. Then, it activated port A to drive the syringe to aspirate the carrier with the desired volume. After that, it activated port C to actuate the valve at position 6 and sending empty syringe. Then, it again activates port C to actuate the chloroform (valve at position 3) was firstly aspirated into the extraction coil. Next, the solasodine standard solution (valve at position 2) was introduced. Then, methyl orange (valve at position 4) was aspirated and the extraction was done by programming the syringe control to aspiration and dispensed modes. After extraction in the extraction coil, the solution was propelled to the separating chamber,

where the separation between aqueous and chloroform phases occurred. The chloroform phase containing ion-association compound was re-aspirated into the extraction coil for transportation to a detector. While the PC was sending the empty syringe command through port A, it activated port D and received absorbance signals from the spectrophotometer and drive the plot module to plot the SIA grams on the screen. The maximum peak heights were also detected at 420 nm and displayed in this process. The time required to analyze one sample was approximately 5 min.

2.1.6 Experimental Protocol

Experimental protocol as shown in the FIAlab® for Windows software

Fill syringe

Loop Start # 1

SyringePump Flow rate (microliter/sec) 100

SyringePump Valve In

SyringePump Delay Until Done

SyringePump Aspirate (μL) 2500

SyringePump Valve Out

SyringePump Delay Until Done

Standard/sample and reagents to extraction coil

Loop Start # 8

SyringePump Flow rate (microliter/sec) 100

Valve port 3

SyringePump Aspirate (μL) 75

SyringePump Delay Until Done

Valve port 4

SyringePump Aspirate (μL) 12.5

SyringePump Delay Until Done

Valve port 2

SyringePump Aspirate (μL) 37.5

SyringePump Delay Until Done

Loop End

SyringePump Valve In

SyringePump Fill

SyringePump Delay Until Done

Extraction

Loop Start # 3

SyringePump Valve Out

Valve port 1

SyringePump Flow rate (microliter/sec) 100

SyringePump Dispense (μL) 1000

SyringePump Delay Until Done

SyringePump Valve Out

Valve port 1

SyringePump Aspirate (μL) 1000

SyringePump Delay Until Done

SyringePump Valve Out

Valve port 1

SyringePump Flow rate (microliter/sec) 100

SyringePump Aspirate (μL) 1000

SyringePump Delay Until Done

SyringePump Valve Out

Valve port 1

SyringePump Dispense (μL) 1000

SyringePump Delay Until Done

Delay (sec) 30

Send standard/sample to detector

Valve port 1

SyringePump Flow rate (microliter/sec) 100

SyringePump Aspirate (μL) 250

SyringePump Delay Until Done
SyringePump Valve In
SyringePump Fill
SyringePump Delay Until Done
SyringePump Valve Out
Valve port 6
SyringePump Flow rate (microliter/sec) 50
SyringePump Empty
SyringePump Delay Until Done

Clean extraction coil

Valve port 1
SyringePump Flow rate (microliter/sec) 100
SyringePump Aspirate (µL) 1000
SyringePump Delay Until Done
SyringePump Valve In
Valve port 5
SyringePump Empty
SyringePump Delay Until Done
SyringePump Valve In
Valve port 1
SyringePump Dispense (µL) 1500
SyringePump Delay Until Done
SyringePump Aspirate (µL) 1500
SyringePump Delay Until Done
SyringePump Valve In
Valve port 5
SyringePump Empty
SyringePump Delay Until Done

Loop End

CHAPTER III

RESULTS AND DISCUSSION

3.1 Sequential Injection Analysis with Lab-at-Valve (SI-LAV) for the Determination of Solasodine

A novel sequential injection analysis with Lab-at-Valve (LAV) semi-automated system on-line liquid-liquid extraction is demonstrated for spectrophotometric determination of solasodine in various *Solanum* species fruits was developed. The main proposed was liquid-liquid extractive determination of solasodine using methyl orange as colorimetric reagent. After optimization of the system, sample, reagent and organic solvent were sequentially aspirated into an extraction coil connected to the center of a selection valve, where extraction took place by flow reversal. The aqueous and organic phases were separated in a lab-at-valve unit attracted to one of the ports of the selection valve. The absorption of ion pair solasodine methyl orange complex in the organic phase was measured spectrophotometrically at 420 nm. The method performances, including reproducibility, linearity, sensitivity and accuracy, were also evaluated. The proposed method is simple, reproducible and accurate. It was successfully applied to the determination of solasodine in *Solanum aculeatissimum Jacq.*, *Solanum violaceum Ortega.*, *Solanum melongena Linn.* and *Solanum indicum Linn.* fruits in Solanaceae family.

3.1.1 Preliminary Study

Solasodine has heterocyclic nitrogen but has no conjugated double bonds in its structure. Therefore it does not give absorption in UV range. After hydrolysis it yields aglycone which can be complexed with methyl orange forming an ion-pair complex (solasodine methyl orange complex). The coloured complex is extracted into chloroform and has its maximum absorption at 420 nm[(104)].

3.1.1.1 Spectral Characteristics

The absorption spectrum of ion-pairing solasodine-methyl orange complex in chloroform was scanned over the wavelength of 240 - 600 nm (Figure 32). The yellow colour of solasodine-methyl orange complex formed at pH 3-4 which showed maximum absorption at 420 nm.

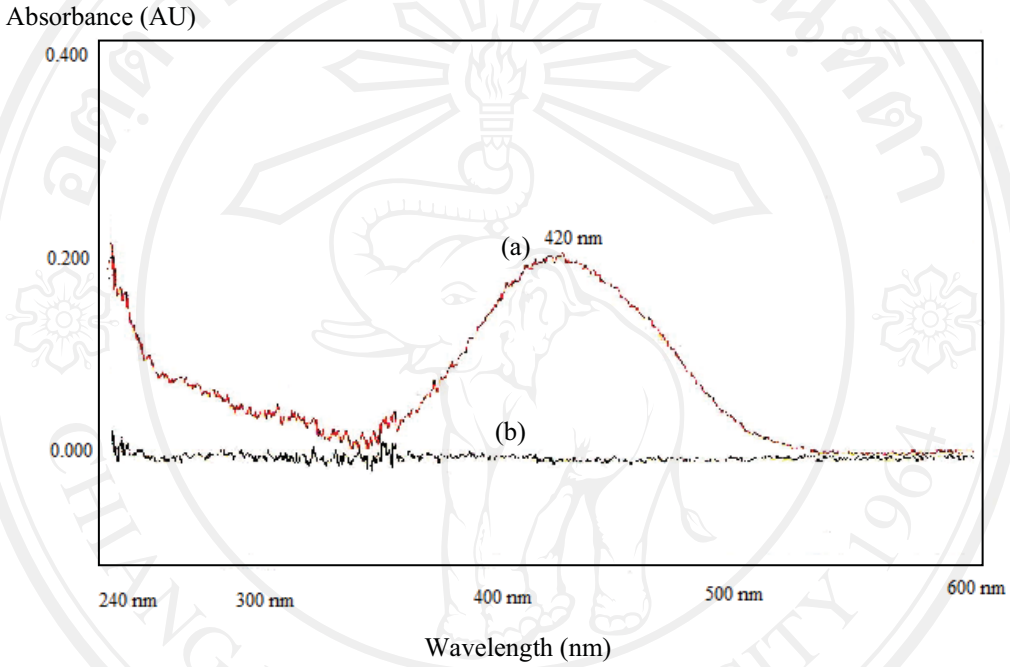

Figure 32 Absorption spectra of solasodine complex solution (a) and reagent blank solution (b)

3.1.2 Sequential Injection Manifold Design

The developed SIA manifold (Figure 31) was arranged using the following equipment: a FIAlab® 3000 system consists of a syringe pump and a six-port selection valve, which is connected to a four-port switching box. A Spectrophotometer equipped with a flow cell over the wavelength range 320 - 1000 nm. The syringe pump was connected to the center of the selection valve via the extraction coil and a separating chamber type conical shape modified from a 1.0 mL pipette tip was placed at port-1 of the selection valve. The absorbance signal of the colored complex was passed through a flow-through cell in a spectrophotometer. An absorbance signal can be retrieved directly from a spectrophotometer via the RS232 interface. The

absorbance signal was measured at 420 nm through a 10 mm path length flow cell. All electrical devices of the manifold were connected to computer controlled by means of a home-made program written in Microsoft Visual Basic 6.0.

3.1.3 Optimization of the Experimental Parameters

The conditions for the determination of solasodine were optimized by studying the influences of various parameters such as operational sequence, the number of flow reversals, reagent/carrier flow rates and reagent concentrations of the respective measurements. The optimal value for each parameter was judging from maximum response of the detector, minimum noise of the baseline and relative standard deviation.

3.1.3.1 Aspiration Order of Reagents and Sample

In reactions involving multiple zone penetrations, it is essential to examine the aspiration order of reagents and sample[(123)]. The sequence order of operation is important factors that determine the time and efficiency that the aqueous and organic phases are in contact for improving the extraction efficiency (Table 22). Two sequential orders as shown in Figure 33 were examined. It can be seen that the operational sequence in which the sample, reagent and organic solvent were sequentially aspirated as small segments (see Figure 33b) provided a higher slope of calibration graph (a plot of absorbance *vs.* solasodine concentration). This could be due to a higher degree of contact between the two phases. In addition, the sensitivity increased with increasing number of the flow reversals. It was found that a higher sensitivity could be reached by using a repeatedly segmented sequence, although 4 cycles of the flow reversal was performed. Therefore, the chloroform was firstly aspirated into the extraction coil, then the standard/sample solution was introduced, and methyl orange was then aspirated. The extraction step was performed in the extraction coil by programming the syringe control unit to aspiration and dispensed modes. The extraction process took place in the extraction coil. After that, the aqueous and organic phases were propelled into the separating unit where separation of the two phases occurred. The ion-association complex was left for 30 s and the

absorption was measured spectrophotometrically at 420 nm. The separating unit was then cleaned before starting the next determination.

Table 22 Aspiration Order of Reagents and Sample

Sequential order	Sensitivity* (AU/µg mL^{-1})
Methyl orange - Solasodine - Chloroform	0.906
Methyl orange - Chloroform - Solasodine	1.531
Chloroform - Methyl orange - Solasodine	3.102
Chloroform - Solasodine - Methyl orange	1.950
Solasodine - Methyl orange - Chloroform	1.531
Solasodine - Chloroform - Methyl orange	1.340

*Mean of three determinations

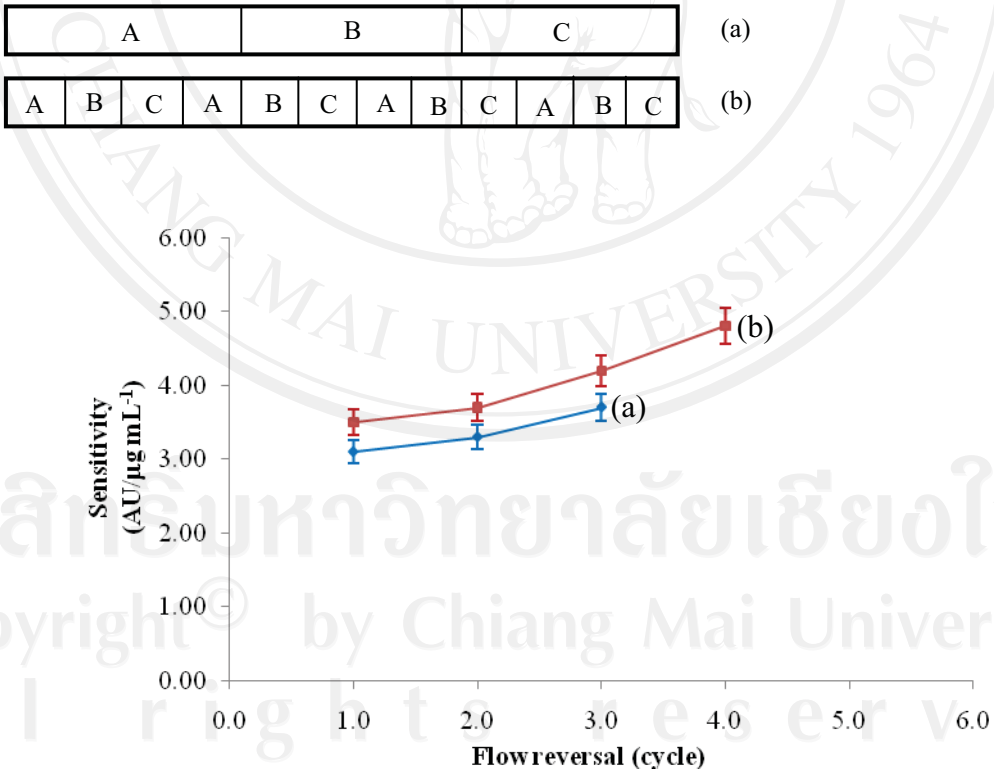

Figure 33 Effect of Sequence order of the SIA-LAV system for the semi-automated liquid-liquid extraction of solasodine: A, Chloroform; B, methyl orange; and C, sample

3.1.3.2 Sample, Reagent and Organic Solvent Aspiration Volumes Optimizations

To minimize the consumption of reagent volumes while maintaining the best results, both of sensitivity and precision of the procedure, thus these parameters were optimized. The volume of organic solvent, sample and methyl orange solutions were studied.

3.1.3.2.1 Organic Solvent Aspiration Volume

The influence of the organic solvent volume was investigated in the range of 300 - 700 µL (Table 23). It was found that the maximum response was obtained at a volume of 600 µL for chloroform volume and it gave the best sensitivity (Figure 34).

Table 23 Effect of chloroform volume

Chloroform volume (µL)	Sensitivity* (AU/µg mL^{-1})
300	0.007
400	0.042
500	0.040
600	0.054
700	0.018

*Mean of three determinations

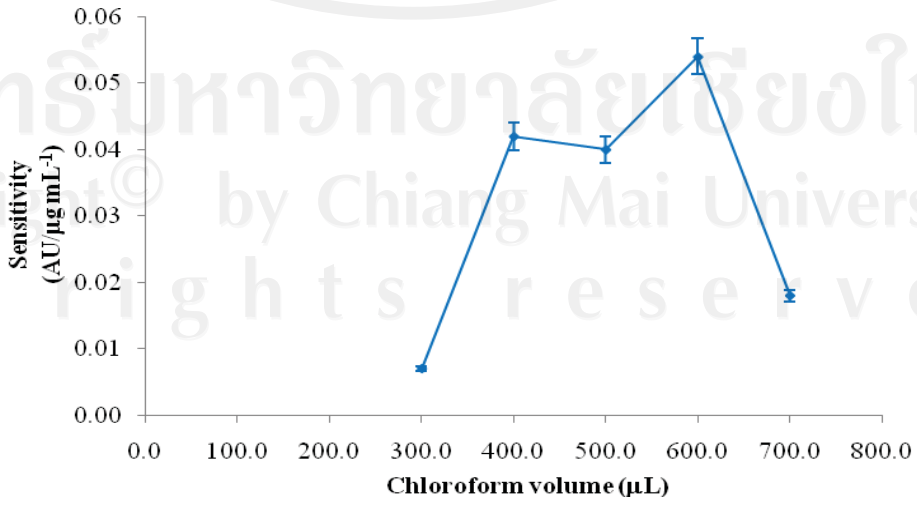

Figure 34 The influence of the organic solvent volume (chloroform)

3.1.3.2.2 Sample and Reagent Volumes

The influence of the sample and methyl orange solutions were studied between 50 - 350 μL. The volume of chloroform was kept constant at 600 μL. It was found that the maximum response was obtained at a volume of 300 μL for sample volume and it gave the best sensitivity (Figure 35). For methyl orange volume, it was found that as the aspiration of methyl orange volume increased the sensitivity increased up to 100 μL (Figure 36). A methyl orange volume of 100 μL was chosen as an optimum reagent volume for subsequent measurements.

3.1.3.2.3 Volume of Sending Sample to Detector

The volumes of sending sample to detector were investigated from 50 - 400 μL s^{-1} at every 50 μL s^{-1} interval. It was found that the maximum response was obtained at a volume of 250 μL for sending sample to detector and it gave the best sensitivity.

Table 24 Effect of sample volume

Solasodine volume (μL)	Sensitivity* (AU/μg mL^{-1})
50	0.049
100	0.101
300	0.103
350	0.071

*Mean of three determinations

Table 25 Effect of methyl orange volume

Methyl orange volume (μL)	Sensitivity* (AU/μg mL^{-1})
50	0.071
100	0.103
300	0.101
350	0.049

*Mean of three determinations

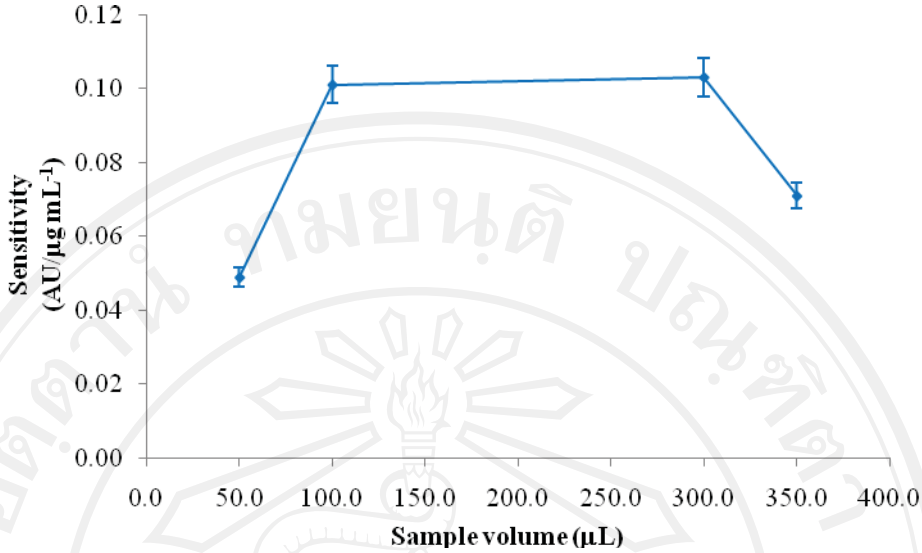

Figure 35 The influence of the solasodine volume

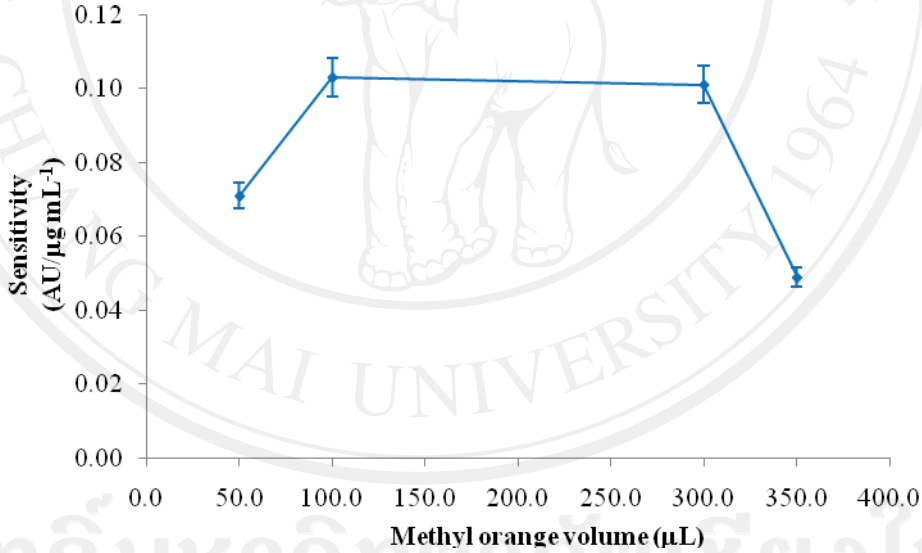

Figure 36 The influence of the methyl orange volume

3.1.3.3 Flow Rate

In any flow-based analysis procedure the response is dependent on the reagent and sample flow rates and thus it is necessary to optimize them to achieve the greatest sensitivity, sample throughput, etc.

3.1.3.3.1 Optimization of Sample and Reagents Flow Rates

It was obvious that the flow rate of aspiration of sample and reagents were significant with the peak height. The sample and reagents flow rates were investigated from 50 - 100 μL s^{-1} at every 10 μL s^{-1} interval while the flow rate of sending sample to detector kept constant at 100 μL s^{-1}. It was found that the flow rate increases with the peak height increases up to 100 μL s^{-1}.

Table 26 Effect of flow rate of sample and reagent on sensitivity

Flow rate of sample (μL s^{-1})	Sensitivity*; (AU/μg mL^{-1})	Correlation coefficient (r^2)	Flow rate of reagent (μL s^{-1})	Sensitivity*; (AU/μg mL^{-1})	Correlation coefficient (r^2)
50	0.0716	0.9978	50	0.0840	0.9973
60	0.2753	0.9971	60	0.2840	0.9952
70	0.4094	0.9954	70	0.4389	0.9973
80	0.4959	0.9945	80	0.4836	0.9972
90	0.6554	0.9825	90	0.6232	0.9974
100	0.8181	0.9820	100	0.8074	0.9969

*Mean of three determinations

3.1.3.3.2 Flow Rate of Sending Sample to Detector

The flow rates of sending sample to detector were investigated from 25 - 125 µL s^{-1} at every 25 µL s^{-1} interval while the flow rate of aspiration of sample and reagent were kept constant at 100 µL s^{-1}. It was observed that the peak height increased with increasing in the flow rate up to 50 µL s^{-1}. Thus, a flow rate of 50 µL s^{-1} was chosen and used for subsequent measurements.

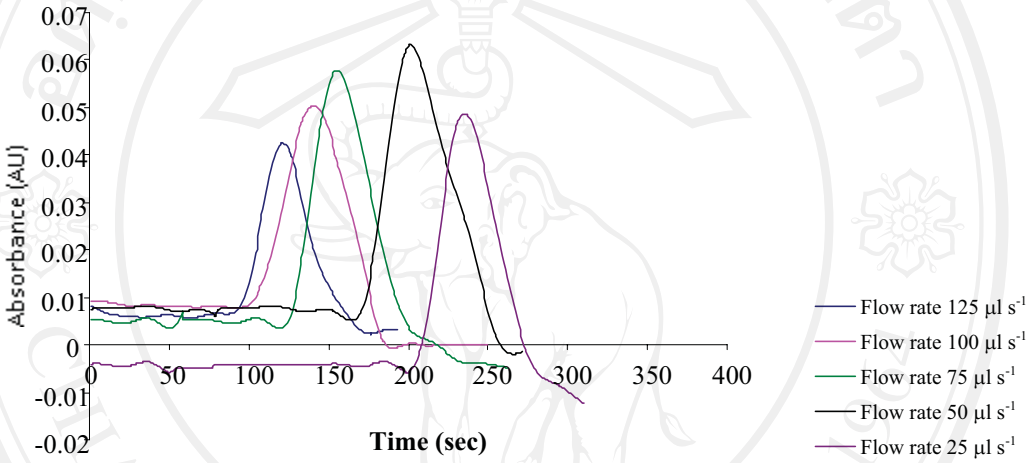

Figure 37 The flow rates of sending sample to detector

3.1.3.3.3 The Effect of Methyl Orange Concentration

The effect of methyl orange concentrations was studied in the range 0.01 - 0.06%. The slopes of the calibration equation indicated that the sensitivity increased with increasing the methyl orange concentration; $y = 1.3472x + 0.0114$, $y = 2.8797x - 0.0143$, $y = 4.2181x - 0.0337$, $y = 4.5184x - 0.0381$, $y = 3.9994x - 0.0168$ and $y = 3.9238x - 0.0542$ where y is absorbance in AU and x is percentage of methyl orange concentration (%), for methyl orange concentrations of 0.01%, 0.02%, 0.03%, 0.04%, 0.05% and 0.06%, respectively. The concentration of methyl orange at 0.03% gave the best sensitivity hence this concentration was chosen for further works (Figure 38).

3.1.3.4 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 50-300 cm and 0.508-1.521 mm i.d. respectively (Table 27). The results obtained showed that a coil length with the internal diameter of 250 cm and 0.635 mm respectively, gave the highest sensitivity and was used in all subsequent experiments.

Table 27 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)
50	0.0606	0.9952	0.508	0.0923	0.9987
100	0.0826	0.9963	0.635	0.1016	0.9994
150	0.08244	0.9978	1.521	0.0867	0.9969
200	0.0816	0.9963			
250	0.1647	0.9985			
300	0.1103	0.9983			

*Mean of three determinations

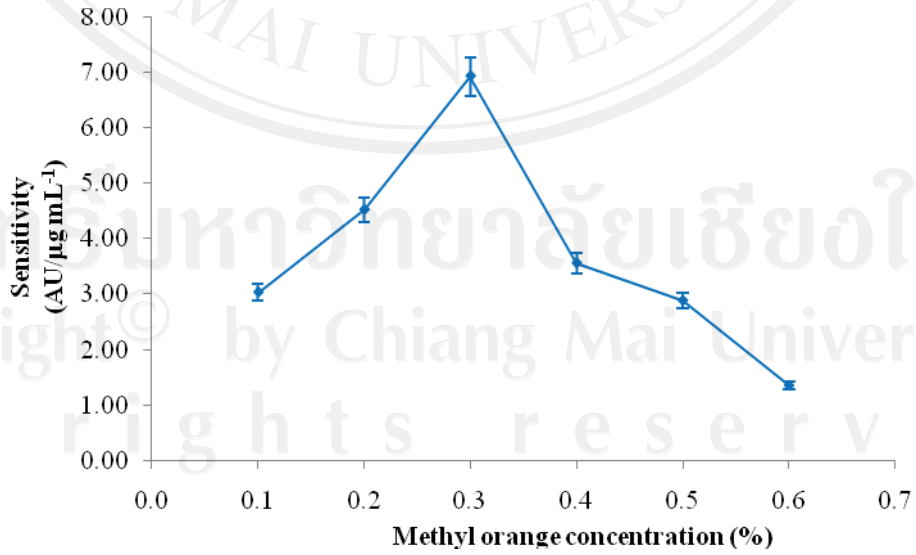

Figure 38 The effect of methyl orange concentration

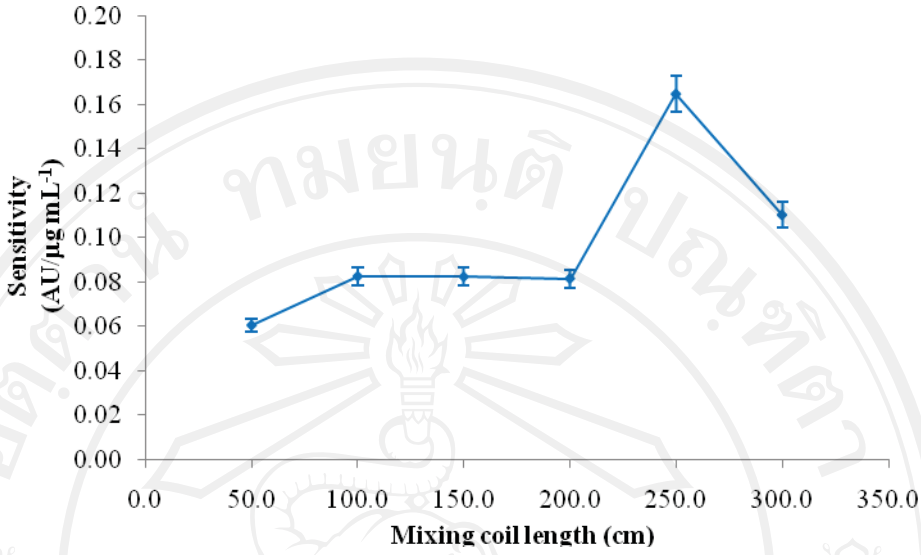

Figure 39 The effect of mixing coil length

3.1.4 Analytical Characteristics

In Figure 40 shows the calibration graph and typical SI-grams of solasodine. Using the selected conditions, the analytical characteristics of the proposed SIA-LAV system were evaluated by examining the linear range, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and sampling frequency.

3.1.4.1 Linearity of Calibration Graph

The linearity of calibration graph was determined using the optimal experimental parameters in Section 2.1.6. Seven standard solutions ranging from 10.0 - 60.0 µg mL^{-1} in concentration, in three replicates each, were injected into the SI system. The calibration graph was obtained by plotting the absorbance of the solutions against the standard concentrations. Linear calibration graph over the concentration range 10.0 - 60.0 µg mL^{-1} of solasodine was obtained with the regression equation; $y = 0.0108x + 0.0202$ and a correlation coefficient of 0.9947 (Figure 40).

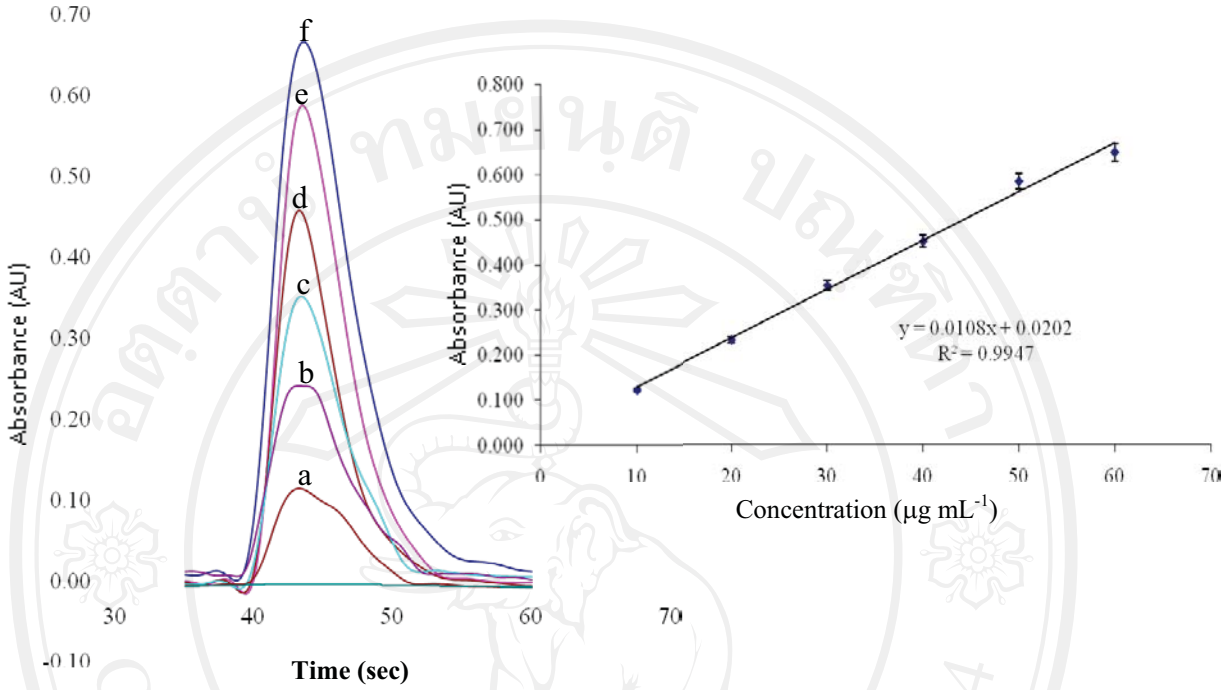

Figure 40 Typical SI-grams and calibration graph of solasodine: a = 10, b = 20, c = 30, d = 40, e = 50, f = 60 µg mL^{-1} respectively

3.1.4.2 Detection Limit and Quantification Limit

Limit of detection (LOD) of solasodine was estimated from the calibration curve using the expression 3.3SD/S where SD is standard deviation of the blank (or the intercept of the calibration curve) and S is the slope of calibration curve and limit of quantitation (LOQ) = 10SD/S[123].

The limit of detection (LOD) was found to be 1.41 µg mL^{-1}. The limit of quantitation (LOQ) value was found to be 4.28 µg mL^{-1}.

3.1.4.3 Precision and Accuracy

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). The precision was evaluated by

assaying six replicate injections of 10, 20 and 30 μg mL^{-1} of solasodine. The repeatability was evaluated each sample on the same day under the same experimental conditions, 2.85%, 1.96% and 3.52% respectively. The intermediate precision was evaluated by assaying each sample on three different days. The intermediate precisions were found to be 3.35%, 1.12% and 5.86% respectively (Table 28).

Table 28 Precision study for solasodine

Solasodine (μg mL^{-1})	Intra-day precision*		Inter-day precision*	
	Concentration (μg mL^{-1})	% R.S.D	Concentration (μg mL^{-1})	% R.S.D
10	9.87	2.85	10.02	3.35
20	19.92	1.96	19.67	1.12
30	29.26	3.52	30.04	5.86

* Each value is the average of six determinations

The recoveries were determined by using standard addition method. Solasodine (10, 20 and 30 μg mL^{-1}) were added and mixed with known aliquots of sample solutions, the sample was extracted and analyzed using the proposed method. The mean recovery (n=3) of solasodine in *Solanum aculeatissimum Jacq.*, *Solanum violaceum Ortega.*, *Solanum melongena Linn.* and *Solanum indicum Linn.* were found to be 95.39%, 97.03%, 98.96% and 95.29% respectively (Table 29). Sample throughput of 12 h^{-1} can be achieved.

Table 29 Percentage recoveries of solasodine contents in *Solanum* species by the SIA-LAV proposed method

Sample	Solasodine content (μg mL^{-1})*		% Recovery*
	Added	Found	
Solanum aculeatissimum Jacq.	10	9.61	96.10
	20	19.27	96.36
	30	28.11	93.71
average			***95.39***
Solanum violaceum Ortega.	10	8.73	87.34
	20	19.92	99.62
	30	31.24	104.13
average			***97.03***
Solanum melongena Linn.	10	8.25	82.52
	20	21.30	106.50
	30	31.18	103.93
average			***97.65***
Solanum indicum Linn.	10	8.99	89.92
	20	19.67	98.37
	30	30.44	101.47
average			***96.58***

* Each value is the average of three determinations

3.1.5 Application

The proposed SI method was applied to the determination of solasodine in *Solanum aculeatissimum Jacq.*, *Solanum violaceum Ortega.*, *Solanum melongena Linn.* and *Solanum indicum Linn.* in Solanaceae family. The extracts of *Solanum* were determined using the sample preparation steps and the optimum conditions as mentioned above. The contents of solasodine are shown in Table 30. Results compared favorably with those obtained by the reported colorimetric method[(104)]. The analytical of this proposed method for the studied solasodine has been compared to those from earlier investigation and summarized in Table 31.

Table 30 Comparative determination of solasodine contents in various *Solanum* species using the SIA-LAV method and the spectrophotometric method[104]

Samples	Amount of solasodine found (mg g^{-1})		*t*-values [c]
	SIA-LAV proposed method [a]	Spectrophotometric method[29] [b]	
Solanum aculeatissimum Jacq.	4.70	4.42	1.93
Solanum violaceum Ortega.	0.84	0.71	1.67
Solanum melongena Linn.	1.40	1.36	0.48
Solanum indicum Linn.	0.67	0.63	0.71

[a] Mean ± SD of five determinations. The calibration equation; y = 0.0109x + 0.0163

[b] Mean ± SD of five determinations. The calibration equation; y = 0.0076x + 0.0181

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

Table 31 Comparison of the proposed method with selected earlier reported methods

Techniques	Sample	Concentration range (μg mL^{-1})	% Recovery	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	References
Spectrophotometric method	*S. laciniatum*	100-4000	98.87	-	-	(104)
	S. avicular		99.75			
Ion-paring RP-HPLC	*S. ptycanthum*	-	100.85	-	-	(90)
RP-HPLC	*S. linnaeanum*	4-100	-	1.60	-	(96)
	S. melongena					
Non-aqueous capillary electrophoresis	*S. elaeagnifolium*	50-500	101.27	3.0	9.0	(99)
SIA with lab-at-valve	*S. aculeatissimum*	10-60	95.39	1.41	4.28	Proposed method
	S. violaceum Ortega.		97.03			
	S. melongena		97.65			
	S. indicum		96.58			

CHAPTER IV

CONCLUSION

4.1 Sequential Injection Analysis with Lab-at-Valve (SI-LAV) for the Determination of Solasodine

Sequential injection analysis with lab-at-valve approach for alternative simple on-line liquid-liquid semi-automated extraction was exploited. A simple fabricated lab-at-valve unit, a separating chamber, attached at one of the ports of a conventional multi-position selection valve offers an on-line automated extraction in a micro-scale. Therefore, consumption of sample, reagent and organic solvent also waste generation are tremendously reduced. The developed SIA-LAV system is precise, selective, accurate and robust for determination of solasodine in *Solanum* species.

Solasodine has heterocyclic nitrogen but has no conjugated double bonds in its structure. Therefore it does not give absorption in UV range. After hydrolysis it yields aglycone which can be complexed with methyl orange forming an ion-pair complex (solasodine methyl orange complex). The coloured complex is extracted into chloroform and has its maximum absorption at 420 nm.

The SIA manifold described in the Figure 31 was used. The SI operation steps were as follows: firstly, the manifold lines were washed with water and all the reagents were filled into the ports of the selection valve. Then suitable volume of the reagents were sequentially aspirated and shaken as zone in the holding coil. Finally, these zones were propelled through a reaction coil. A zone penetration occurred. The absorbance of a product zone was continuously monitored at a wavelength of 420 nm.

A series of experiments were conducted to establish the optimum analytical variables. Under the optimum conditions, a linear calibration graph over the range of 10.0 - 60.0 μg mL^{-1} of solasodine was obtained with the regression equation; $y = 0.0108x + 0.0202$ and a correlation coefficient of 0.9947. The detection limit (LOD) was found to be 1.41 μg mL^{-1}. It is calculated as three times of the standard deviation. The quantification limit (LOQ) value was found to be 4.28 μg mL^{-1}. The R.S.D of

intra-day and inter-day precisions were found to be 1.96 - 3.52% and 1.12 - 5.86% respectively. The recoveries were determined by using standard addition method. Solasodine standards were added and mixed with known aliquots of sample solutions, the sample was extracted and analyzed using the proposed method. The mean recovery of solasodine in *Solanum aculeatissimum Jacq.*, *Solanum violaceum Ortega.*, *Solanum melongena Linn.* and *Solanum indicum Linn.* were found to be 95.39%, 97.03%, 98.96% and 95.29% respectively. The method was successfully applied to the determination of solasodine in medicinal plants with a sample throughput rate of 12 sample h^{-1}. Results compared favorably with those obtained by the reported colorimetric method[104].