การพัฒนาวิธีตรวจวัดสารประกอบซูดานเรด ในอาหารโดยใช้เทคนิคทางเคมีไฟฟ้า

นางสาว วนิดา วอนสวัสดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี ภาควิชาเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549

METHOD DEVELOPMENT FOR DETERMINATION OF SUDAN RED IN FOOD USING ELECTROCHEMICAL TECHNIQUE

Miss Wanida Wonsawat

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Chemistry

Department of Chemistry

Faculty of Science

Chulalongkorn University

Academic Year 2006

Thesis Title	Method development for determination of sudan red in food using electrochemical technique
By	Miss. Wanida Wonsawat
Field of Study	Chemistry
Thesis Advisor	Associate Professor Orawon Chailapakul, Ph.D.
Thesis Co-Advisor	Luxsana Dubas, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master's Degree

.....Dean of the Faculty of Science
(Professor Piamsak Menasveta, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Sirirat Kokpol, Ph.D)

.....Thesis Advisor
(Associate Professor Orawon Chailapakul, Ph.D.)

.....Thesis Co-advisor
(Luxsana Dubas, Ph.D.)

.....Member
(Assistant Professor Fuangfa Unob, Ph.D.)

.....Member
(Assistant Professor Boosayarat Tomapatanaget, Ph.D.)

วนิดา วอนสวัสดิ์ : การพัฒนาวิธีตรวจวัดสารประกอบซูดานเรด ในอาหารโดยใช้เทคนิคทางเคมีไฟฟ้า (METHOD DEVELOPMENT FOR DETERMINATION OF SUDAN RED IN FOOD USING ELECTROCHEMICAL TECHNIQUE) อ. ที่ปรึกษา: รศ. ดร. อรวรรณ ชัยลภากุล, อ.ที่ปรึกษาร่วม: อ. ดร. ลักษณา ดูบาส

งานวิจัยนี้เป็นการพัฒนาวิธีวิเคราะห์สีสังเคราะห์กลุ่มสารประกอบเอโซ (ซูดาน 1, ซูดาน 2, ซูดาน 3 และ ซูดาน 4) ในตัวอย่างอาหารโดยใช้เทคนิคการตรวจวัดทางเคมีไฟฟ้าด้วยขั้วไฟฟ้ากลาสสิคาร์บอนและขั้วไฟฟ้าคาร์บอนดัดแปรด้วยคาร์บอนนาโนทิวป์ จากการศึกษาปฏิกิริยาออกซิเดชันของสีซูดานทั้ง 4 ชนิดโดยใช้ขั้วไฟฟ้าคาร์บอนและระบบการตรวจวัดแบบไซคลิกโวลแทมเมทรีพบว่าให้ผลของไซคลิกโลแทมโมแกรมที่ชัดเจน การศึกษาตัวแปรที่เหมาะสมสำหรับการวิเคราะห์โดยใช้การตรวจวัดแบบแอมเพอโรเมทรีที่ประยุกต์ใช้ร่วมกับระบบโฟลว์อินเจคชันที่มีขั้วไฟฟ้ากลาสสิคาร์บอนเป็นอุปกรณ์การตรวจวัด วิธีการนี้ให้ค่าขีดจำกัดต่ำสุดของการตรวจวัดเท่ากับ 0.01, 0.10, 0.025, และ 0.01 ppm สำหรับซูดาน 1, ซูดาน 2, ซูดาน 3 และ ซูดาน 4 ตามลำดับจากนั้นได้ทำการแยกและวิเคราะห์ปริมาณสีซูดานทั้งสีชนิดด้วยเทคนิคไฮเพอฟอร์มานซ์ลิควิดโครมาโทกราฟีร่วมกับการตรวจวัดแบบแอมเพอโรเมทรีเปรียบเทียบการทดลองระหว่างขั้วไฟฟ้ากลาสสิคาร์บอนและขั้วไฟฟ้าดัดแปรด้วยคาร์บอนนาโนทิวป์ ในระบบนี้ใช้คอลัมน์ Inertsil C18 สภาวะที่เหมาะสมสำหรับการตรวจวัด คือ อะซิโทไนไตรล์และ 20 มิลลิโมลาร์ อะซิเตตบัฟเฟอร์ในอัตราส่วน 90 ต่อ 10 โดยปริมาตรที่อัตราเร็ว 1 มิลลิลิตรต่อนาที พบว่าช่วงความเข้มข้นที่เป็นเส้นตรง คือ 0.01 ถึง 150.0 และ 0.005 ถึง 25.0 ppm ที่ขั้วไฟฟ้ากลาสิคาร์บอนและขั้วไฟฟ้าคาร์บอนดัดแปรด้วยคาร์บอนนาโนทิวป์ ตามลำดับ ค่าการคืนกลับอยู่ในช่วง 93.74 ถึง 104.56 และ 93.98 ถึง 104.09 เปอร์เซ็นที่ขั้วไฟฟ้ากลาสิคาร์บอนและขั้วไฟฟ้าคาร์บอนดัดแปรด้วยคาร์บอน นาโนทิวป์ตามลำดับ ด้วยค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ไม่เกิน 10 เปอร์เซ็นต์ งานวิจัยนี้ได้ทำการประยุกต์ใช้กับตัวอย่างเครื่องดื่มและซอสพริกโดยผลที่ได้จากวิธีที่เสนอมีประสิทธิภาพดีให้ผลการวิเคราะห์อยู่ในช่วงที่ยอมรับได้

ภาควิชา.....เคมี..... ลายมือชื่อนิสิต.....วนิดา วอนสวัสดิ์.....

สาขาวิชา.....เคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....อรวรรณ ชัยลภากุล.....

ปีการศึกษา.....2549..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....ลักษณา ดูบาส.....

477 24498 23 : MAJOR CHEMISTRY
KEY WORD: SUDAN I / SUDAN II / SUDAN III / SUDAN IV/ AMPEROMETRIC DETECTION / HPLC / CARBON NANO TUBES /

WANIDA WONSAWAT: METHOD DEVELOPMENT FOR DETERMINATION OF SUDAN RED IN FOOD USING ELECTROCHEMICAL TECHNIQUE. THESIS ADVISOR: ASSOC. PROF. ORAWON CHAILAPAKUL, Ph. D., THESIS CO-ADVISOR: LUXSANA DUBAS, Ph. D.

The purpose of this research is to develope a method for analysis synthetic azo dye compound (sudan I, sudan II, sudan III, and sudan IV) in food sample. The electrochemical analysis was investigated using cyclic voltammetry at glassy carbon and CNT modified glassy carbon electrodes. It was found that four sudan dyes provided well-defined cyclic voltammogram at both electrodes. The amperometric waveform parameters were optimized at GC electrode by FIA system. The significantly low detection limits of 0.01, 0.10, 0.025, and 0.01 ppm for sudan I, sudan II, sudan III and sudan IV were obtained, respectively. High performance liquid chromatography with amperometric detection was investigated for separation and quantitative determination of four sudan dyes. Comparison experiments were carried out using GC and CNT modified GC electrodes. The chromatography was performed using a commercially available Innertsil C18 column, with the optimum conditions: acetonitrile and 20 mM acetate buffer (90:10; %v/v) at flow rate 1 mL $min.^{-1}$ Sudan dyes were detected at 0.95 and 0.85 V at GC and CNT modified GC electrodes, respectively. The linear concentration ranges of four sudan dyes were 0.01 to 150.0 and 0.005 to 25.0 ppm at GC and CNT modified GC electrode, respectively. The recoveries were in the ranges of 93.74 to 104.56 and 93.98 to 104.09% at GC and CNT modified GC electrodes, respectively with %RSD < 10%. The proposed method was further applied to analyse soft drink and chilli sauce samples. The results from proposed method gave the acceptable percent recovery values.

Department……..Chemistry…….Student's signature..... Wanida Wonsawat
Field of study……...Chemistry..... Advisor's signature.....
Academic year …… 2006……..... Co-advisor's signature ……

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and sincerest appreciation to my advisor, Associate Professor Dr. Orawon Chailapakul, who always gives the great opportunity for my research throughout three years. She has consistently provided me with invaluable guidance, profound assistance and forbearance discerning suggestion, critical proofreading, encouragement and especially sincere forgiveness for my mistakes throughout the research. In addition, she has supported moral principles, knowledge and various experiences to me, until I obtaine great successful science education today.

Deepest gratitude to my thesis co-advisor, Dr. Luxsana Dubas, for her suggestion, Assistant Professor Dr. Fungfa Unob and Assistant Professor Dr. Boosayarat Tomapatanaget for critrical proofreading, comments and suggestion. I would like to thank Miss Montra Piriyapittaya for her help in using HPLC agilent.

This research was financially supported by The 90 th Anniversary of Chulalongkorn University (Ratchadphiseksomphot Endowment Fund).

My deepest thanks go to the staffs of the department of chemistry, Chulalongkorn University, the members in electroanalytical chemistry research group for their helpfulness and enjoyable time all the way through. Special thanks go to Miss Sarawadee Korsrisakul for her sincere friendship and cheerful willingness, Miss Auchana Preechaworapon for her suggestion and encouragement, Miss Weena Siangproh and Miss Kanokporn Boonsong for her kind advices in this thesis.

Finally, I am affectionately thankful to my father and mother for their heartful unlimited support, enthusiasm support, stand by for my success and best understanding throughout my study.

CONTENTS

PAGE

PAGE

PAGE

PAGE

LIST OF TABLES

TABLE **page**

TABLE **page**

LIST OF FIGURES

FIGURE **page**

FIGURE **page**

FIGURE **page**

FIGURE **page**

LIST OF ABBREVIATIONS AND SYMBOLS

HPLC	High Performance Liquid Chromatography
FIA	Flow Injection Analysis
GC	Glassy Carbon Electrode
EC	Electrochemical Detection
mg	milligram
mL	milliliter
kg	kilogram
μm	micrometer
μL	microliter
i.d.	Internal diameter
R^2	Correlation coefficient
LOD	Limit of Detection
LOQ	Limit of Quantitation
TLC	Thin- Layer Chromatography
BAS	Bioanalytical System, Inc.
WE	Working Electrode
RE	Reference Electrode
CE	Counter Electrode
Ag/AgCl	Silver / Silver Chloride Reference Electrode
i	Current (A)
i_{pa}	Anodic peak current (A)
i_{pc}	Cathodic peak current (A)
E_{pa}	Anodic peak potential (V)
E_{pc}	Cathodic peak potential (V)
RSD	Relative Standard Deviation
UV	Ultraviolet
LC/MS	Liquid Chromatography/ Mass Spectrometry
MS/MS	Mass Spectrometry/ Mass Spectrometry
CI	Chemical ionization
API	Atmospheric pressure ionization

APCI	Atmospheric pressure chemical ionization
ESI	Electrospray ionization
ppm	Part per million
ppb	Part per billion
M.W.	Molecular weight
t_R	Retention time
R_s	Resolution
%	Percentage
°C	Degree celsius
AOAC	Association of Official Analytical Chemists
TBAP	Tetrabutyl ammonium phosphate
ODS	Octadodeccylsilane
mM	millimolar
μM	micromolar
SPC	Screen Print Carbon
SWNT	Single-walled carbon nanotubes
MWNT	Multi-walled carbon nanotubes

CHAPTER I

INTRODUCTION

1.1 Introduction and literature reviews

The food market has changed rapidly with increasing of procession foods. For this reason, the food industries try to produce the attractive food with quality and price to the consumers [1]. The uses of colors to make foodstuffs for aesthetically and psychologically attractive have been known for many centuries. Synthetic colors added in the foods to replace the natural color that lost in the processing are interesting by consumer. Food colorant is allowed to use in food, so sudan dyes are important for food industries. Several sociological, technical and economic factors had influenced in food industry two decades ago.

The Imported Food Sampling and Surveillance Project were set up in 2003 when food products were contaminated with illegal dyes, especially sudan dyes in chili products which imported form India. The effect of this founding brought to write an approved protocols and considerated that the imported food have to get a certificate of a licensed disposal operator.

Sudan dyes are fat-soluble synthetic industrial azo dyes [2, 3]. Sudan dyes (see Figure 1.1) which is red coloring agents is used in a variety of household products, including waxes, textile, printing, cosmetic, drug, food-processing industries, shoe, floor polishes, paper colorants and fabric. Not only they are used in household products but they are also used extensively in laboratories as either biological stains or pH indicators [4]. It is well known that sudan I, sudan II, sudan III, and sudan IV have been classified as carcinogens to humans by International Agency for Research on Cancer (IARC) [2, 5, 6].

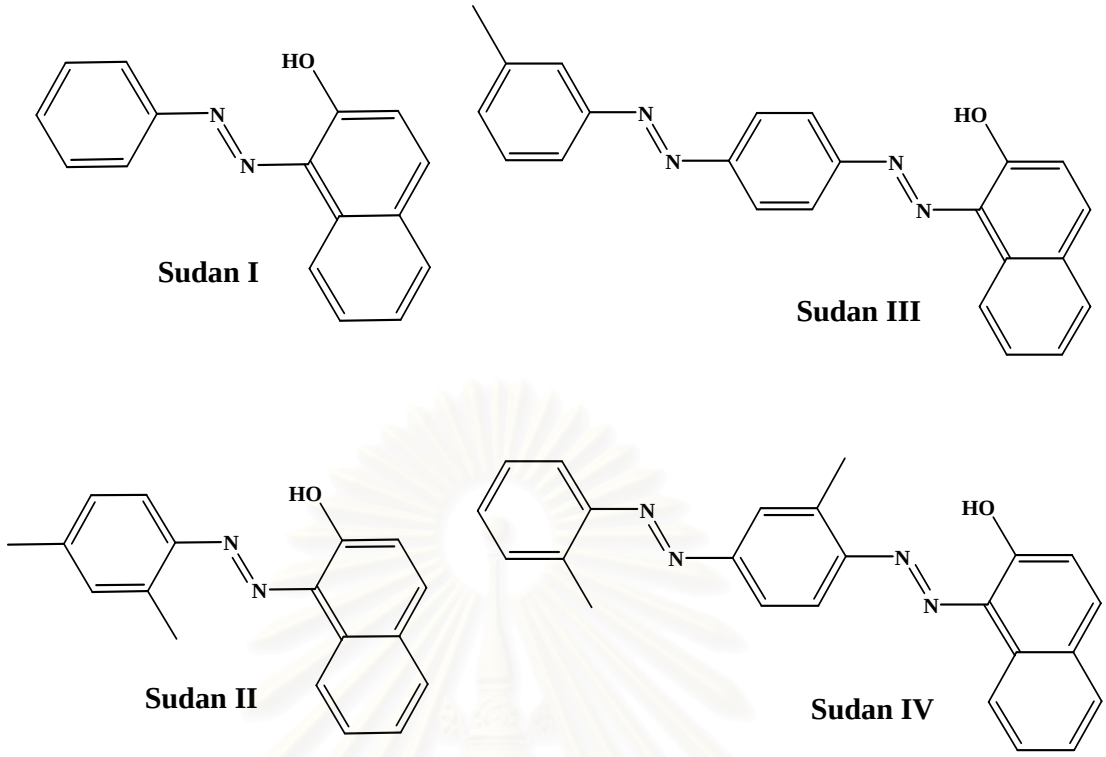


Figure 1.1 Chemical structures of Sudan I, Sudan II, Sudan III and Sudan IV

From 2,057 samples, drawn at 224 local markets in 56 districts of Uttar Pradesh, 32% contained artificial colors in 61.6% were non-permitted. In the artificially colored uneatable, 39% sweets and all the samples of powdered turmeric, as well as chilli contained non-permitted colors. Seven grade non-food dyes were used include metanil yellow, orange II, rhodamine B, Blue VRS, auramine, sudan I, sudan III and sudan IV [7].

Sudan dyes have started recently to appear in certain hot chili products that was imported from India. The first problem was unearthed in May 2003, when French authorities detected the dye sudan I in Indian hot chili. In response, the European Commission was implemented immediately by an EU-wide monitoring program to test the presence of sudan I in imported chili and chili products and drew up legislation requiring that contaminated products had been destroyed. Since June 2003, the exporters or food manufacturers must have a certificate of analysis shown that there are no in chili powders and products contained chili powder [8].

Azo dye compounds can be metabolized by intestinal anaerobes to produce mutagens and/or carcinogens. Sudan dyes or water-insoluble azo dyes can be metabolized to amines by enzymes in the liver, which is suspected carcinogenic compounds and linked to the development of cancer in laboratory animals. The mutagenic of sudan azo dyes were degraded to products base. Sudan I is a mammalian carcinogen but it is not mutagenicity [9].

Sudan I, l-phenylazo-2-hydroxynaphthalene, caused carcinogenic livers in rat and the urinary bladder mammals [9]. From the reported in vitro, sudan I can be oxidized to the form which bind to calf thymus DNA [10]. From the investigation, it found that the product of the carcinogen azo dye, sudan I, were detoxicated which bind to nucleic acids after activation by peroxidase [11]. Sudan II can be reduced to *ρ*-phenylenediamine and aniline carcinogenic aromatic amines [12]. The effect of azo dye sudan III on hepatic drug metabolism is interesting, especially, which a number of implicated reports for the prevention of chemical carcinogenesis in rats. Sudan III were investigated to enzyme activities in microsomes [13]. M.Vahentin et al., have investigated the utility of sudan III [14]. It is a bis-azo dye, which undergoes a two-step electrochemical reaction in both non-aqueous and aqueous media. They studied the relationships between the kinetic and thermodynamic properties of the surface EE electrode reactions by the square-wave voltammetry (SWV). The fat-soluble bis-azo dyes, sudan III and sudan IV, are the parent compounds in a number of azo colourings. Various toxic effects of these compounds have been reported in animals, ranging from non-malignant atypical epithelial cell, if it is injected into the ears of rabbits to different degrees of liver damage [3]. The optical data storage and electro-optic modulators were carried out with sudan IV based poly (alkyloxymethacrylate) films with different chain lengths of methylene group [15].

Today the chili products are important economic section in south-east asia especially Thailand. The most markets of these countries are the United States and Europe, which have regulated to control quality of imports. The residues of sudan dyes may be found in the chilli products and the levels of sudan dyes residues may be unacceptable for the international markets [16]. Therefore, the fast and accurate identification and quantification of the methods for detection of sudan dyes at low levels are intensively required.

Several methods testing the presence of sudan dyes in food products have been proposed. Francesco Puoci et al., developed method for detecting sudan I by synthesis of molecularly imprinted polymers (MIRs) for solid phase extraction (MISPE) using sudan I as a template [17], capillary electrophoresis [18], and flow through sensor spectrophotometry [19], spectrophotometry [20], HPLC-UV detector [21], HPLC-DAD [17], HPLC-PDA [5,22], and HPLC with chemiluminescence detection [23]. Recently, the HPLC method coupled with mass spectrometry (MS) has been utilized for the analysis of sudan dyes and reported to be more sensitive than UV absorbance detection, such as HPLC-MS with MS/MS [24], LC–ESI-MS/MS [25,26], HPLC–APCI-MS [27,28]. HW. Chen et al., [29] developed fast direct detection of sudan dyes in chili powder and found that the proposed method provided lows LOD and LOQ (pg/mL) without any pretreatments. This research reported the separation and detection of seven sudan dyes with isocratic HPLC conditions [30].

The official methods used for the determination of sudan dyes in food are complicated, time-consuming and non-specific. Therefore, the fast, simple, less expensive, sensitive and specific analytical methods are required for identification and quantitation of sudan dyes. Electroanalytical techniques have become widespread practical for analysis using flowing systems such as flow injection analysis (FIA) and high performance liquid chromatography (HPLC) due to its high sensitivity, selectivity, low cost and relatively short analysis time when compare with the other techniques. The other essential property is low dispersion, which provides minimization of detector dead volume [31-35]. The use of amperometric detection is also adequate for the quantitation of electroactive substances in simple matrices. Hence, it can be used directly to determine sudan dyes in food by high performance liquid chromatography couple with electrochemical detector (HPLC-EC).

The current explosion of interest in carbon nanotubes (CNT) began with the discovery by Iijima in 1991 [36]. CNT as the novel carbon material have been applied to biological and electrochemical fields [37, 38]. The novel properties, such as high surface area, electrical conductivity, good chemical stability, and the promotion ability of electron transfer reactions when used as electrode material in electrochemical devices [39]. Besides, CNT modified electrode also shows good

electrocatalytic ability to biomolecules. Thus, they can be used to improve a sensitivity and selectivity for the electrochemical detection [40, 41]. In this research, the results obtained from electrochemical oxidation of four sudan dyes at GC electrode was compared with those obtained at CNT modified GC electrode.

1.2 Objective and scopes of the research.

The objective of this study is to develop an analytical method for the determination of sudan azo dyes (sudan I, sudan II, sudan III and sudan IV) in food samples. The high performance liquid chromatography (HPLC) coupled with amperometric detection is used for this research.

For the amperometric detection system, a glassy carbon electrode (GC), a silver/silver chloride (Ag/AgCl) and a platinum wire (Pt) are working electrode, reference electrode and counter electrode respectively, The scopes of this study are:

1) To investigate the electroactive reaction of sudan azo dyes (sudan I, sudan II, sudan III and sudan IV) by cyclic voltammetry at GC electrode.
2) To optimize the parameters for amperometric detection of sudan dyes at GC electrode by flow injection analysis (FIA) and high performance liquid chromatography (HPLC) system.
3) To establish the optimal condition for high performance liquid chromatography (HPLC) system.
4) To validate the developed method.
5) To compare the developed method to the standard method.
6) To compare the results obtained from GC electrode and CNT modified GC electrodes.

CHAPTER II

THEORY

2.1 Electrochemical techniques

Electroanalytical chemistry encompasses a group of quantitative analytical methods that are based upon the electrical properties of an analytical solution when it is made part of an electrochemical cell [42]. Electroanalytical techniques are capable of producing exceptionally low detection limits and a wealth of characteristic information describing electrochemically addressable systems. Such information includes the stoichiometry and rate of interfacial charge transfer, rate of mass transfer, extent of adsorption, and equilibrium constants for chemical reactions.

Electroanalytical methods have certain general advantages over other types of procedures. First, electrochemical measurements are often specific for a particular oxidation state of an element, whereas most other analytical methods are capable of revealing only the total concentration. The second important advantage of electrochemical methods is inexpensive of the instrumentation. The third feature of certain electrochemical methods is giving the information about activities rather than concentrations of chemical species [43].

Now, there are various electroanalytical methods that have been used for wide range applications. These methods are divided into bulk methods and interfacial methods which is wider usage than the former ones. Interfacial methods are the methods in which the reactions occur at the interface between the surface of electrode and the thin layer of solution near the surfaces.

Static and dynamic methods are the major subgroups of the interfacial methods based upon the presence and absence of current in electrochemical cell. Dynamic interfacial methods are the methods based on the presence of the current.

For the controlled- potential, potential is controlled while the other variables. The advantages of these methods are high sensitivity, portability, wide dynamic range, wide range of working electrode that can be used, low consumption of sample volumes, and low limit of detection (LOD).

In this research, the voltammetry and amperometry were used. The details of these methods are described as the following:

2.1.1 Voltammetry

Electroanalytical methods depend on the measurement of current as a function of applied potential are called voltammetric methods [44]. Conditions employed encourage polarization of the indicator or working electrode.

Voltammetry is based on the measurement of current in an electrochemical cell under conditions of concentration polarization in which the rate of oxidation or reduction of the analyte. These phenomenons are limited by mass transfer of the analyte to the electrode surface.

Voltammetry is widely used in analytical, inorganic, physical, and biological chemistry for fundamental studies of oxidation and reduction processes in various media. Figure 2.1 shows the common waveform of the voltammetry.

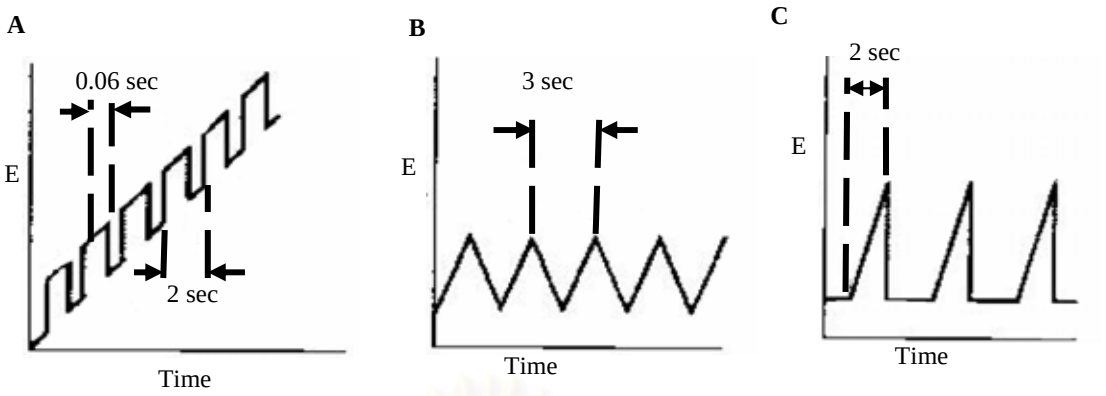


Figure 2.1 Potential-time waveforms are used in various electrochemical techniques. Wave form A: square, B: triangular, and C: linear potential-time patterns are use in square wave, cyclic voltammetry, and linear sweep, respectively.

2.1.2 Cyclic voltammetry

Cyclic voltammetry (CV) is a widely used electroanalytical technique, such as study of adsorption processes on surfaces, the detection of reaction intermediates, and the observation of follow-up reaction of products formed at electrode and electron transfer mechanisms at electrode surfaces [45]. The current response of a small stationary electrode in an unstirred solution is measured, when potential is applied as a function of time.

Important parameters for cyclic voltammetric technique are the cathodic peak potential, E_{pc}; the anodic peak potential, $E_{pa;}$ the cathodic peak current, $i_{pc;}$ and the anodic peak current, $i_{pa.}$ The parameters of this technique are illustrated in Figure 2.2 for a reversible electrode reaction; i_{pa} and i_{pc} are approximately equal in absolute value but opposite in sign. Moreover, at 25 °C, the difference in peak potentials, ΔEp is expected to be

$$\Delta E\text{p} = \left| E_{pa} - E_{pc} \right| = \frac{0.059}{n}$$

Where n is the number of electrons involved in the half-reaction. For irreversible processes, the individual peak is reducing in size because of slow electron transfer

[46]. While an electron transfer reaction may appear reversible at a slow sweep rate, increasing the sweep rate may lead to increase values of ΔEp [46,47].
Quantitative information obtained from the Randles-Sevcik equation at 25 °C is

$$i_p = 2.686 \times 10^5 n^{3/2} AcD^{1/2} \nu^{1/2}$$

Where i_p is the peak current in ampere, A is an electrode area in cm^2, D is the diffusion coefficient in $cm^2\ s^{-1}$, c is the concentration in mol cm^{-3}, and ν is the scan rate in V s^{-1}.

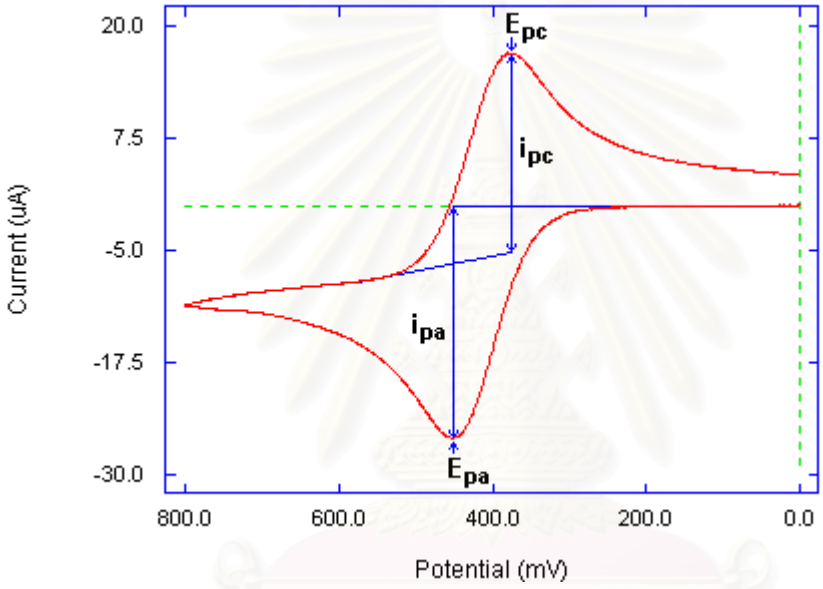


Figure 2.2 A typical cyclic voltammogram showing the important peak parameters

2.1.3 Amperometry

Amperometry is a simplest technique which a constant potential is applied to electrochemical cell. This technique is based on measuring the limiting current at a constant applied potential. The current is proportional to the concentration gradient at the electrode surface. For FIA/HPLC amperometric detection, the applied potential of an analyte is determined by hydrodynamic voltammetry.

2.2 Electrochemical cell

An electrochemical cell consists of at least two electrodes and one electrolyte. There are always three electrodes in the electroanalytical cell. The first electrode is the indicate electrode also known as the working electrode. This electrode which the electrochemical phenomena being investigated takes place. The second electrode is the reference electrode. The potential of this electrode has to be constant potential. The third electrode is the counter or auxiliary electrode which serves as a source for electrons so that current can be passed from the external circuit through the cell.

2.3 Working electrodes

The working electrode is the electrode which the analyte is oxidized or reduced. Electrolysis current passes between the working electrode and a counter electrode. The potential between the working electrodes versus a reference electrode is controlled. The dimensions of the working electrode are kept small to enhance its tendency to become polarized. There are made from inert metal such as platinum, gold, pyrolytic graphite and glassy carbon.

2.3.1 Glassy carbon (GC)

Glassy carbon (GC) has been used for many electrochemical techniques. It was use to measure the concentration and to detect the presence of electrochemical species, because of its good electrical and thermal conductivity, low density, corrosion resistance, low thermal expansion, low elasticity, and high purity. In addition, carbon materials can be produced in a variety of structures such as powders, fibers, large blocks, and thin solid sheets. Furthermore, carbon materials are generally available at low-cost. The most common shape for glassy carbon electrodes are rod, typically 0.5 cm or less in diameter. However, rectangular plates and circular disks of glassy carbon are also used for electrodes. In these researches, carbon electrode is used as a detector for liquid chromatography [48].

Glassy or vitreous carbon is a more recent addition to the growing list of the solid carbon electrodes [47]. The preparation of glassy carbon electrodes is difficult to machine because of hardness. Glassy carbon is produced by thermal degradation of selected organic polymers, such as resins of furfuryl alcohol, phenol formaldehyde, acetone-furfural, or furfural alcohol-phenol copolymer. Generally, starting with the coke, it is heated to remove volatiles. Then after further preliminary treatments, the amorphous carbon is transformed to graphite by heating at 2500°C to 3000°C. The physical properties of glassy carbon depend on the maximum temperature of heat treatment. Glassy carbon with satisfactory properties for many applications can be produced at 1800°C. The glassy carbon appears and shows fracture behavior similar to that of glasses. The glassy carbon is sealed in an inert or nonconductive medium (e.g. PTFE, epoxy resin, and glass) and the exposed end is polished to a flat. The electrochemical pre-treated glassy carbon electrode for the amperometric detection can be enhanced and stabilized the electrode response [48]. Several stages in a metallographic polishing procedure of GC electrode with an electrochemical pretreatment have been investigated. From the investigation, the S/B ratios obtained from HPLC with amperometric detection depend on the polishing procedure. The degree of adsorption and deactivation may be reduced by a subsequent electrochemical pretreatment [49]. One of the first applications for glassy carbon electrodes was reported by Zittle and Miller in the mid – 1960s. Van der Linder and Dieker also reviewed the use of glassy carbon as the working electrodes in electroanalytical chemistry. It was reported that glassy carbon electrodes have the widest potential range among many carbon electrodes (see Table 2.1) or other solid electrodes.

Table2.1 Potential limits for various carbon electrodes in different electrolytes [50]

Electrolyte(25°C)	Potential limit (V vs. SCE)	
	Anodic	Cathodic
	Glassy Carbon	
1 M HCl	0.9	-1.1
Phosphate buffer, pH 6	1.4	-1.5
1 M NaOH	0.5	-1.6
0.2 M $LiClO_4$ in acetonitrile	3.0[a]	-2.6[a]
	Carbon Paste	
0.1 M H_2SO_4	1.34	NR
0.1 M HCl	1.07	NR
Acetate buffer, pH 4.85	1.24	NR
2 M KCl	0.93	NR
Phosphate buffer, pH 6.80	1.26	NR
	Pyrolytic Graphite	
0.1N H2SO4	1.19	-0.24
0.1 M HCl	1.12	-0.32
0.1 M NaOH	0.68	-0.34

[a] 0.1 M Ag/Ag^+ Reference electrode

NR: Not reported

2.3.2 Carbon nanotube (CNT) -ionic liquid gel modified glassy carbon

CNT was discovered by Sumio Iijima in 1991. CNT is a nanoscopic structure made of carbon atoms in the shape of a hollow cylinder. The cylinders are typically closed at their ends by semi-fullerene-like structures. The diameter is in the range of 0.5 nm [50]. CNT is generally classified into two types, one is single-walled carbon nanotube (SWNT) and the other is multi-walled carbon nanotube (MWNT). SWNT,

which is seamless cylinders each made of a single grapheme sheet show in Figure 2.3. MWNT consists of two or more seamless grapheme cylinders concentrically arranged (Figure 2.4). They show many unique characteristics, such as large surface area and high electrical conductivity. It is well known that CNT have capability to promote electron transfer reaction and improve sensitivity in electrochemistry, thus they are widely used to prepare electrodes [51].

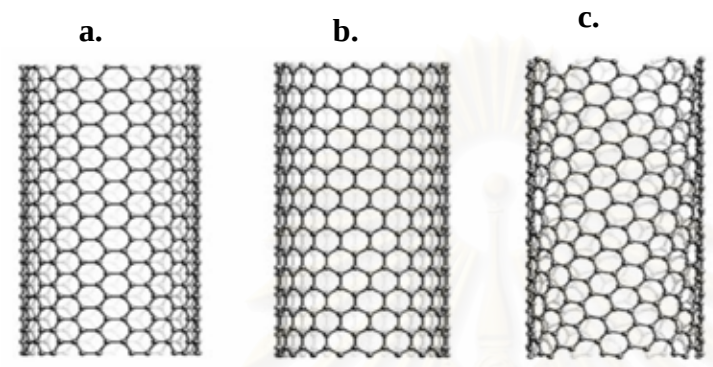


Figure. 2.3 Computer-generated images of single-wall carbon nanotubes: (a) armchair type, (b) zig-zag type, and (c) helical type.

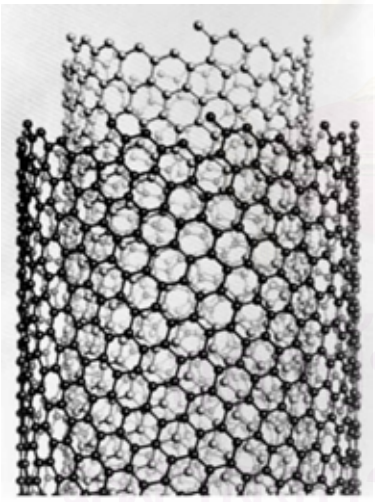


Figure. 2.4 Multi-wall carbon nanotubes

2.4 Flow injection analysis

Flow injection analysis (FIA) was first described in the mid 1970s. The great advantage of the FIA is easy for additional components, which are added to the system to achieve an analytical objective. This makes FIA simple, automated and capable of having a high sampling rate, low consumption of reagents and samples, better repeatability, good precision, high sensitivity, great selectivity, as well as relative low cost of the instrumentation. FIA technique is based on the injection of a liquid sample into a continuous carrier steam of a suitable liquid. So, analysis time can be greatly reduced due to the high sample throughput of flow systems. The simplest flow injection analyzer consists of a pump, which is used to push the carrier stream though a narrow tube; an injection port, which a well-defined volume of a sample solution is injected into the carrier stream in a reproducible manner and reproducible timing of its movement from the injection point toward the detector [53].

2.5 FIA Components

2.5.1 Sample and reagent transport system

The carrier solution in FIA is moved through the system by a peristaltic or HPLC pump. The flow rate of the peristaltic pump is controlled by the speed of the motor and the inside diameter of the plastic tubing.

2.5.2 Sample injector

The injector of FIA is similar to HPLC analysis. The injected sample volumes are between 1 and 200 μL. A typical method for introducing sample is syringe injection. Thus, the satisfactory way of sample introduction is upon sampling loops. The time span between the sample injection, S and the peak maximum, which yields the analytical readout as peak height, H is the residence time, T. Then, the sample zone is transported toward a detector that continuously records electrode potential.

2.5.3 Detectors

There are many types of the FIA detectors such as fluorometry, spectrophotometry, and electrochemistry [54]. A typical recorder output has the form of a peak height, H or area, A. It is related to the concentration of the analyte. Alternative automatic procedures based on flow injection technique with amperometric detectors have been widely suggested in pharmaceutical, food, forensic and clinical sciences. However, a simple and cheap electrochemical methodology has never been reported for sudan dyes. The use of amperometric detection is sensitive enough for the quantitation of electroactive substances in simple matrices. Therefore, it could be possible to determine sudan dyes directly in food sample. From the Figure 2.5a and b is a flow diagram of the simplest type of FIA couple with amperometric systems.

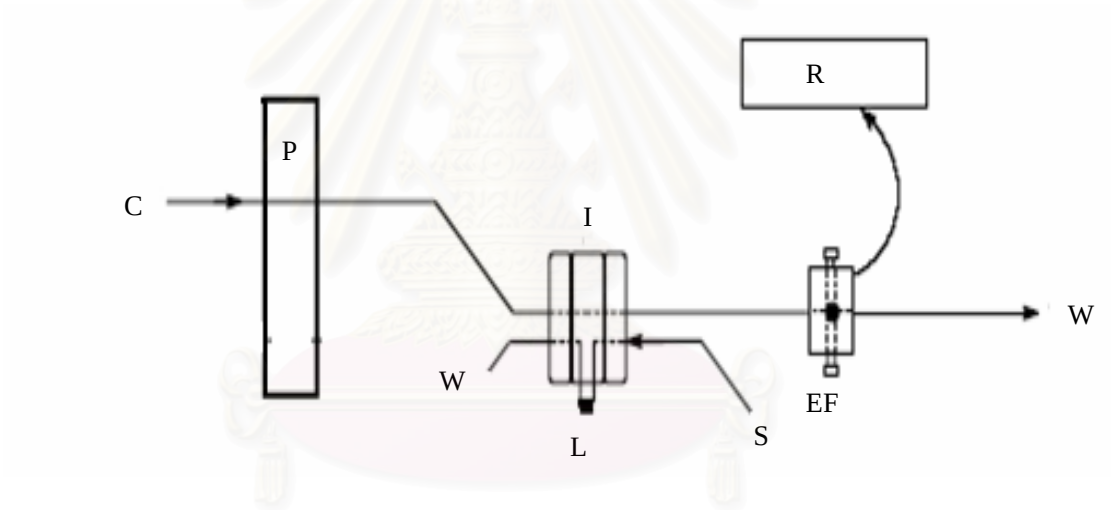


Figure 2.5a Schematic diagram of the flow system used for evaluation of the GC electrode for Sudan dyes determination. P; peristaltic pump; I; manual injector; S; sample, L; sample volume, C; carrier solution, EFC; electrochemical flow cell, R; Potentiostat (recorder) and W: waste [55].

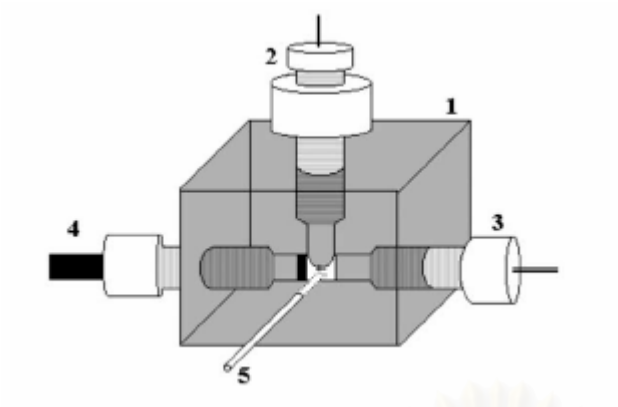


Figure 2.5b Schematic diagram of the electrochemical flow cell used in the amperometric measurements in flow injection system. 1. Polyurethane resin block, 2.Reference electrode (Ag/AgCl), 3.Stainless steel electrode, 4.GC electrode, and 5.Polyethylene tubing (flow) [55].

2.6 High performance liquid chromatography (HPLC)

In many analytical laboratories, HPLC is the most widely use for the analytical separation in many analytical laboratories. The reasons for the popularity of the method are sensitive, ready adaptable for accurate quantitative determinations [43]. The schematic diagram of a HPLC is shown in Figure 2.6.

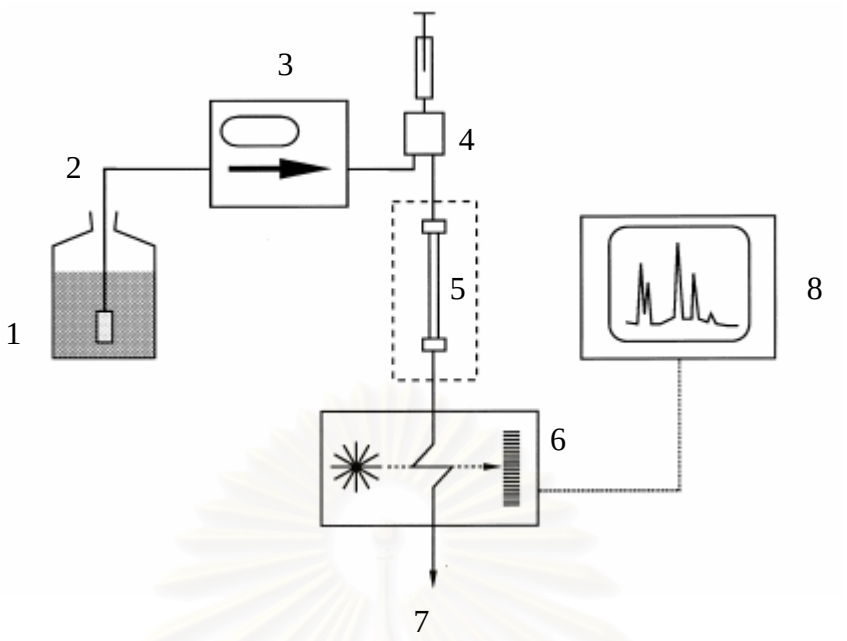


Figure 2.6 Schematic diagram of an HPLC unit. 1; solvent reservoir; 2; transfer line with frit; 3; pump; 4; sample injection; 5; column; 6; detector; 7; waste; 8; data acquisition.

a.) A solvent reservoir

In general, the reservoir made of glass or stainless steel contains 200 to 1000 mL of a mobile phase prior introduce into pump. The dissolved gas in the mobile phase are generates bubbles in the column, the pump check valve and the detector.

b.) HPLC pumps

For analytical proposes HPLC pumps should provide flow rates range from 0 to 10 mL min^{-1}. There are two types of pump in common use for HPLC analysis. One is a pneumatic pump, which the necessary high pressures are achieved by pneumatic amplification. The other is a syringe pump. The syringe pump is strongly constructed with a plunger that is driven by a motor.

c.) HPLC sample valves (injector)

A sample loop of the injector is the most widely use for the introduction of sample solution into HPLC system. The injection part commonly consists of an injection valve and the sample loop. The sample is typically dissolved in the mobile phase before it is injected into the loop via the injection valve. Loop volumes range between 10 μL to 500 μL. The injection valve is more precise than a syringe injection. The volumes used must be minuscule to prevente band broadening and overloading columns. The sample injection is typically automated.

d.) HPLC columns (Stationary phase)

HPLC columns are packed with very fine particles (usually a few microns in diameter). The stationary phase must therefore have much smaller particle size, 3 – 10 μm, to reduce the effective depth of the mobile phase. The very fine particles are required to attain the low dispersion that gives the high plate. Plate counts in excess of 25,000 plates per column are possible with modern columns. The separation in HPLC columns achieve by the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The main consideration with HPLC is the much wider variety of solvents and packing materials. The HPLC columns are containing various type of stationary phase such as; size-exclusion, normal phase, ion exchange and reveres phase. The choice of the suitable chromatographic conditions for a HPLC analysis depends on two factors: the molecular sizes and polarities of the analytes. To separate hydrophobic compound reverse phase chromatography can be used. The stationary phase of this mode consists of silica based packing with n-alkyl chains covalently bound. The stationary-phase materials with methyl, hexyl, octyl, doeicosanyl (C_{22}) and phenyl side chains are also commercially available. The popularity of the octadecylsilyl-(ODS) substituted silica (C_{18}) come initially from a comparison of the efficiency and selectivity of a series of phases with different chain lengths [56].

e.) Mobile phase

The mobile phase in HPLC refers to the solvent being continuously flowed to the column or stationary phase. In the isocratic elution, compounds are eluted using constant mobile phase composition. This type of elution is both simple and inexpensive. The mobile phase in the electrochemical detector is buffer to provide a relatively high concentration of ions in the solution. This enables the mobile phase to act as a supporting electrolyte for electrochemical reaction as well.

f.) HPLC Detectors

The HPLC detector is the function for monitoring analyte from the column. There are many types of the HPLC detectors. Electrochemical detector (EC) for HPLC is interesting because of the low detection limit, high sensitivity, and reproducibility. The linear dynamic range is wider than the optical detectors [48]. Electrochemical detectors measure compounds that undergo oxidation or reduction reactions in solution by the addition or removal of electrons at an electrode surface. These electrochemical reactions take place when a positive or negative potential is applied to the solution. Electrochemical techniques that can be applied to HPLC are amperometry, coulometry and conductometry [43]. In the experiment, a fixed potential is applied to the solution. The number of mole of analytes can be determined by monitoring the total of coulombs (current x time).

Electrochemical cell for HPLC (HPLC-EC) in this work is the thin-layer cell. All the commercially available HPLC-EC detectors contain three electrodes. The auxiliary electrode directly across from the working electrode helps to minimize iR drop between the two electrodes. As a result, a wider linear dynamic range is achieved. The reaction takes place at the working electrode. Usually, working electrodes are made of glassy carbon, which is highly resistant to organic mobile phases. Gold and platinum electrodes can also be used and have been found to be preferable for some samples. The working electrode is held at a fixed potential versus a silver/silver chloride (Ag/AgCl) or calomel reference electrode. Finally, counter (or auxiliary) electrode made from stainless-steel is used as a source or sink for electrons so that current can be passed from the external circuit through the cell [56].

When this electrode is placed in a flowing stream of mobile phase, it will generate a background current due to any oxidation or reduction of the mobile phase or contaminants. If an analyte passes the working electrode, it will be oxidized (or reduced) by the working electrode. The background current will be increased. The selectivity of electrochemical detection is obtained by choosing the appropriate potential of the working electrode for the analyte(s). The optimal operating potential is a function of the analyte structure and the nature of the working electrode material. A plot of hydrodynamic voltammograms can be created from data, where the current vs. applied potential defines the best situation for a fixed set of separation-detector conditions.

2.7 Sample preparation

Sample preparation is an essential part of HPLC analysis to provide a reproducible and homogeneous solution that is suitable for injection onto the column. The aims of sample preparation are to contain free interference and to prevent the column from insoluble material [57].

Liquid-liquid extraction (LLE), known as solvent extraction and partition is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids. This technique is a method for separate compounds based on their solubility preferences for two different immiscible liquids. The hydrophilic compounds prefer the polar aqueous phase, whereas more hydrophobic compounds will be found mainly in the organic solvent. Therefore, an analyte is extracted into the organic phase from an aqueous sample, but similar approaches are used when the analyte is extracted into an aqueous phase. Analytes extracted into the organic phase are easily recovered by evaporation of the solvent, while analytes extracted into the aqueous phase can often be injected directly to a reversed-phase HPLC column. An extraction is an equilibrium process with limited efficiency; amounts of the analyte can remain in both phases. Chemical equilibrium involving changes in pH, ion pairing, complexation, and can be used to enhance analyte recovery and/or the

elimination of interferences [56]. The LLE organic solvent is chosen for the following characteristics:

- Low solubility in water (< 10%)
- Low volatility
- Enhance the recovery of the analyte in the organic phase.

2.8 Distribution constant and distribution ratio

The organic reagents are important because it can be extracted readily from water into virtually immiscible organic solvents. The selectivity of a given reagent may be improved by the addition of another equilibrium step (extraction) in the separation [58]. For a nonionic solute that exists in the same molecular form in the two phases the distribution equilibrium of a solute A in this single definite form between water and an organic phase is described by

$$\text{A (water)} \Leftrightarrow \text{A (organic)}$$

$$(K_D)_A = \frac{[A]_{org}}{[A]_{aq}}$$

Where K_D is the distribution constant for A, the subscript org denote the organic phase, and the subscript aq denotes water. The limiting value of K_D at zero ionic strength is a true constant for a particular species under specified condition.

CHAPTER III

EXPERIMENTAL

3.1 Chemical and Reagents

3.1.1 Sudan I (Sovent Yellow), (Sigma – Aldrich)
3.1.2 Sudan II (Sigma – Aldrich)
3.1.3 Sudan III (Fluka)
3.1.4 Sudan IV (Sovent Red 24), (Sigma – Aldrich)
3.1.5 Acetonitrile (HPLC Grade, Merck)
3.1.6 Ethanol (HPLC Grade, Merck)
3.1.7 Acetic acid (BDH)
3.1.8 Ammonium acetate (Riedel de Haën)
3.1.9 A standard buffer solution pH 4 and pH 7 (Metrohm)
3.1.10 Methanol (HPLC Grade, Merck)
3.1.11 Multi-wall carbon nanotube (MWNTS, A gift from Peking University)
3.1.12 Ionic liquid of 1- octyl-3-methylimidazolium hexafluorophosphate (OMIMPF6, A gift from Peking University)

3.2 Instruments and Equipments

3.2.1 An Autolab potentiostat (PGSTAT 30, Methom)
3.2.2 A water Model 510 solvent delivery system (Water Associates Inc, Milford, MA, U.S.A.)
3.2.3 A Rhedyne injection valve, Model 5100 (Altech), with a 20 μL stainless steel injection loop (0.5 mm. i.d.)
3.2.4 Milli – Q water system, (Millipore, Bedfold, MA, USA, R≥18.2 MΩ)
3.2.5 Centrifuge (Cole Parmer)
3.2.6 Mobile phase filter set included 300 mL glass reservior, glass membrane holder, 1,000 mL flask and metal clip (Millipore, USA)
3.2.7 Stainless steel electrode
3.2.8 Autopipette and tips (Gilson, Germany)

3.2.9 Inertsil-ODS3 C18 (5 μm, 4.6 mm x 25 cm, GL Science)

3.2.10 A glassy carbon (GC) electrode (0.07cm^2 , Bioanalytical System Inc.)

3.2.11 A silver/silver chloride (Ag/AgCl) electrode (Bioanalytical System Inc.)

3.2.12 A platinum wire (Bioanalytical System Inc.)

3.2.13 A polish set of 0.05 μm alumina powder (Metrohm)

3.2.14 A thin layer flow cell (Bioanalytical System Inc.)

3.2.15 A Teflon cell gasket (Bioanalytical System Inc.)

3.2.16 Peek tubing (0.25mm i.d.) abd connecting (Upchurch)

3.2.17 Teflon tubing (1/10 inch i.d., Upchurch)

3.2.18 A cutting set (Altech)

3.2.19 A pH meter (Metrohm)

3.2.20 A sonicator (USA, A006651)

3.2.21 An analytical balance (Metler, AT 200)

3.2.22 Erlenmeyer flasks 10 ,100 and 250 mL

3.2.23 Separatory funnels 500 mL

3.2.24 Volumetric flasks 10, 25, 50 and 100 mL

3.2.25 Beakers 10, 25, 50, 500 and 1,000 mL

3.2.26 Vortex mixer (Model VTX-3000L, Mixer uzusio LMS.CO, LTD)

3.2.28 Filters membrane (0.2 μm, 47 mm, Whatman)

3.2.29 Syringe filter, PTFE 13 mm, 0.45 μm (Chrom Tech, Inc.)

3.2.30 Volumetric flask 5.00, 10.00, 50.00, 250.00 and 500.00 mL

3.2.31 Beaker 10, 50, 150, 250 and 600 mL

3.2.32 A glass filter set (250 mL funnel, 1 L flask, glass base and tube cap and spring clamp) for HPLC mobile phase filtration (KONTES)

All glassware were washed with detergent, and rinsed with double distilled water and acetonitrile before use.

3.3 Preparation of chemical solutions

3.3.1 Sudan I – IV standard stock solutions

Each standard stock solution of 1,500 μM four sudan dyes was prepared by weighting sudan I 3.72 mg, sudan II 4.15 mg, sudan III 5.29 mg and sudan IV 5.71 mg, dissolved each dyes with acetonitrile, and after that transferred

each dyes into 10.00 mL volumetric flask. The acetonitrile solution was used for diluting to the mark. All of the standard solutions were stored at 4° C and protected from the light.

3.3.2 Working standard solutions

3.3.2.1 Working standard solutions for cyclic voltammetry

The standard solution of sudan dyes was prepared by pipetting of each stock standard solution (1,500 µM) into a 5 mL volumetric flask and adjusted to 5 mL with carrier solution. The working standard solutions contained 100 µM of each sudan dyes. The solutions were protected from the light.

3.3.2.2 Working standard solutions of sudan I – IV for flow injection analysis

The standard solution of four sudan dyes was prepared by pipetting of each stock standard solution (1,500 µM) into a 5 mL volumetric flask and adjusted to 5 mL with carrier solution. The solutions were protected from the light. The concentrations for FIA are shown in Table. 3.1.

Table 3.1 The concentration of working standard solution of sudan I, sudan II, sudan III and sudan IV for FIA.

Analyte	Concentration of working standard solution (ppm)
Sudan I	0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0,10.0,12.0,15.0, 20.0, 25.0
Sudan II	0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0,10.0,12.0,15.0, 20.0, 25.0,50.0, 75.0, 100.0
Sudan III	0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0,10.0,12.0,15.0, 20.0, 25.0,50.0, 75.0, 100.0,150.0
Sudan IV	0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0,10.0,12.0,15.0, 20.0, 25.0,50.0, 75.0, 100.0

3.3.2.3 The mixed working standard solutions of four sudan dyes for HPLC – EC analysis

The mixed standard solution of four sudan dyes was prepared by appropriate diluting either 1,500 µM stock solution with mobile phase into 5 mL volumetric flask and kept in an airtight container. The final concentration of each sudan dye was shown in Table. 3.2.

Table 3.2 The concentration of mixed standard solution of sudan I, sudan II, sudan III and sudan IV for HPLC-EC

No.	Concentration			
	Sudan I	Sudan II	Sudan III	Sudan IV
1	0.005	0.005	0.005	0.005
2	0.01	0.01	0.01	0.01
4	0.025	0.025	0.025	0.025
5	0.05	0.05	0.05	0.05
6	0.10	0.10	0.10	0.1
7	0.25	0.25	0.25	0.25
8	0.50	0.50	0.50	0.5
9	1.0	1.0	1.0	1.0
10	2.5	2.50	2.50	2.5
11	5.0	5.0	5.0	5.0
12	10.0	10..0	10.0	10.0
13	-	12.0	15.0	15.0
14	-	-	25.0	25.0
15	-	-	30.0	30.0
16	-	-	50.0	50.0
17	-	-	60.0	60.0
18	-	-	100.0	100.0
19	-	-	110.0	110.0
21	-	-	120.0	120.0
22	-	-	130.0	130.0
23	-	-	-	150.0

3.3.3 Preparation of samples

The commercial samples were purchased in big trades: 3 brands of soft drinks and 1 brand of chili sauce.

3.3.3.1 Sample preparation of soft drink samples

Five milliters of soft drink sample was pipeted into four beakers. Each 0, 0.5, 1.0 and 1.5 ppm of the mixed standard solution of sudan dyes at the final volume was added into beaker and mixed rapidly with vortex mixer, respectively. Next step, a portion of clarified solution was removed and evaporated at 30 °C on hot plate stirrer until remained 1 mL of sample solution. The remaining sample solution was extracted with 5 mL of acetonitrile by vortex mixer for 1 minute. After extracted, it was clarified by centrifugation at 4,000 rpm for 5 minutes at room temperature, dried solvent with nitrogen gas, and added 1 mL of mobile phase. The solution was mechanically shaken for a minute, then transferred into a 5 mL volumetric flask and diluted to the final volume. A sample solution was filtered through a 0.45 µm PTFE syringe filter membrane and degassed for 1 minute with a sonicator before injection into the HPLC – EC system.

3.3.3.2 Sample preparation of chili sauce sample

One gram of chili sauce samples was weighed into 5 mL volumetric flask and added with 0, 0.5, 1.0, and 1.5 ppm of the mixed standard solution of sudan dyes calculate at the final volume, respectively. Each sample was extracted with 5 mL of acetonitrile by vortex mixer for 1 minute. After extracted, it was centrifuged at 4,000 rpm for 5 minutes at room temperature. A portion of clarified solution was removed and evaporated until it dried. Then this part was dissolved with 1 mL of mobile phase and transferred into 1 mL HPLC – bottle. Sample solution was filtered through a 0.45 µm PTFE syringe filter membrane.

3.3.4 Carrier solution / Mobile Phase for FIA and HPLC – EC

20 mM acetate buffer solution was prepared by dissolving 15.42 mg of ammonium acetate and 1.0 mL of glacial acetic acid in 100 mL volumetric flask and diluted to volume by Milli – Q water.

The mobile phase was prepared by mixing 100 mL of acetate buffer solution and 900 mL of acetonitrile solution (HPLC glade) in 1.0 L volumetric flask. Then,

the solution was mixed thoroughly to complete dissolution, filtered through a 0.45 μm nylon membrane with mobile phase filter set and finally degassed by ultrasonic bath.

3.4 Procedure

3.4.1 Cyclic voltammetry

Cyclic voltammetric investigations of the electrochemical behaviors of four sudan dyes were studied in a electrochemical cell. The voltammograms were recorded using an autolab potentiostat 100. The platinum wire was used as the counter electrode and an Ag / AgCl was used as the reference electrode. The GC as the working electrode was polished to a mirror with 1.0 and 0.3 μm alumina powder, washed by Milli – Q water, and sonicated with ethanol and water prior to use. The electrochemical measurements were carried out in a faradaic cage to reduce electronic noise at room temperature.

3.4.2 Flow injection analysis

Flow injection amperometric measurements were performed using HPLC pump and a manual injector. Amperometric detection was carried out in a thin-layer flow cell equipped with a GC electrode. The determination of each sudan dyes was performed at fixed optimal potential for each sudan dyes. Four injections were carried out for each concentration.

3.4.3 High performance liquid chromatographic optimization

HPLC-EC system for analysing solution of mixed sudan dyes was equipped with a C18 column. The appropriate mobile phase, acetonitrile and acetate buffer was developed by varying percentage of acetonitrile and the buffer solution. The separation of four sudan dyes was tested with mixed standard. A mixed standard solution of four sudan dyes; i.e. sudan I, sudan II, sudan III and sudan IV was injected. The injection volume was 20 μL and the detector was amperometric detector.

3.4.4 Fabrication of the modified electrode

The GC disc working electrode (4 mm diameter, China) was polished before use with 1 μm and 0.3 μm alumina powder. After rinsing, the electrode was placed in an ultrasonic cleaner for 5 min, first with ethanol and then with deionized water, to remove any traces of impurities. To dry the surface of the GC electrode, it was flowed with N_2. A mixture of 12 mg MWNTs and 0.2mL OMIMPF6 was ground with an agate mortar for about 20 min, and a black gel was formed [59]. Then, the GC electrode was spread with carbon nanotube gel and placed on a smooth glass slide, and the gel was mechanically attached to the electrode surface. Finally, after the gel on the electrode surface was smoothed with a spatula to leave a thin gel film on the GC electrode surface, the gel, which modified the glassy carbon electrode (denominated as CNT electrode in this research), was fabricated.

3.5 Calibration and linearity

Each concentration of the mixed standard solution of sudan dyes 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 150.0, 200.0, 250.0 and 300.0 ppm was injected in duplicate. The calibration curve was plotted between the peak areas and the concentrations.

3.6 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ are defined as the concentrations that provide a current response higher 3 times than the noise ($S/N \geq 3$) and 10 times than the noise ($S/N \geq 10$), respectively. They are determined by various concentrations under HPLC conditions.

3.7 Precision and accuracy

For intra-day precision, the repeated analysis of spiked samples is studied in one day. For inter-day precision, the repeat of analysis of spiked samples is studied on different days. The spiking concentration at levels 0.5, 1.0, and 1.5 ppm were used in this study and each level was repeated in triplicate.

3.8 Applications

The HPLC-EC using the CNT electrode was applied to detect four sudan dyes in soft drink. In this research, the HPLC-EC study of sudan dyes in soft drink samples has been carried out at CNT modified GC selectrode comparison with GC electrode. Three types of soft drinks were purchased from the big trade.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Cyclic voltammetric investigation

The electrooxidation of sudan dyes was investigated by cyclic voltammetry. The cyclic voltammograms for 100 μM standard solutions of four sudan dyes with the corresponding background current of acetonitrile and 20 mM acetate buffer (90:10; v/v) at GC electrode show well-defined, irreversible peaks obtained for the oxidation of sudan I in Figure 4.1. The results obtained from the other sudan dyes, sudan II, sudan III and sudan IV were analogous to the sudan I response and are shown in appendix A. The obtained electrochemical results for all analytes are shown in Table 4.1. The results indicate that the sensitivity of sudan I is higher than sudan II, sudan III and sudan IV, respectively.

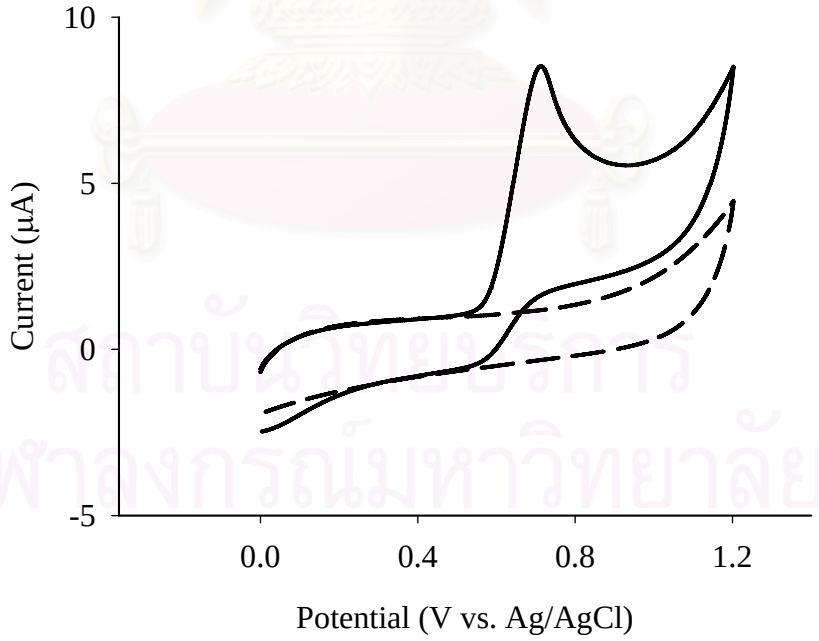


Figure 4.1 Cyclic voltammograms for 100 μM sudan I in acetonitrile and 20 mM acetate buffer (90:10; v/v) (solid line) together with the corresponding background current (dash line) at a GC electrode. The scan rate was 50 mV/s; electrode area, 0.07 cm^2.

In the presence of these analytes, the anodic waves were observed on the positive scan beginning at ca. 0.0 to +1.2 V vs. Ag/AgCl for four sudan dyes. The cathodic peak was not observed for four sudan dyes.

Table 4.1 The electrochemical data of 100 µM sudan I, II, III and IV at GC electrode

Analytes	E_p^{ox}* (V vs. Ag/AgCl)	I_p^{ox} ** (µA)	S/N[a]
Sudan I	0.729	7.083	5.358
Sudan II	0.717	5.307	4.014
Sudan III	0.748	3.555	2.689
Sudan IV	0.726	3.416	2.584

*Potential of the oxidation peak

**Current of the oxidation peak

[a]Calculated from I_p^{ox} / background current

4.1.1 Scan rate dependence study

The effect of the scan rate on the electrochemical behaviors of sudan I, sudan II, sudan III and sudan IV were investigated by variation of the scan rate from 10 to 300 mV/s. The relationship between the current responses versus the square root of the scan rate ($v^{1/2}$) is highly linear ($r^2 > 0.99$) for four sudan dyes as shown in the insets of Figures 4.2, 4.3, 4.4, and 4.5, respectively. From these results, it can be concluded that the diffusion process controls the transportation of these analytes.

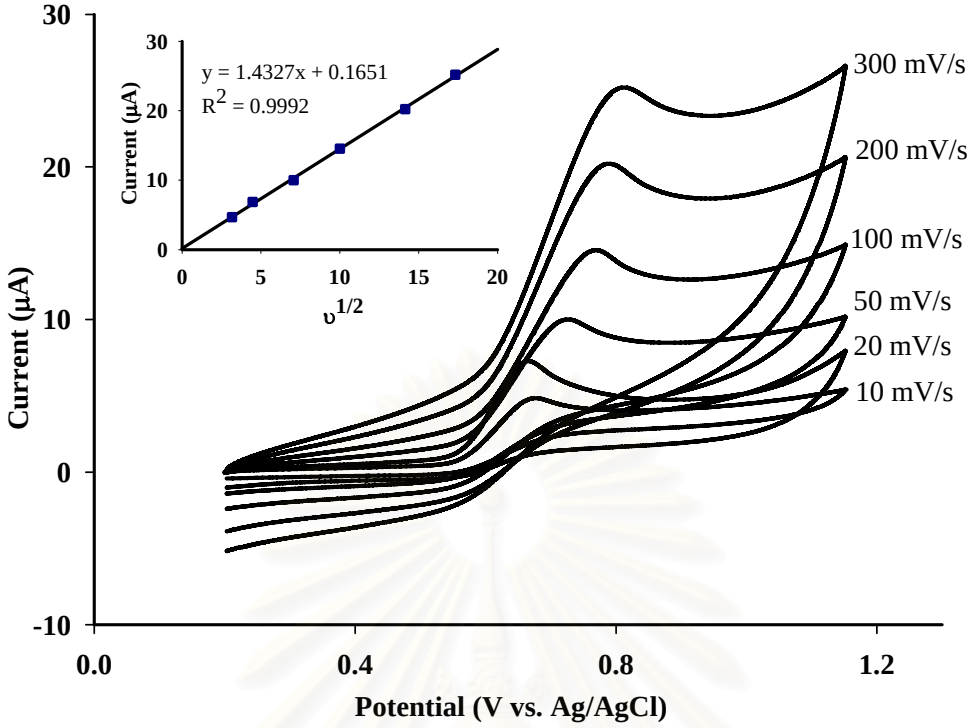


Figure 4.2 Cyclic voltammograms for 100 µM Sudan I in solution of acetonitrile and 20 mM acetate buffer (90:10; v/v) using various scans rates and a GC electrode. The inset shows the relationship of the current response versus the square root of the scan rate ($v^{1/2}$)

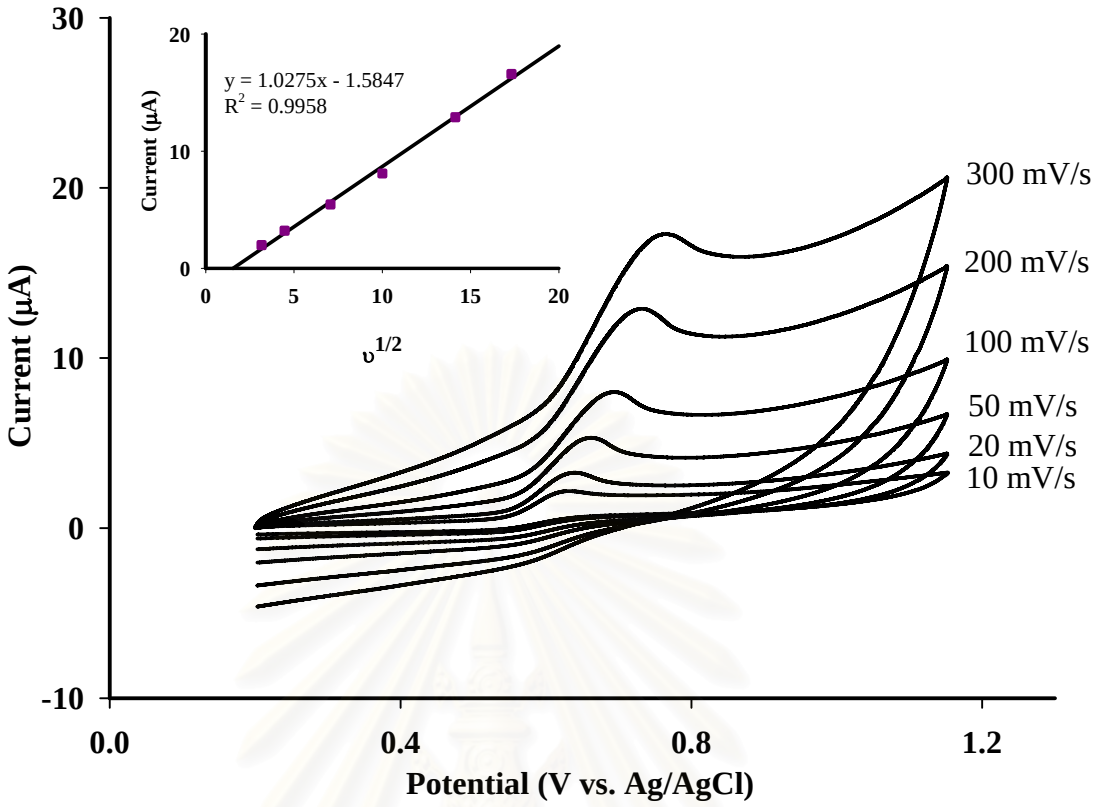


Figure 4.3 Cyclic voltammograms for 100 µM sudan II in a solution of acetonitrile and 20 mM acetate buffer (90:10; v/v) using various scan rates and a GC electrode. Inset shows the relationship of the current responses versus the square root of the scan rate ($v^{1/2}$)

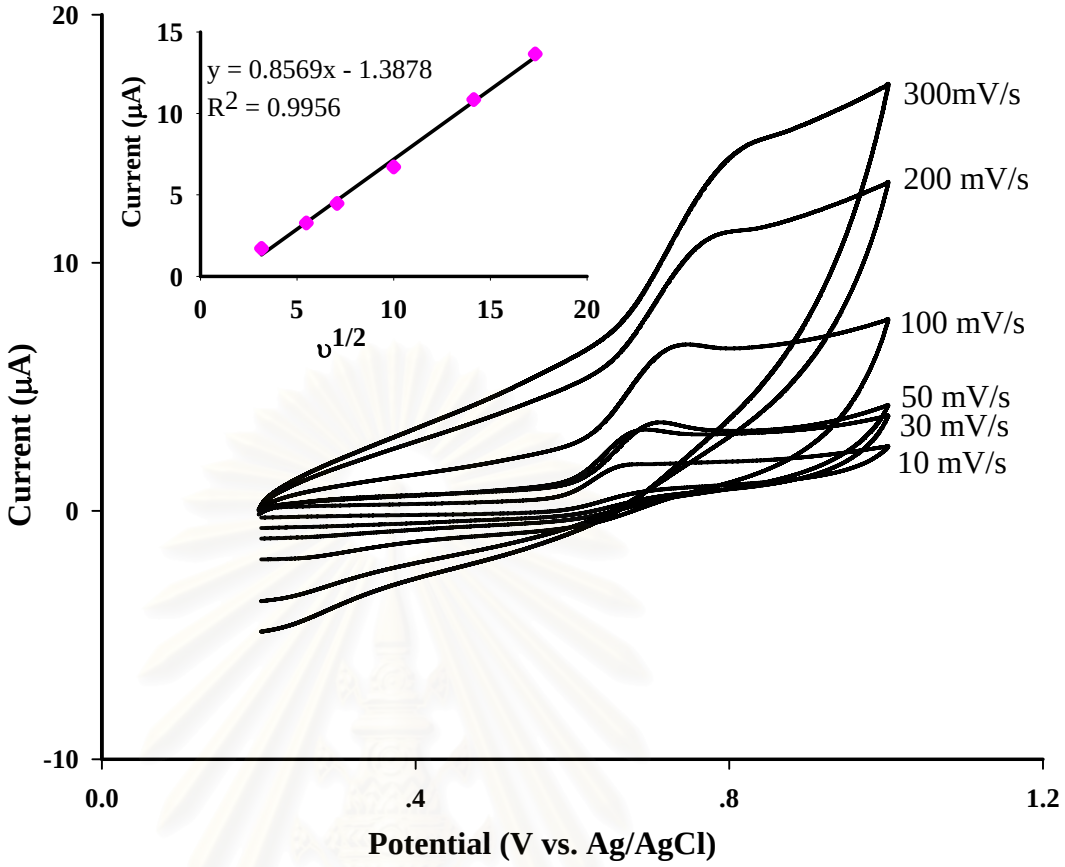


Figure 4.4 Cyclic voltammograms for a dependence study of various scan rates on 100 µM sudan III in acetonitrile and 20 mM acetate buffer (90:10; v/v) solution at GC electrode. Inset shows the relationship of the current response versus the square root of the scan rate ($v^{1/2}$).

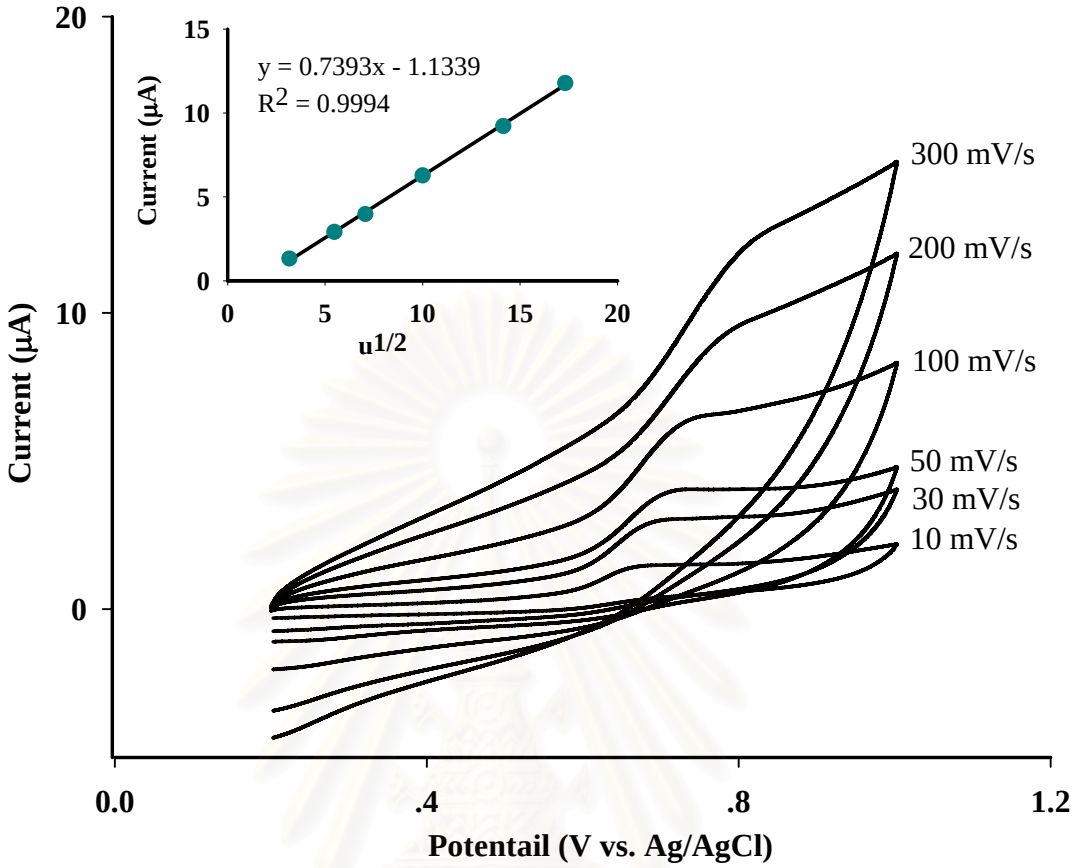


Figure 4.5 Cyclic voltammograms for a dependence study of various scan rates on 100 µM Sudan IV in acetonitrile and 20 mM acetate buffer (90:10; v/v) solution at a GC electrode. The inset shows the relationship of the current response versus the square root of the scan rate ($v^{1/2}$).

4.2 Flow injection analysis study

A flow-injection method for the determination of four sudan dyes based on electrochemical oxidation at the GC electrode is presented.

4.2.1 Hydrodynamic voltammetric study

The optimum potential was investigated by hydrodyanamic voltammetry. A hydrodynamic voltammogram was obtained from the average of three standard injections of 20 µL of 10 ppm of each sudan dye solution in the flow

injection system at increasing values of the applied potential, from 0.4 to 1.4 V vs. Ag/AgCl in the flow injection system. The carrier solution was acetonitrile and 20 mM acetate buffer (90:10; v/v). Figure 4.6 shows the hydrodynamic analysis of four sudan dyes. The results show the maximum S/B ratio at 0.8, 0.9, 0.95, and 0.8 V vs. Ag/AgCl for sudan I, sudan II, sudan III, and sudan IV, respectively. Hence, these potentials were used for quantitative flow injection analysis.

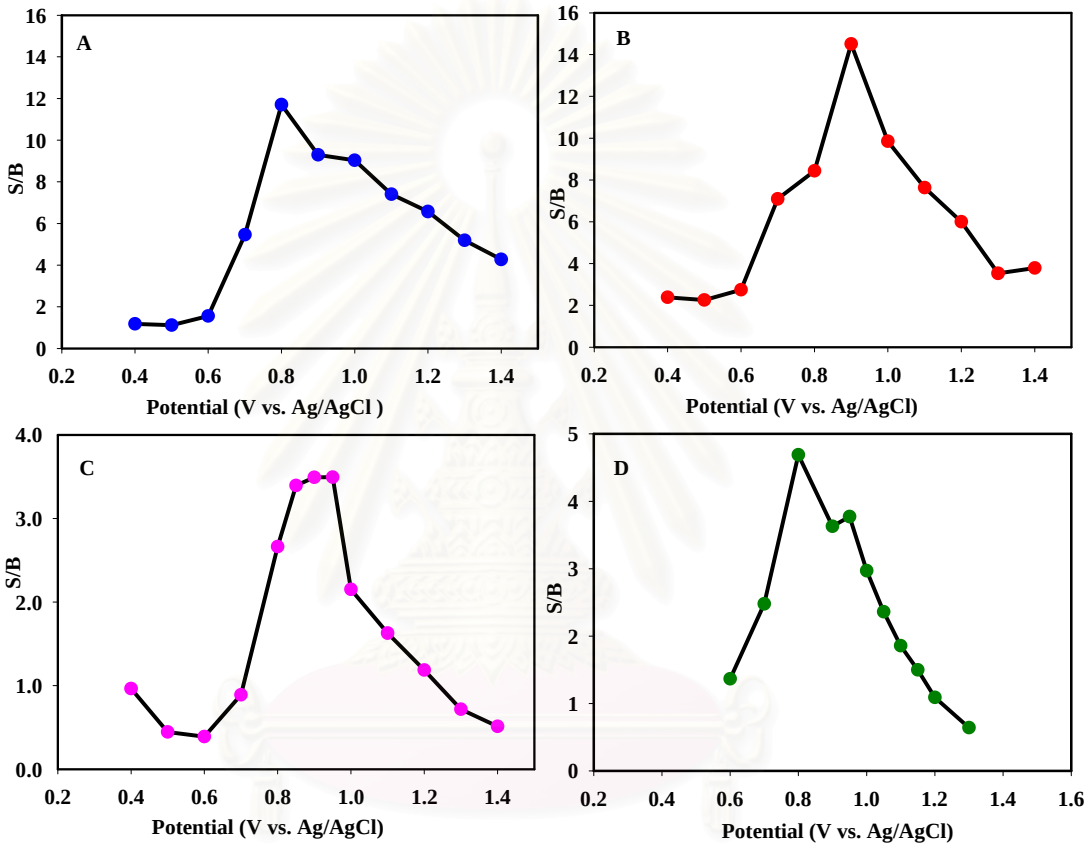


Figure 4.6 Hydrodynamic voltammetric results of signal-to-background for 10 ppm of each sudan dyes. The average peak current obtained from injections (n=3) of (A) sudan I (B) sudan II (C) sudan III and (D) sudan IV in carrier solution of acetonitrile and 20 mM acetate buffer (90:10; v/v) The flow rate was 1 mL min^{-1}.

4.2.2 Analytical performance using the flow injection system

4.2.2.1 Calibration and linearity study

From the chosen optimum potential**,** the calibration curves were separately constructed for each sudan dye standard solution in the concentration range of 0.01 to 120 ppm as shown in Figures 4.7a-d. Each point of the calibration graph corresponds to the mean value from three replicated injections. The regression analysis of each sudan dye standard solution is summarized in Table 4.2. Linear range of FIA analysis was in the range from 0.05-5, 0.25-10, 0.05-10 and 0.025-10 ppm for sudan I, sudan II, sudan III, and sudan IV, respectively.

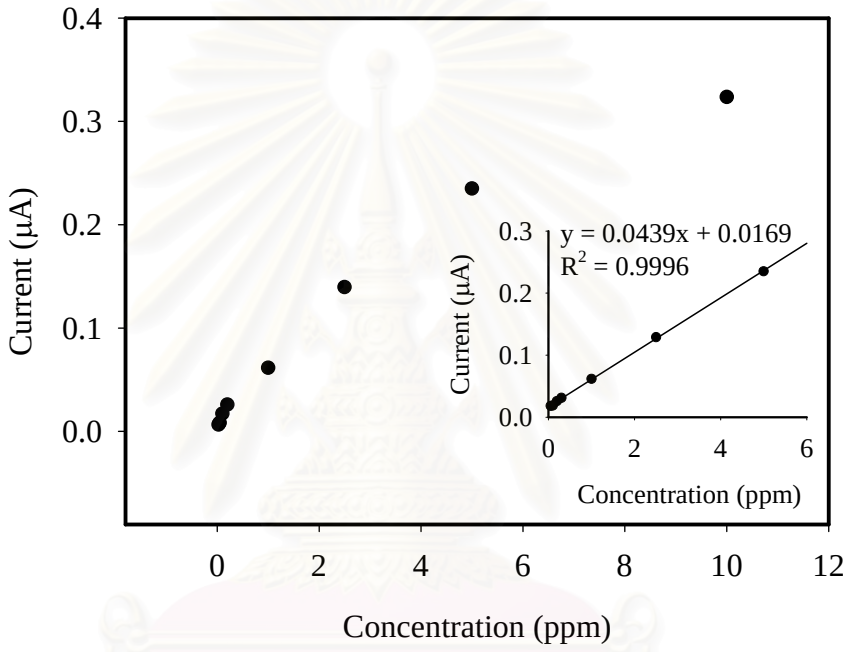


Figure 4.7a The relationship between the current response and the concentrations of sudan I in the carrier stream of acetonitrile and 20 mM acetate buffer (90:10; v/v). The flow rate is 1 mL min^{-1}. A linear range is shown in the inset.

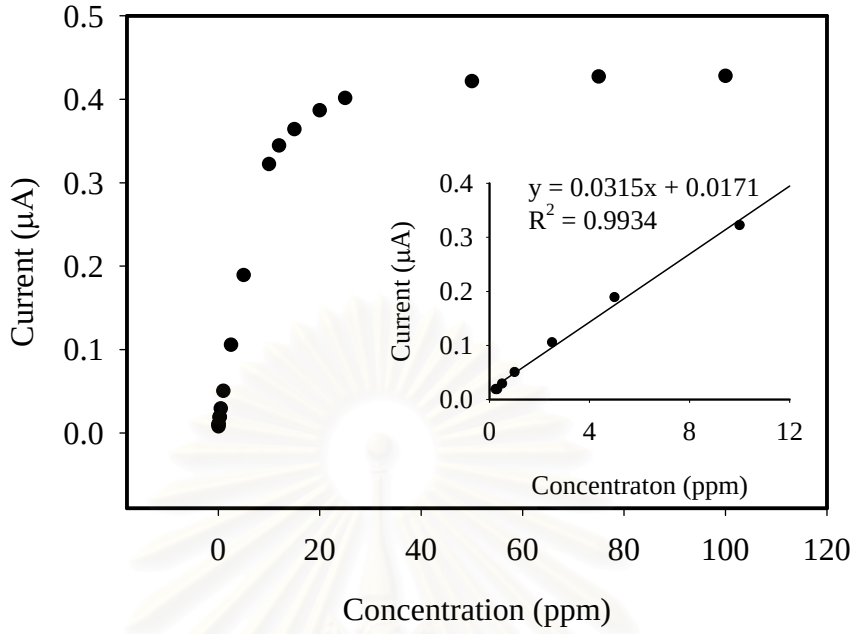


Figure 4.7b The relationship between the current response and the concentrations of sudan II in the carrier stream of acetonitrile and 20 mM acetate buffer (90:10; v/v). The flow rate is 1 mL min^{-1}. A linear range is shown in the inset.

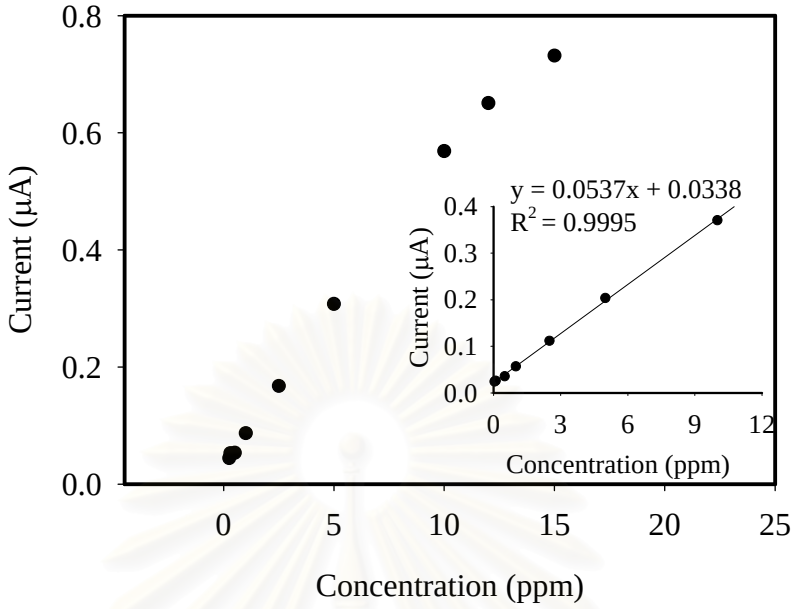


Figure 4.7c The relationship between the current response and the concentration of sudan III in the carrier stream of acetonitrile and 20 mM acetate buffer (90:10; v/v). The flow rate was 1 mL min^{-1}. A linear range is shown in the inset.

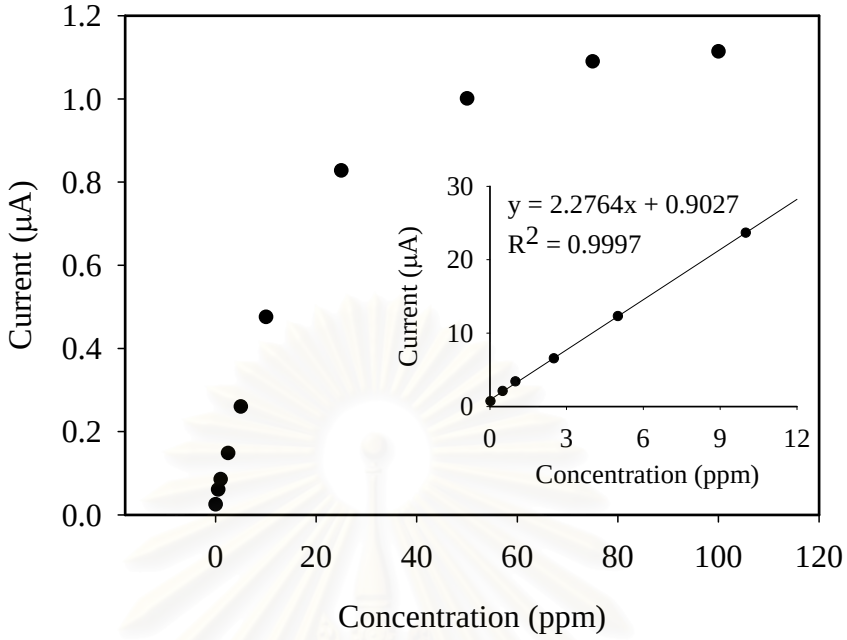


Figure 4.7d The relationship between the current response and the concentration of sudan IV in the carrier stream of acetonitrile and 20 mM acetate buffer (90:10; v/v). The flow rate was 1 mL min^{-1}. A linear range is shown in the inset.

4.2.2.2 LOD and LOQ

The detection limit was investigated by examining various concentrations of sudan I, sudan II, sudan III, and sudan IV from 0.01 ppm to 120 ppm. The LOD is defined as the concentration that provides a signal-to-noise ratio of 3 (3S/B). The LOQ was determined under the definition of ten times the signal to noise ratio (10S/B). The current signal increases with the concentration increase as seen with sudan II in Figure 4.8. From these results, the detection limits obtained from this proposed method were 0.01 ppm for sudan I and sudan IV, 0.10 ppm for sudan II and 0.025 ppm for sudan III. The data are summarized in Table 4.2.

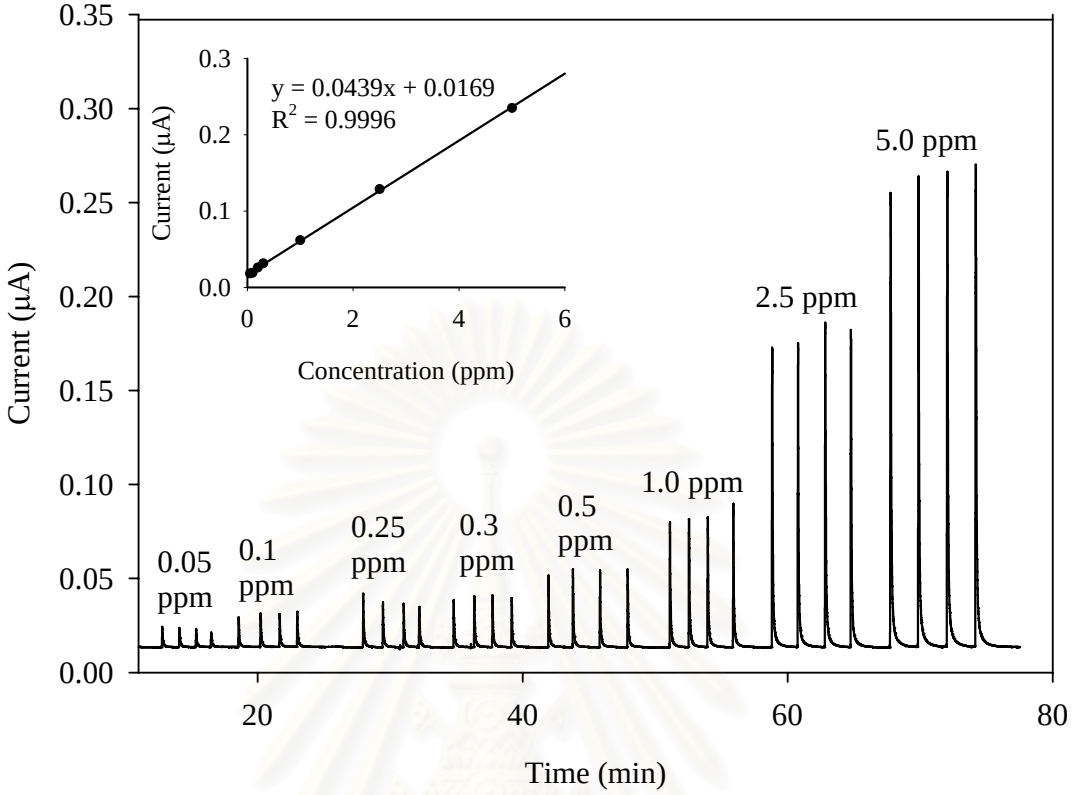


Figure 4.8 Flow injection signals of sudan I standard in carrier stream of acetonitrile and 20 mM acetate buffer (90:10; v/v). The flow rate was 1 mL min^{-1}.

Table 4.2 Regression analysis of parameters (R^2), linear range (LR), limit of detection (LOD) and limit of quantification (LOQ) of sudan I, sudan II, sudan III, and sudan IV by FIA with amperometric detection using GC electrode.

	SUD I	SUD II	SUD III	SUD IV
LR (ppm)	0.05-5.0	0.25-10.0	0.05-10.0	0.025-10.0
R^2	0.9996	0.9934	0.9995	0.9997
LOD (ppm)	0.01	0.10	0.025	0.01
LOQ (ppm)	0.05	0.25	0.05	0.025

4.3 The results of HPLC amperometric method development

4.3.1 The optimal parameters of HPLC separation

Optimum conditions are necessary for the quantitative analysis of the four sudan dyes (sudan I, sudan II, sudan III, and sudan IV), to enhance sensitivity and efficiency of separation. Optimization of HPLC with amperometric detection were established by varying one parameter at a time, fixing other parameters constant and observing its effect on peak width and resolution.

4.3.1.1 Mobile phase

Effect of mobile phase type and mobile phase composition are important for HPLC analysis. The type of solvent and mobile phase for analysis of sudan dyes was investigated from prior published results [2]. The optimum solvent for sudan I, sudan II, sudan III and sudan IV was acetonitrile because of its ready solubility. The results are shown in Table 4.3.

Table 4.3 Solubility of sudan I, sudan II, sudan III and sudan IV in 100g methanol, ethanol, and acetonitrile at 25 °C

Analytes	mg/100g		
	Methanol	Ethanol	Acetonitrile
Sudan I	1.98	1.00	1.98
Sudan II	0.38	0.75	1.82
Sudan III	0.26	0.45	1.14
Sudan IV	0.18	0.22	0.65

The mobile phase composition was investigated by various ratios of acetonitrile and buffer solution. This isocratic elution was developed to separate sudan dyes in this study. The results are collected in Table 4.5 and are also shown in Figures 4.9, 4.10, and 4.11. From the result, conditions D have a longer time for separation than condition E. Thus, the best separation of sudan I, sudan II,

sudan III and sudan IV was obtained under condition E when the mobile phase was composed of acetonitrile: 20 mM acetate buffer in the ratio of 90:10 (%v/v). The HPLC chromatographic conditions for sudan I, sudan II, sudan III and, sudan IV detection are described in Table 4.6 and shown by the chromatogram in Figure 4.11. The selectivity of the appropriate mobile phase from HPLC optimized conditions and optimum potential can be determined by resolution and retention time values as shown in Table 4.4, the results from other condition are shown in Appendix D.

Table 4.4 Retention time and resolution of sudan I, sudan II, sudan III and sudan IV

Analytes	Retention time : t_R (min)	Resolution :R_s
Sudan I	4.7	10.0
Sudan II	7.8	6.20
Sudan III	10.9	10.13
Sudan IV	19.2	-

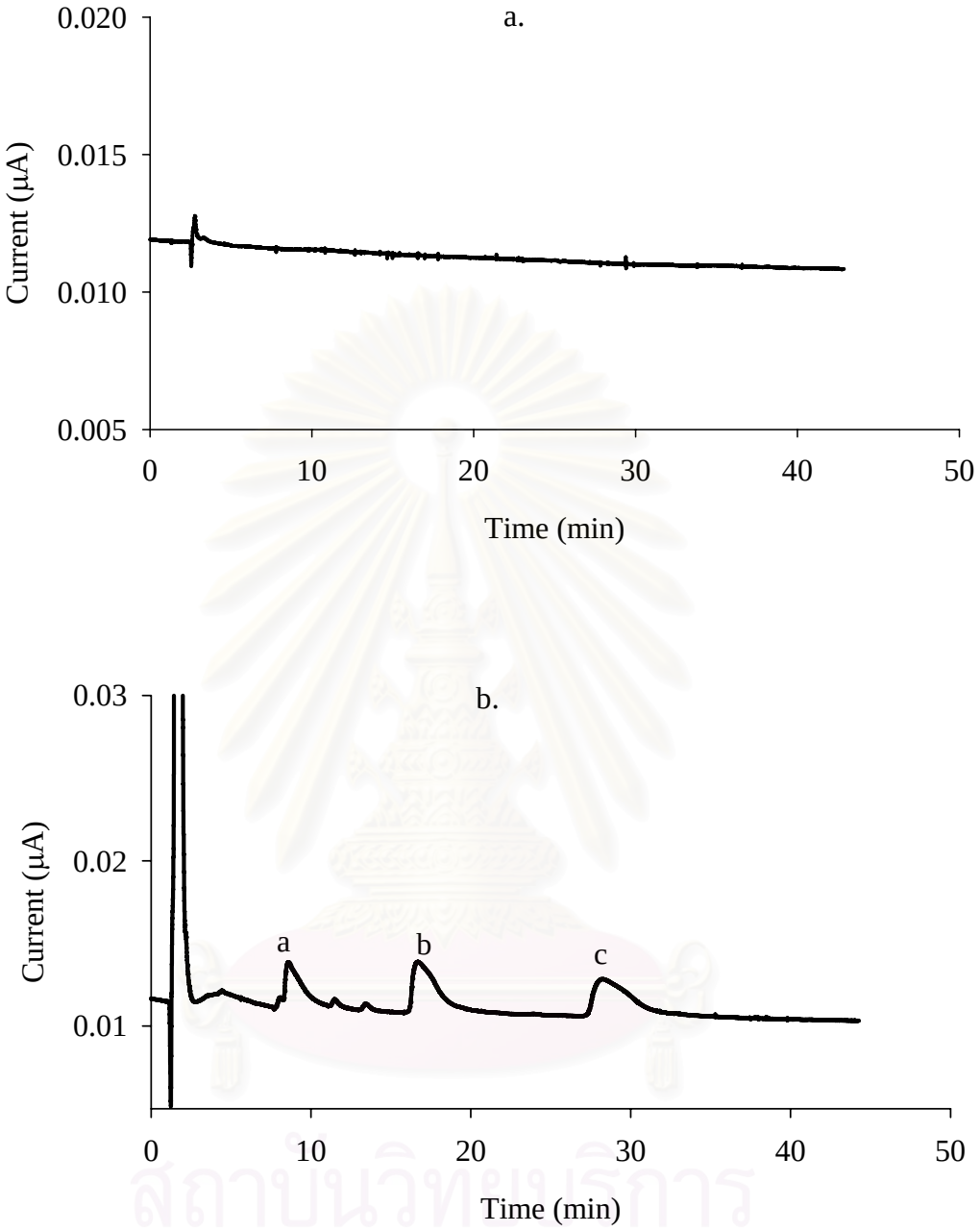


Figure 4.9 Chromatograms of 10 ppm mixed standard of sudan I, sudan II, sudan III and sudan IV at a GC electrode. The mobile phase was; *a.* acetonitrile: H_2O (80:20 %v/v), *b.* acetonitrile: H_2O and 0.05 M TBAP (80:20 %v/v). The injection volume was 20 μL, and flow rate was 1 mL min^{-1}.

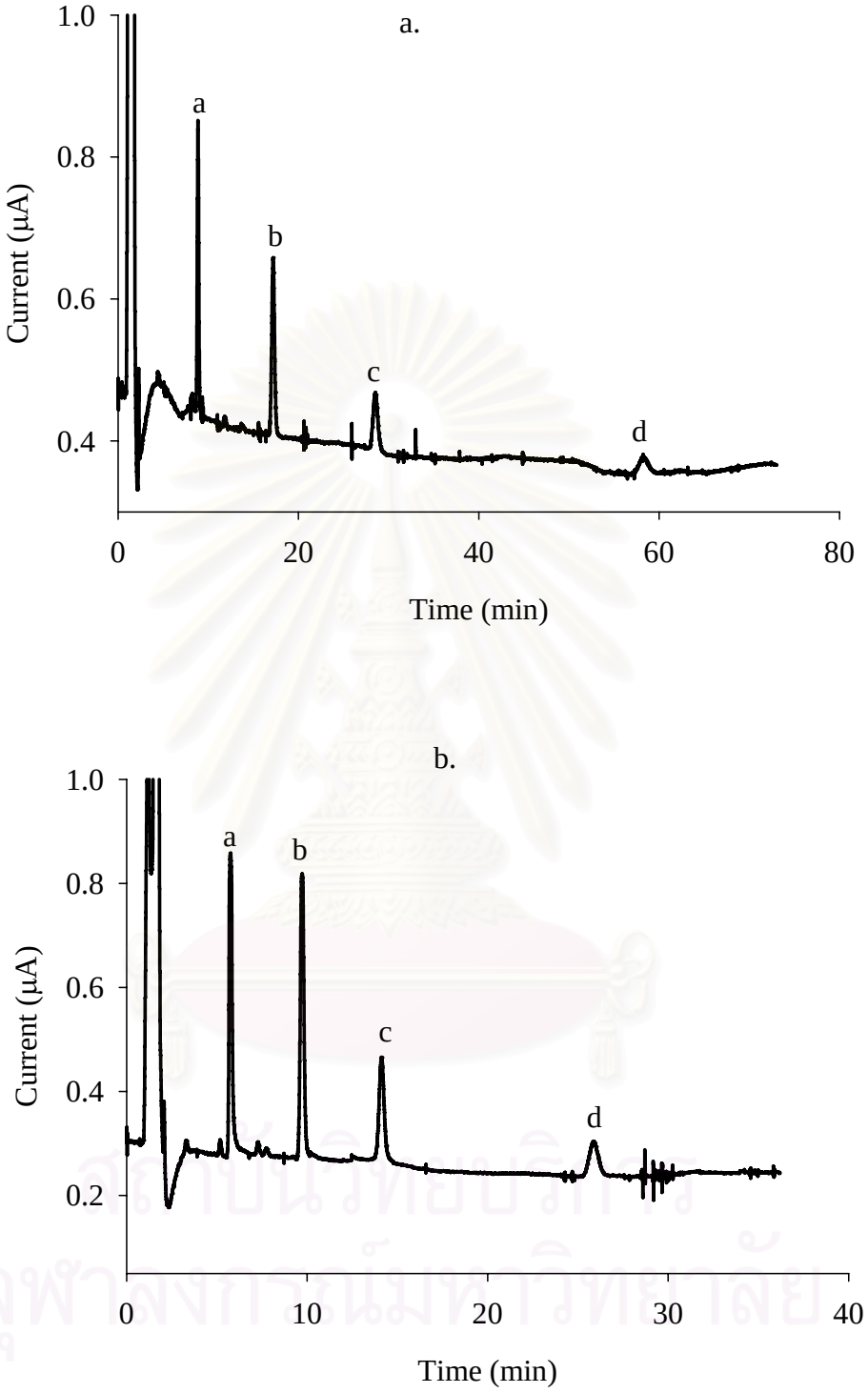


Figure 4.10 Chromatogram of 10 ppm mixed standard of sudan I, sudan II, sudan III and sudan IV at GC electrode. The mobile phase was; *a.* acetonitrile: 0.1% formic acid (80:20 %v/v), *b.* acetonitrile: 0.1% formic acid (90:10 %v/v). The injection volume was 20 μL, and flow rate was 1 mL min^{-1}.

Table 4.5 The mobile phase investigation: Inertsil ODS-3 C18 HPLC packed column (GL science Inc., 4.6 mm x 250mm, 5 μm), flow rate 1 mL/min, column temperature 25°C and elution mode was isocratic.

Investigation conditions	Mobile phase
Condition A	Acetonitrile : H_2O (80 : 20 %v/v)
Condition B	Acetonitrile : H_2O (80 : 20 %v/v) (dilute working solution with mobile phase containing 0.05 M TBAP)
Condition C	Acetonitrile : 0.1 % formic acid (80 : 20 %v/v)
Condition D	Acetonitrile : 0.1 % formic acid (90 : 10 %v/v)
Condition E	Acetonitrile : 20 mM acetate buffer (90 : 10 %v/v)

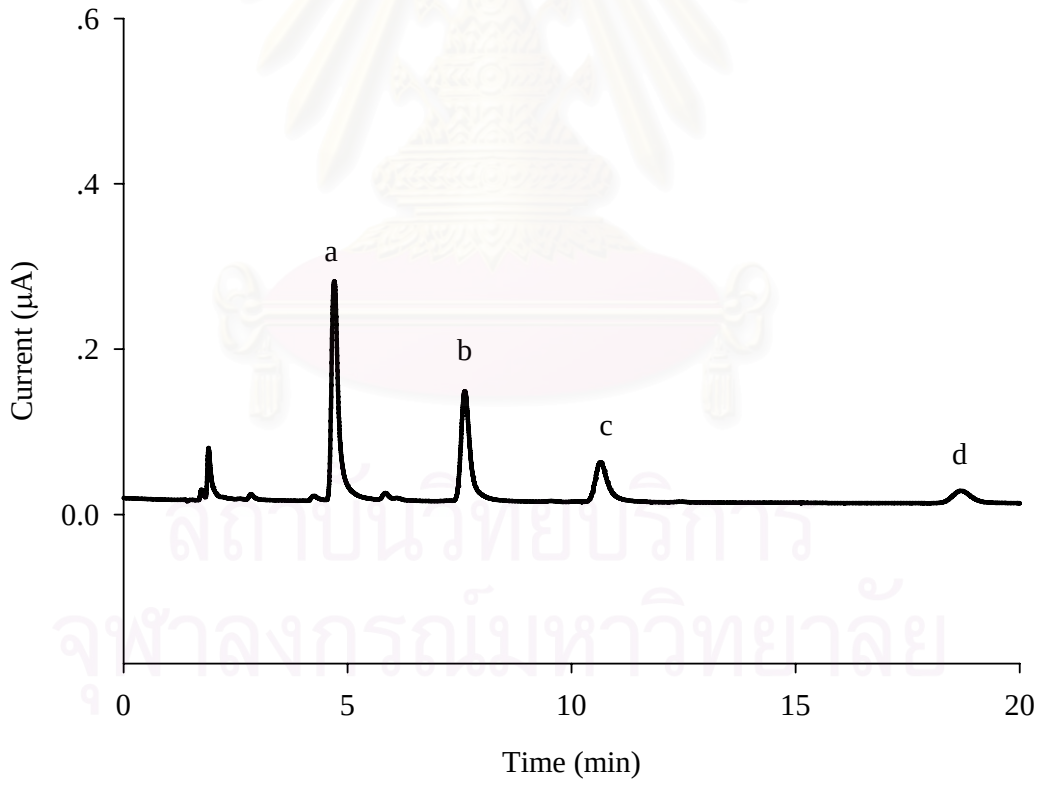


Figure 4.11 Chromatogram of a mixture containing 10 ppm concentration of standard a) sudan I, b) sudan II, c) sudan III and d) sudan IV at GC electrode. The mobile phase was acetonitrile: 20 mM acetate buffer (90:10 %v/v). The injection volume was 20 μL, and flow rate 1 mL min^{-1}

Table 4.6 The HPLC chromatographic conditions for sudan I, sudan II, sudan III and sudan IV detection

HPLC parameters	HPLC conditions
Analytical column	Inertsil ODS-3 C18 HPLC packed column (GL science Inc., 4.6 mm x 250mm, 5 μm)
Mobile phase	Acetonitrile : 20 mM acetate buffer (90 : 10 %v/v)
Flow rate	1 mL/min
Injection volume	20 μL
Elution mode	Isocratic
Column temperature	Room temperature (25°C)
Detector	Amperometric detector

4.3.1.2 Working potential optimization on GC electrode

For an electrochemical detector, the sensitivity of analyte detection depends on the efficiency of the analyte on the electrode surface. Hence, the optimum potential of four sudan dyes in the HPLC system were investigated by injection of 1.0 ppm mixed standard sudan dyes solution and used a potential range from +0.7 to +1.3 V versus Ag/AgCl at the GC electrode. The results of optimum potential waveform of four sudan dyes are shown in Figure 4.12. The oxidation current increased, which increased the potential until a potential of 0.95 V versus Ag/AgCl for sudan I, sudan II and sudan III and 0.90 V versus Ag/AgCl for sudan IV. So, the potential at 0.95 V versus Ag/AgCl was selected for analysis of four sudan dyes in HPLC system by GC electrode.

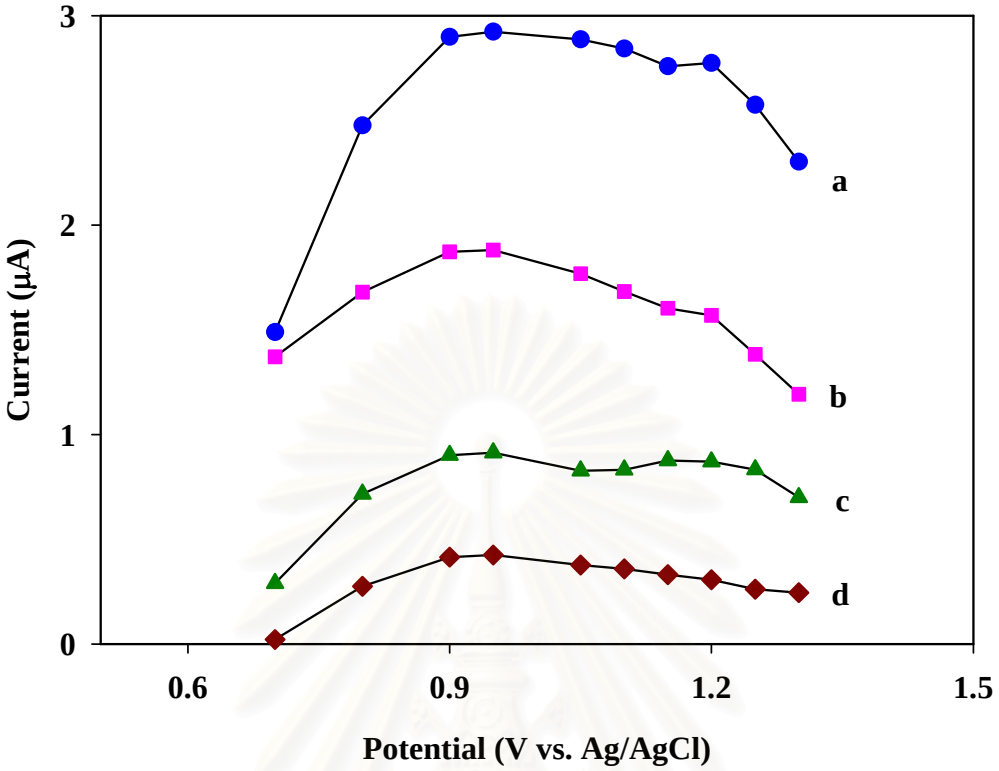


Figure 4.12 HPLC-EC response as a function of detection potential for 1 ppm of a) sudan I, b) sudan II, c) sudan III and d) sudan IV in acetonitrile and 20 mM acetate buffer (90:10; %v/v) at a GC electrode. The injection volume was 20 μL and the flow rate was 1 mL min^{-1}.

4.3.2 Calibration and Linearity

The current response of the four sudan dyes varied linearly with standard concentrations over a range of 0.01-150 ppm. Calibration curves of the four sudan dyes were obtained from the relationship of the peak area versus concentration (ppm). These calibration curves, shown in Figures 4.13, 4.14, 4.15 and 4.16 were obtained from triplicate analyses of each analyte. The slope and y-axis intercept together with correlation coefficient were calculated according to a regression equation in $y = mx + b$ form. The correlation coefficients (R^2), slopes and intercepts of each sudan dyes are summarized in Table 4.7.

Table 4.7 Calibration characteristics of sudan I, sudan II, sudan III and sudan IV by the best HPLC condition

analytes	Slope (μA/ppm)	Intercept	Correlation coefficient (R^2)	Linear range (ppm)
Sudan I	0.4760	+0.9994	0.9994	0.01-15.0
Sudan II	1.4591	+0.0399	0.9989	0.01-12.0
Sudan III	0.0736	+0.1194	0.9970	0.025-120.0
Sudan IV	0.0462	+0.0094	0.9997	0.10-150.0

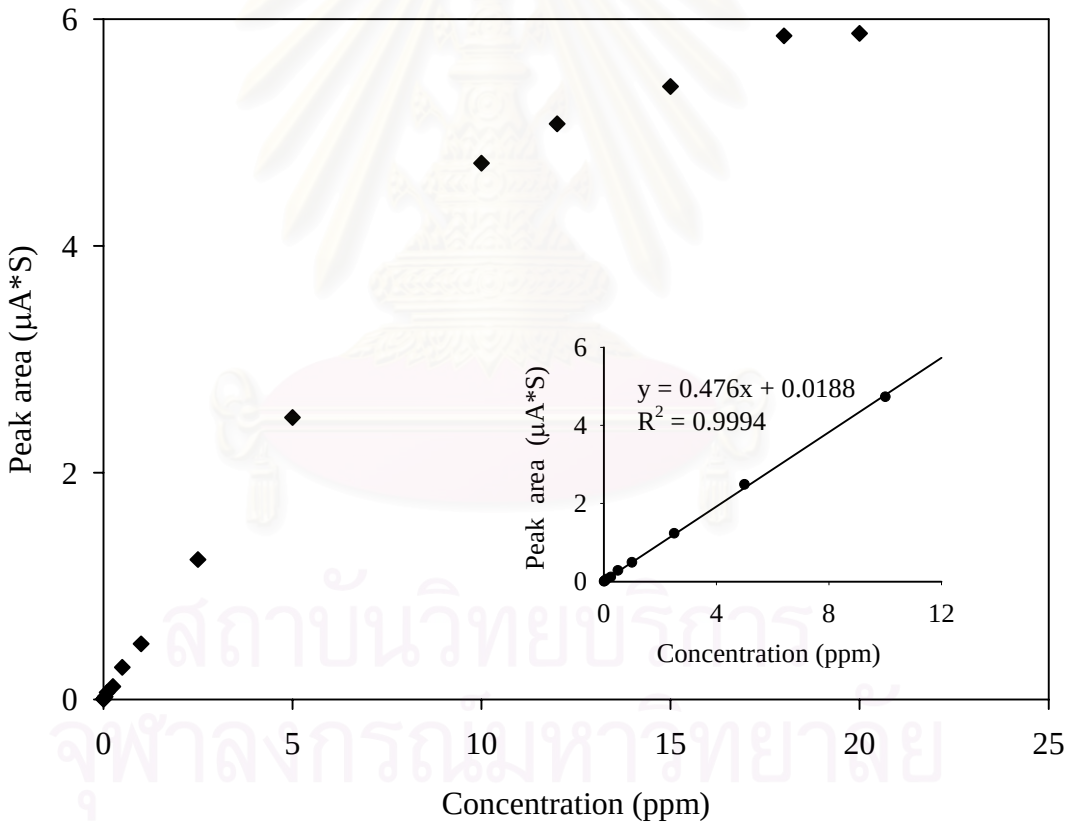


Figure 4.13 Calibration curve of standard sudan I solutions by HPLC-EC using the GC electrode

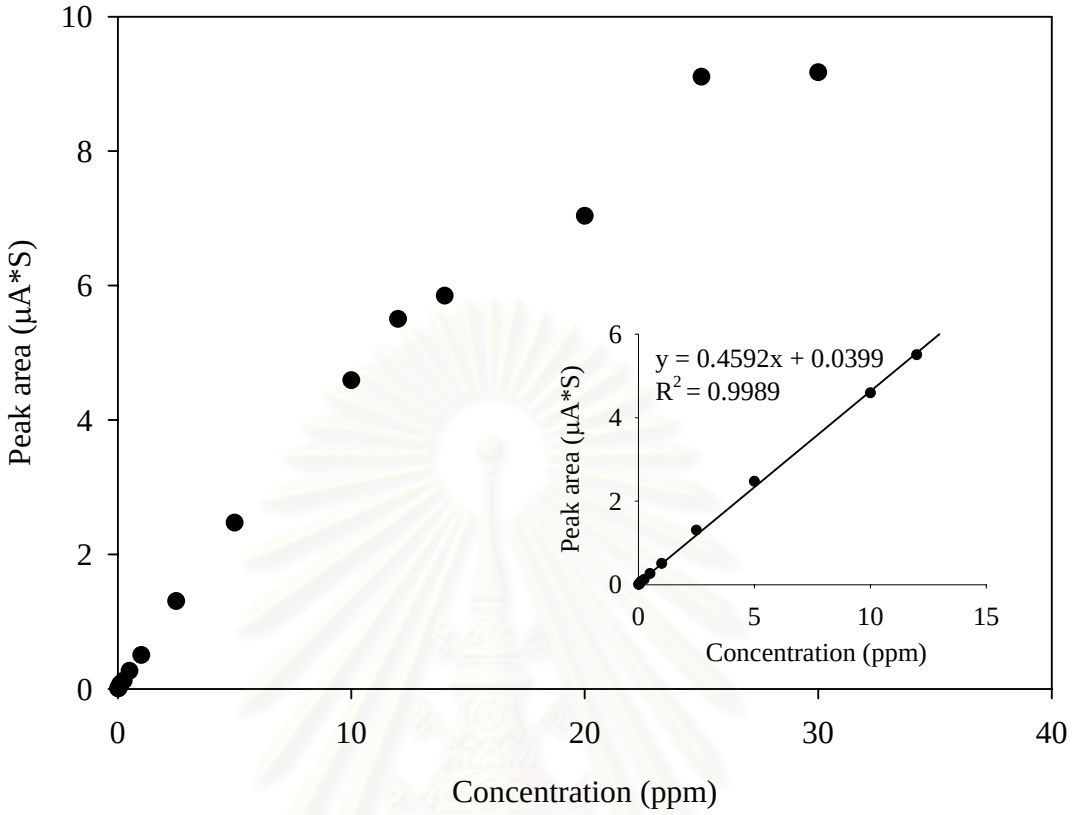


Figure 4.14 Calibration curve of standard sudan II solutions by HPLC-EC using the GC electrode

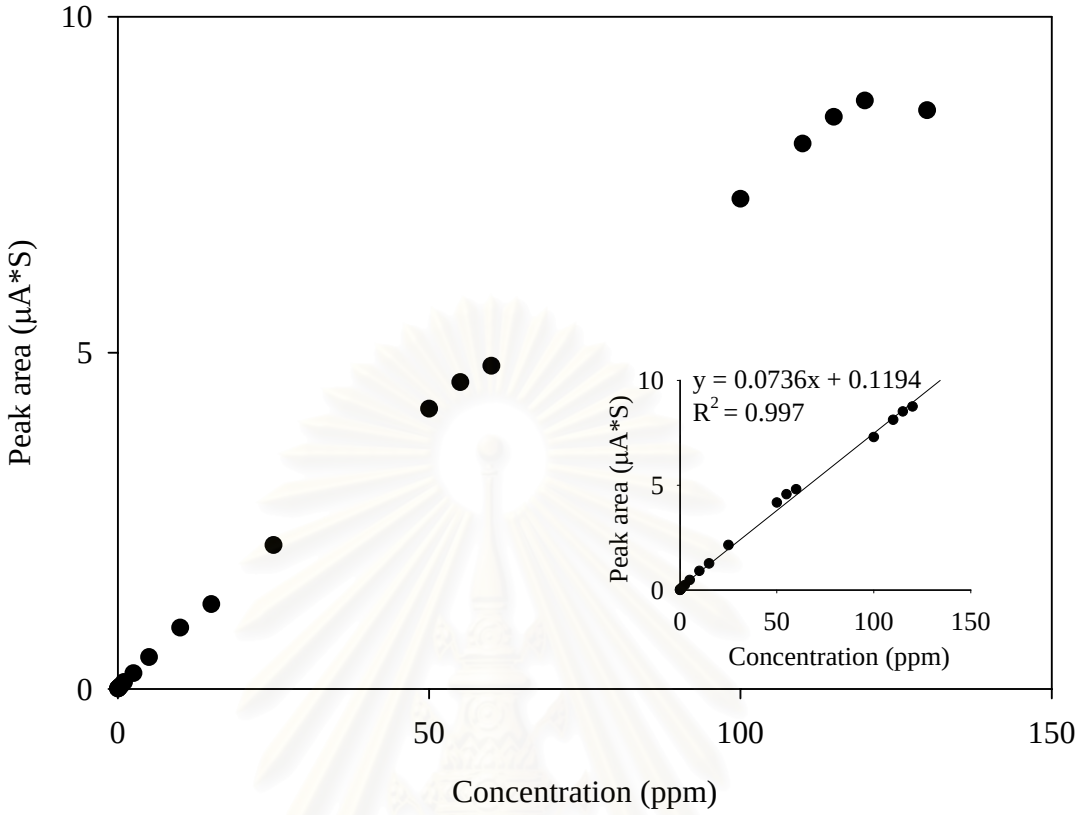


Figure 4.15 Calibration curve of standard sudan III solutions by HPLC-EC using the GC electrode

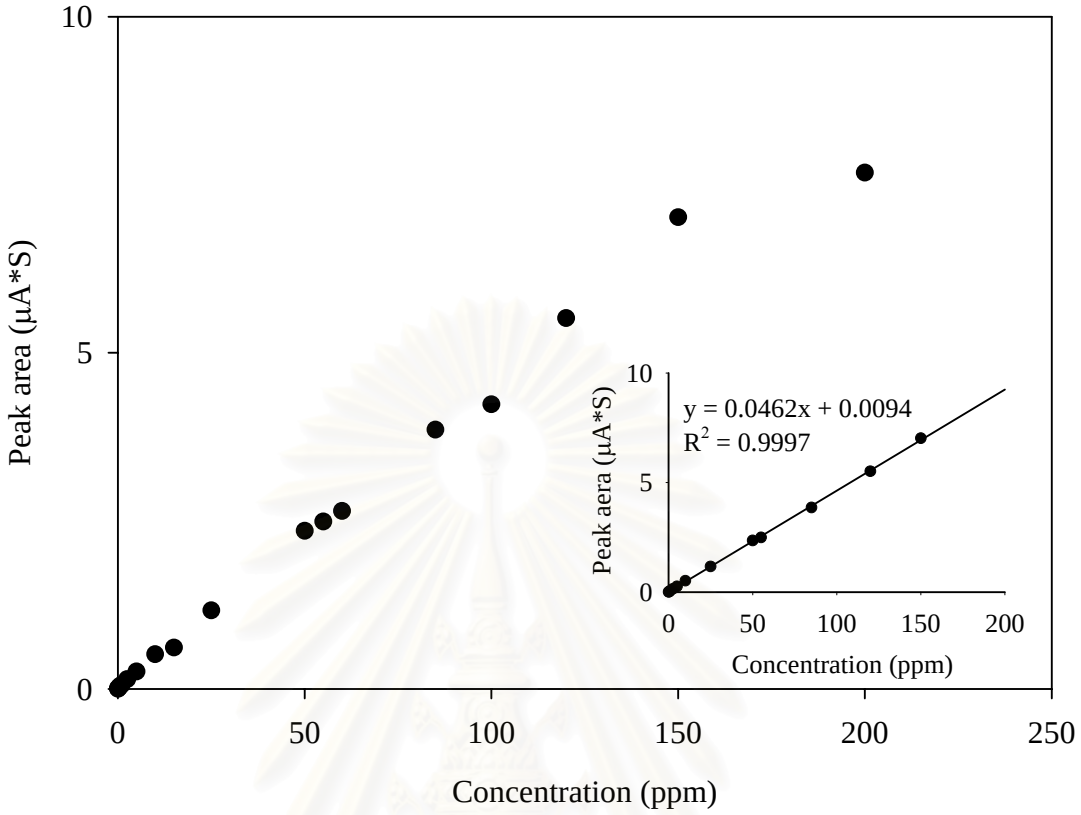


Figure 4.16 Calibration curve of standard sudan IV solutions by HPLC-EC using the GC electrode

4.3.3 LOD and LOQ

The LOD and LOQ were obtained from triplicate analyses of each analyte under the definition of 3 times the signal to noise ratio (LOD = 3S/B) and 10 times signal to noise ratio (LOQ = 10S/B). Table 4.8 shows the summary of LOD and LOQ of all analytes.

Table 4.8 LOD and LOQ of sudan I, sudan II, sudan III and sudan IV at GC electrode

	Sudan I	Sudan II	Sudan III	Sudan IV
LOD (ppm)	0.005	0.005	0.005	0.05
LOQ (ppm)	0.01	0.01	0.05	0.10

4.3.4 Method accuracy and precision

The accuracy of this technique is reported as present recovery at each level, which is determined by comparison between the known amounts added at 1.0 ppm of sudan I, sudan II, sudan III and sudan IV samples (three soft drinks and a chili sauce sample). Percent recoveries of all samples are shown in Table 4.9 and Table 4.10 respectively. The mean values of percent recovery were found in the range of 93.91 to 102.61 % for soft drink samples and 104.74 to 107.00% for chili sauce sample with RSD < 10%, indicating high accuracy for the HPLC-EC method.

Table 4.9 Percent relative recoveries and percent RSD of spiking 1 ppm sudan I, sudan II, sudan III, and sudan IV in soft drink samples (n=3)

Type of Soft drink	Analytes	% Recovery				%RSD
		1	2	3	Mean ± SD	
F-EC	Sudan I	102.87	103.69	101.27	102.61±1.23	1.20
	Sudan II	97.69	101.53	101.60	100.28±2.24	2.23
	Sudan III	101.84	101.14	93.19	98.72±4.80	4.86
	Sudan IV	99.88	98.07	97.78	98.58±1.14	1.15
G-EC	Sudan I	97.04	98.74	98.04	97.36±0.86	0.87
	Sudan II	95.18	98.70	97.61	97.17±1.80	1.86
	Sudan III	100.85	100.72	101.05	100.87±0.17	0.17
	Sudan IV	94.87	95.52	98.79	96.39 ±2.10	2.18
M-EC	Sudan I	94.52	93.84	93.38	93.91±0.58	0.62
	Sudan II	103.12	101.45	96.08	100.22±3.68	3.67
	Sudan III	102.69	102.76	100.38	101.94±1.36	1.33
	Sudan IV	96.90	94.86	98.54	96.77±1.84	1.90

Table 4.10 Percent relative recoveries and percent RSD of spiking 1.0 ppm sudan I, sudan II, sudan III and sudan IV in chili sauce sample (n=3)

Analytes	% Recovery				%RSD
	1	2	3	Mean ± SD	
Sudan I	106.63	107.03	105.03	106.23±1.06	0.99
Sudan II	106.86	107.33	106.81	107.00±0.28	0.26
Sudan III	103.63	108.19	102.41	104.74±3.04	2.91
Sudan IV	104.45	105.22	106.55	105.41±1.06	1.00

4.3.4.1 Intra-day assay

The results were obtained and repeatedly analyzed in one day. The percent recoveries were carried out on three soft drink samples and a chili sauce sample using standard addition of sudan dyes at 0.5, 1.0, and 1.5 ppm. The precision of an analytical method is expressed as relative standard deviation (RSD) of repeated analysis. This work was studied for repeatability by the performance method using the same laboratory and the same equipment. The results of intra-day analysis are summarized in Tables 4.11, 4.12, 4.13 for three soft drink samples and Table 4.14 for a chili sauce sample. They were found that the average recoveries range from 89.47-104.56%, 94.36-102.13%, and 93.74-104.95% for F-soft drink, G-soft drink and M-soft drink samples and 96.40-102.88% for a chili sauce sample, respectively. These recovery values are accepted by the AOAC manual for the Peer Verified Methods program that recommend the acceptable recovery values for method development (in Appendix F). The results obtained from these studies indicate that the method developed in this research provides good accuracy. The experimentally determined %RSD values varied from 0.11-4.94%, 0.17-2.67%, and 0.16-1.90% for F-soft drink, G-soft drink and M-soft drink sample and 0.33-4.53% for in chili sauce sample, respectively. These %RSD values are accepted by the AOAC manual for the Peer Verified Methods program that recommend the acceptable %RSD values for method development (in Appendix F). The results indicated that the method is sufficiently precise at the concentration level of analyte being measured within a day.

Table 4.11 Percent recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in F-ECsoft drink sample (n=3)

Analytes	Spiked standard levels (ppm)	% Recovery				%RSD
		1	2	3	Mean±SD	
Sudan I	0.5	97.60	95.79	95.39	96.26± 1.18	1.22
	1.0	104.67	95.85	97.58	99.36± 4.68	4.71
	1.5	98.85	102.29	101.60	100.91± 1.82	1.80
Sudan II	0.5	97.51	97.42	97.30	97.41± 0.10	0.11
	1.0	98.21	97.56	100.47	98.74± 1.52	1.54
	1.5	101.02	101.38	100.16	100.85± 0.63	0.63
Sudan III	0.5	97.86	100.70	101.36	99.97± 1.86	1.86
	1.0	102.61	94.44	94.17	97.07± 4.79	4.94
	1.5	99.07	102.39	102.45	101.30± 1.94	1.91
Sudan IV	0.5	101.85	102.48	109.34	104.56± 4.16	3.98
	1.0	99.90	99.39	100.26	99.85± 0.44	0.44
	1.5	99.85	99.69	98.86	89.47± 0.53	0.53

Table 4.12 Percent relative recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in G-EC soft drink sample (n=3)

Analytes	Spiked standard levels (ppm)	% Recovery				%RSD
		1	2	3	Mean±SD	
Sudan I	0.5	94.91	94.07	94.10	94.36±0.48	0.51
	1.0	97.04	98.74	98.04	97.94±0.86	0.87
	1.5	101.91	101.22	101.52	101.55±0.35	0.34
Sudan II	0.5	103.58	101.18	101.64	102.13±1.27	1.25
	1.0	95.18	98.70	97.62	97.17±1.80	1.86
	1.5	101.70	100.41	100.90	101.01±0.65	0.64
Sudan III	0.5	93.11	97.61	93.40	94.71±2.51	2.67
	1.0	100.85	100.72	101.05	100.87±0.17	0.17
	1.5	100.39	99.48	100.35	100.08±0.51	0.52
Sudan IV	0.5	98.37	99.95	98.25	98.86±0.95	0.96
	1.0	94.87	95.52	98.79	96.39±2.10	2.18
	1.5	100.53	102.04	100.78	101.12±0.81	0.80

Table 4.13 Percent relative recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in M-EC soft drink sample (n=3)

Analytes	Spiked standard levels (ppm)	% Recovery				%RSD
		1	2	3	Mean±SD	
Sudan I	0.5	92.35	94.74	94.12	93.74±1.24	1.32
	1.0	94.52	93.84	93.38	93.91±0.58	0.62
	1.5	103.26	103.34	103.57	103.39± 0.16	0.16
Sudan II	0.5	107.21	98.23	102.12	102.52± 4.50	4.39
	1.0	103.21	101.45	96.08	100.22± 3.68	3.67
	1.5	97.80	99.55	101.51	99.62± 1.86	1.86
Sudan III	0.5	103.44	102.87	103.46	103.25± 0.33	0.32
	1.0	102.69	102.76	100.38	101.94± 1.36	1.33
	1.5	98.35	98.44	99.46	98.75± 0.62	0.62
Sudan IV	0.5	104.34	103.98	103.67	104.00± 0.33	0.32
	1.0	96.90	94.86	98.54	96.77± 1.84	1.90
	1.5	100.86	101.74	100.35	100.98± 0.70	0.70

Table 4.14 Percent relative recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in chili sauce sample (n=3)

Analytes	Spiked standard levels (ppm)	% Recovery				%RSD
		1	2	3	Mean±SD	
Sudan I	0.5	92.43	101.20	97.20	99.94±4.39	4.53
	1.0	100.00	94.24	98.86	97.70±3.05	3.12
	1.5	100.81	102.44	100.86	101.37±0.92	0.91
Sudan II	0.5	99.05	97.01	104.69	100.25±3.98	3.97
	1.0	101.02	101.27	98.19	100.16±1.71	1.71
	1.5	99.67	99.74	100.28	99.90±0.33	0.33
Sudan III	0.5	101.63	101.30	102.96	101.96±0.87	0.86
	1.0	98.14	97.03	94.03	96.40±2.13	2.20
	1.5	100.72	101.25	102.42	101.46±0.87	0.86
Sudan IV	0.5	103.66	104.47	100.50	102.88±2.10	2.04
	1.0	95.58	100.60	101.03	99.07±3.03	3.06
	1.5	101.32	99.36	99.75	100.14±1.04	1.03

4.3.4.2 Inter-day assay

The results were obtained and repeatedly analyzed on three different days. The percent recoveries were carried out on the three soft drink samples and a chili sauce sample using standard addition of sudan dyes at 0.5, 1.0, and 1.5 ppm. The results of inter-day analysis are summarized in Tables 4.15, 4.16, 4.17 for three soft drink samples, Table 4.18 and Appendix E for the chili sauce sample. They were found that the average recoveries range from 96.49-101.22%, 97.05-100.63%, and 102.22-95.82% for F-soft drink, G-soft drink and M-soft drink samples and 98.18-102.73% for the chili sauce sample, respectively. These recovery values are accepted by the AOAC manual for the Peer Verified Methods program that recommends the acceptable recovery values for method development (in Appendix

F). The results obtained from these studies indicate that the developed in this research provides good accuracy. The precision of the method is expressed as the percent relative standard (%RSD). The experimentally determined %RSD values varied from 0.38-6.89%, 0.66-4.67%, and 0.50-6.18% for F-soft drink, G-soft drink and M-soft drink samples and 0.62-4.80% for the chili sauce sample, respectively. The result indicates that the method is sufficiently precise at the concentration level of analyte being measured within three days.

Table 4.15 Percent recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV with standard addition of: 0.5, 1.0 and 1.5 ppm in F-EC soft drink samples in the first day (between-day, n=3).

Analyte	Spiked standard levels (ppm)	% Recovery				%RSD
		Day 1	Day 2	Day 3	Mean±SD	
Sudan I	0.5	96.26	95.54	97.66	96.49±1.08	1.12
	1.0	99.36	102.61	101.67	101.22±1.67	1.65
	1.5	100.91	99.33	99.52	99.92±0.86	0.86
Sudan II	0.5	97.41	86.56	95.37	93.11±5.76	6.19
	1.0	98.75	100.28	99.93	99.65±0.80	0.81
	1.5	100.85	101.38	100.53	100.92±0.43	0.42
Sudan III	0.5	99.97	97.39	92.55	96.64±3.77	3.90
	1.0	97.07	98.72	100.23	98.67±1.58	1.60
	1.5	101.30	100.68	100.61	100.86±0.38	0.38
Sudan IV	0.5	104.56	96.99	91.15	97.56±6.73	6.89
	1.0	99.85	98.58	104.32	100.92±3.02	2.99
	1.5	99.47	100.91	99.10	99.83±0.96	0.96

Table 4.16 Percent recoveries and percent RSD of sudan I, sudan II, sudan III, and sudan IV at 0.5, 1.0 and 1.5 ppm standard additions in G-EC soft drink samples in the first day (between-day, n=3).

Analyte	Spiked standard levels (ppm)	% Recovery				%RSD
		Day 1	Day 2	Day 3	Mean±SD	
Sudan I	0.5	94.36	102.75	95.49	97.53±4.55	4.67
	1.0	97.94	99.76	103.53	100.41±2.85	2.84
	1.5	101.55	99.81	99.20	100.19±1.22	1.22
Sudan II	0.5	102.13	101.56	95.92	99.87±3.43	3.44
	1.0	97.17	98.83	105.88	100.63±4.63	4.60
	1.5	101.00	100.33	98.26	99.87±1.43	1.43
Sudan III	0.5	94.71	100.49	95.96	97.05±3.04	3.14
	1.0	100.87	101.809	103.71	102.13±1.44	1.42
	1.5	100.07	99.17	98.81	99.35±0.65	0.66
Sudan IV	0.5	98.86	102.29	96.34	99.16±2.99	3.01
	1.0	96.39	99.51	100.87	98.92±2.30	2.32
	1.5	101.12	100.10	99.75	100.32±0.71	0.71

Table 4.17 Percent recoveries and percent RSD of sudan I, sudan II, sudan III, and sudan IV with standard addition of 0.5, 1.0 and 1.5 ppm of analytes in M-EC soft drink sample for the first day (between-day, n=3).

Analyte	Spiked standard levels (ppm)	% Recovery				%RSD
		Day 1	Day 2	Day 3	Mean±SD	
Sudan I	0.5	98.53	95.18	93.74	95.82± 2.46	2.57
	1.0	100.35	106.28	93.91	100.18±6.19	6.18
	1.5	99.99	97.74	103.39	100.37±2.85	2.84
Sudan II	0.5	98.83	98.08	102.52	99.81±2.38	2.38
	1.0	101.07	104.35	100.22	101.88±2.18	2.14
	1.5	99.50	98.25	99.62	99.12±0.76	0.76
Sudan III	0.5	102.44	99.69	103.25	101.80±1.87	1.83
	1.0	100.88	103.84	101.94	102.22±1.50	1.47
	1.5	99.30	98.33	98.75	98.79±0.49	0.50
Sudan IV	0.5	101.57	96.55	104.00	100.71±3.80	3.77
	1.0	100.80	105.59	96.77	101.05±4.42	4.37
	1.5	99.59	97.12	100.98	99.23±1.96	1.97

Table 4.18 Percent recoveries and percent RSD of sudan I, sudan II, sudan III, and sudan IV with standard addition of 0.5, 1.0 and 1.5 ppm of analyte to chili sauce samples for the first days (between-day, n=3)

Analyte	Spiked standard levels (ppm)	% Recovery				%RSD
		Day 1	Day 2	Day 3	Mean±SD	
Sudan I	0.5	102.89	96.94	101.14	100.32±3.05	3.05
	1.0	106.23	97.70	100.43	101.45±4.36	4.29
	1.5	96.91	101.37	99.66	99.31±2.25	2.27
Sudan II	0.5	99.50	100.25	104.95	102.23±3.85	3.77
	1.0	107.00	100.16	100.34	102.53±3.87	3.77
	1.5	96.69	99.90	100.14	99.00±2.00	2.02
Sudan III	0.5	98.82	101.96	93.74	98.18±4.15	4.23
	1.0	104.74	96.40	96.60	99.25±4.76	4.80
	1.5	98.08	101.46	100.64	100.06±1.76	1.76
Sudan IV	0.5	103.29	102.88	102.03	102.73±0.64	0.62
	1.0	105.41	99.07	98.24	100.91±3.92	3.88
	1.5	97.57	100.14	100.63	99.45±1.64	1.65

4.4 Applications

4.4.1 HPLC amperometric method for determination four sudan dyes in soft drink samples using the CNT modified GC electrode

4.4.1.1 Working potential optimization on CNT modified GC electrode

The sensitivity of analyte detection depends on the reaction of analyte on the electrode surface. The CNT modified GC electrode, with unique structural and electronic properties, has been widely used to improve the sensitivity

in the electroanalysis. The optimum potential of four sudan dyes in the HPLC system were investigated by injection of 1 ppm mixed standard sudan dye solution, using a potential range from +0.7 to +1.2 V versus Ag/AgCl at the CNT modified GC electrode. The results of optimum potential waveform of four sudan dyes are shown in Figure 4.17. On the other hand, the current response of four sudan dyes were obtained by CNT modified GC electrode at each potential higher than the GC electrode (Figure 4.12). The oxidation current similarly increased the results from the GC electrode, which increased potential to a maximum of 0.85 V versus Ag/AgCl for sudan I, sudan II and sudan III, and 0.95 V versus Ag/AgCl for sudan IV. So, the potential at 0.85 V versus Ag/AgCl was selected for analysis of the four sudan dyes by HPLC system with CNT modified GC electrode. Therefore, a CNT modified GC electrode can improve the sensitivity of this electroanalysis.

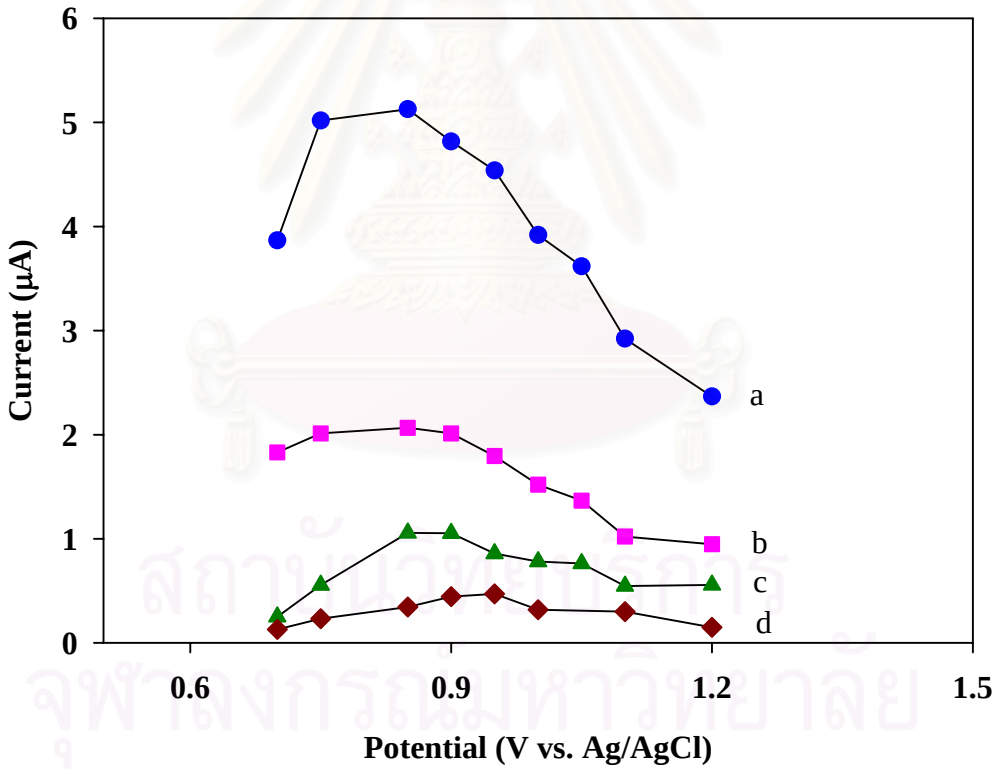


Figure 4.17 HPLC-EC response as a function of detection potential for 1 ppm of: a) sudan I, b) sudan II, c) sudan III, and d) sudan IV in acetonitrile and 20 mM acetate buffer (90:10; %v/v) using a CNT modified GC electrode. The injection volume was 20 μL and the flow rate was 1 mL min^{-1}.

4.4.1.2 Calibration and linearity of the CNT modified GC electrode

The current response of the four sudan dyes varied linearly with standard concentrations over a range of 0.01-100 ppm. Calibration curves of the four sudan dyes show the relationship of the peak area versus concentration. The calibration curves are shown in Figures 4.18, 4.19, 4.20 and 4.21, and were obtained by triplicate measurements of each analyte. The slope and y-axis intercept together with correlation coefficient were calculated according to the regression equation: $y = mx + b$. The correlation coefficient (R^2), linear range, LOD, and LOQ of each sudan dye are summarized in Table 4.19. The chromatograms of the four sudan dyes from GC with CNT modified GC working electrode versus Ag/AgCl are shown in Figure 4.22. It can be seen that the current response of the CNT modified GC electrode is higher than the GC electrode. In this case, the CNT modified GC electrode improved the sensitivity of the electrochemical measurement.

Table 4.19 Linear range, LOD, and LOQ of sudan I, sudan II, sudan III, and sudan IV showing comparisons of using the GC and CNT modified GC electrodes.

	Electrode	Sudan I	Sudan II	Sudan III	Sudan IV
Linear range (ppm)	GC	0.01-15.0	0.01-12.0	0.05-120.0	0.10-150.0
	CNT	0.005-15.0	0.005-20.0	0.05-20.0	0.05-25.0
R^2	GC	0.9994	0.9989	0.9970	0.9997
	CNT	0.9976	0.9989	0.9986	0.9989
LOD (ppm)	GC	0.005	0.005	0.005	0.05
	CNT	0.001	0.001	0.005	0.025
LOQ (ppm)	GC	0.01	0.01	0.05	0.10
	CNT	0.005	0.005	0.05	0.05

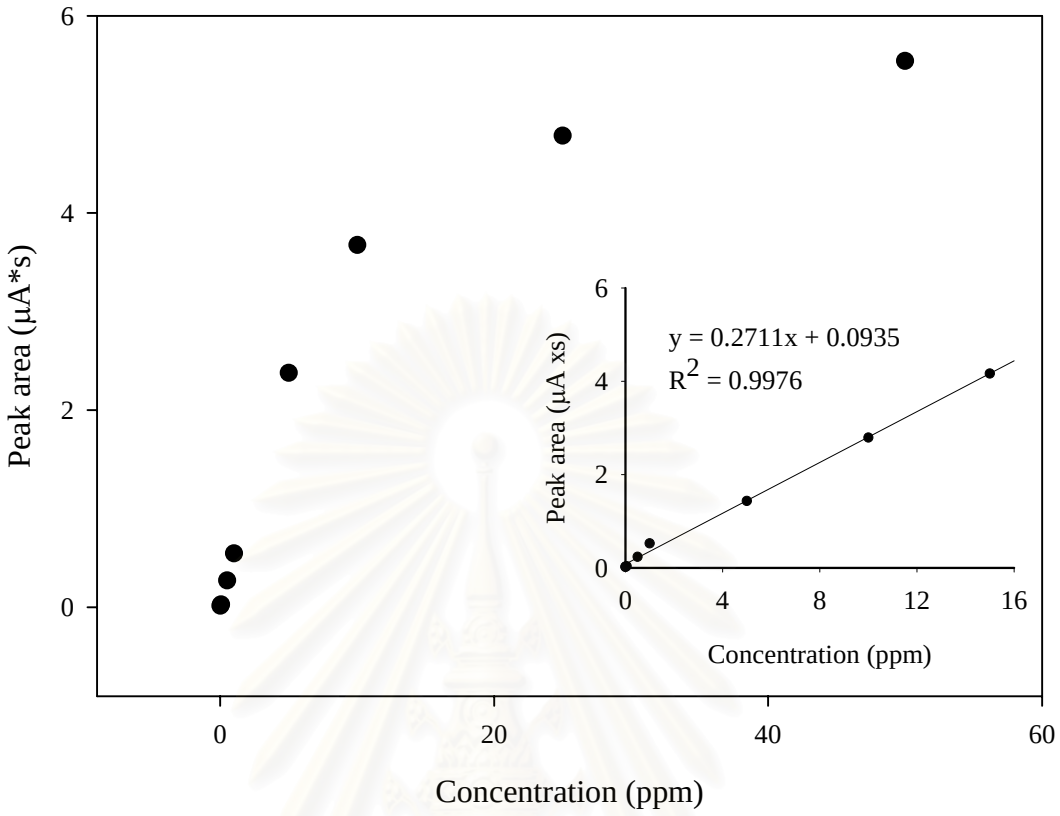


Figure 4.18 Calibration curve of standard sudan I solutions by HPLC-EC using the CNT modified GC electrode

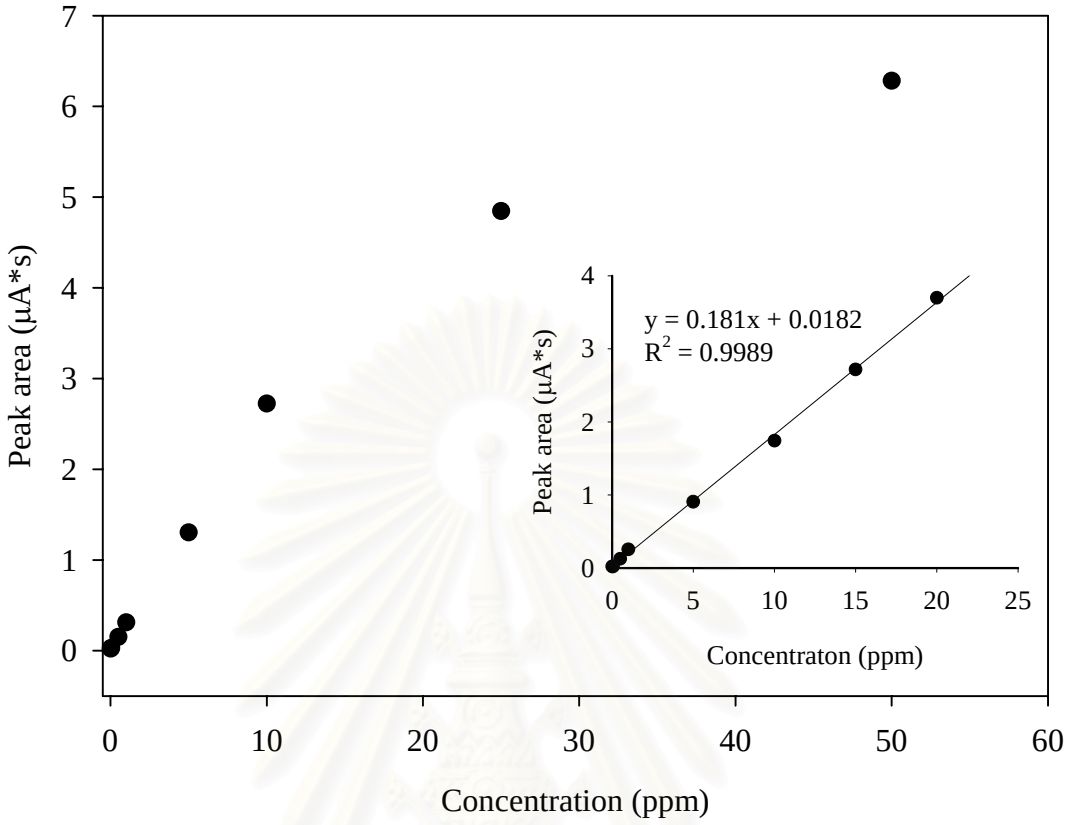


Figure 4.19 Calibration curve of standard sudan II solutions by HPLC-EC using the CNT modified GC electrode

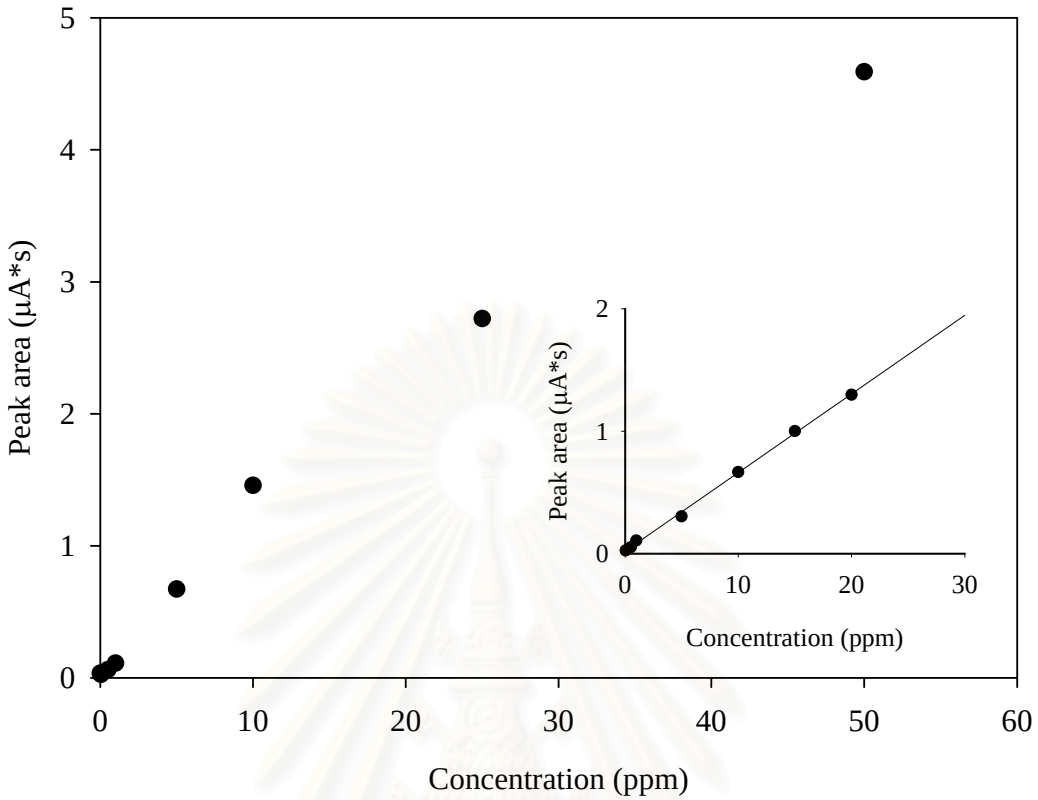


Figure 4.20 Calibration curve of standard sudan III solutions by HPLC-EC using the CNT modified GC electrode

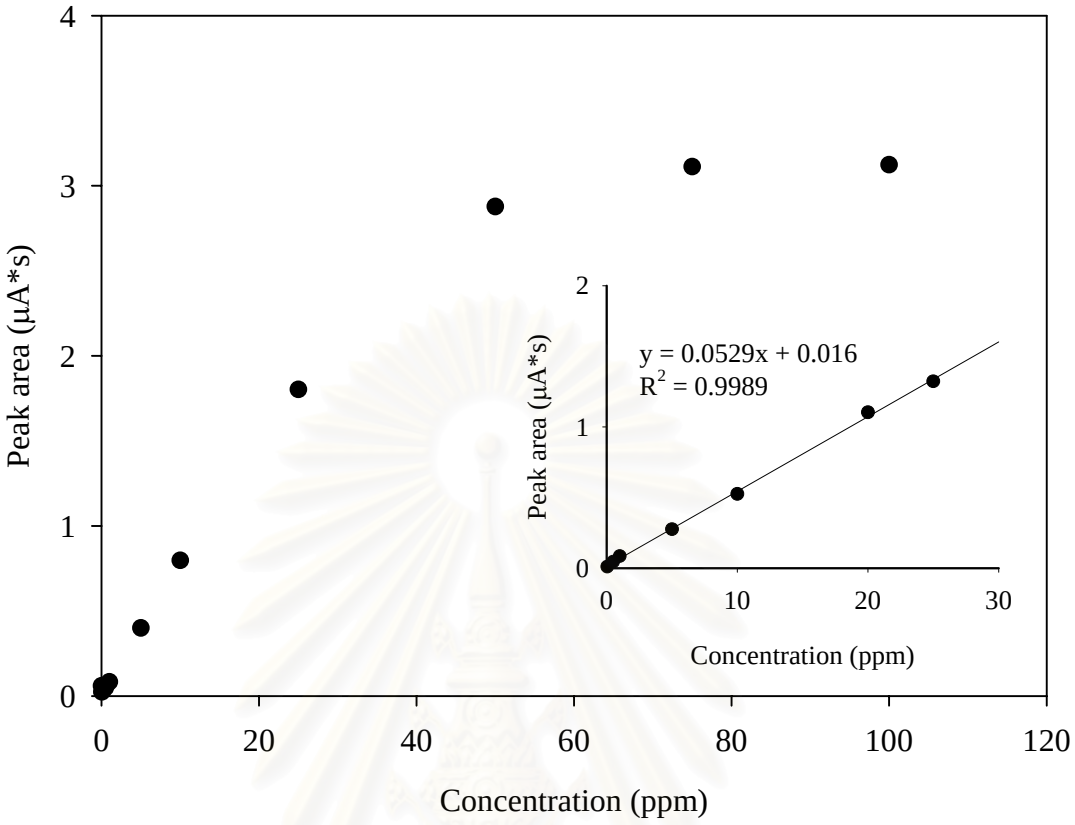


Figure 4.21 Calibration curve of standard sudan IV solutions by HPLC-EC using the CNT modified GC electrode

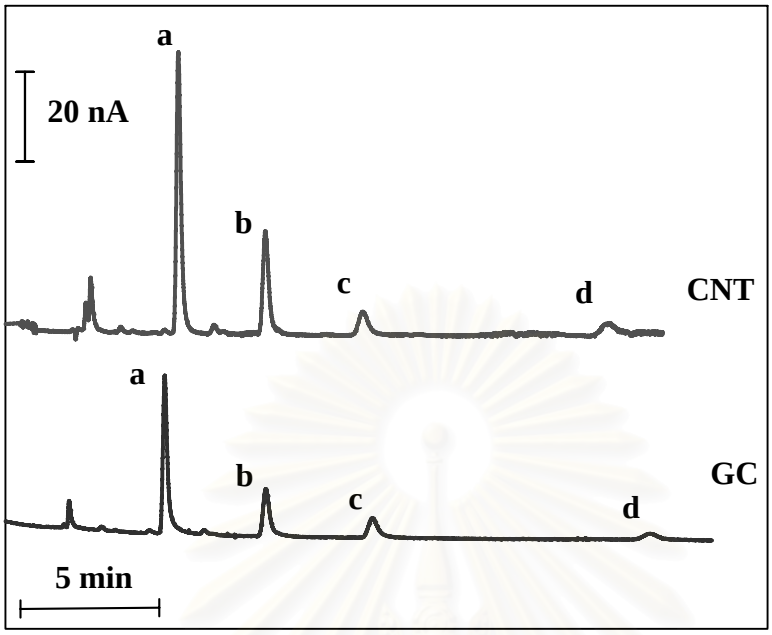


Figure 4.22 HPLC-EC chromatogram of a standard mixture containing 1 ppm concentration of (a) sudan I, (b) sudan II, (c) sudan III, and (d) sudan IV at the GC electrode compared with CNT modified GC electrode. The mobile phase was acetonitrile and 20 mM acetate buffer (90:10; v/v). The injection volume was 20 µL, and the flow rate was 1 mL min^{-1}

4.4.1.3 Analysis of sudan dyes samples

The HPLC amperometric method developed in this study was applied to the assay of sudan dyes in soft drink samples. The separation and detection of sudan dyes in the matrix are demonstrated in Figure 4.23. Figure 4.23A-B shows the chromatograms for blank soft drink analysis at the GC and CNT modified GC electrodes, respectively. The chromatograms of spiking the standard mixture solution are shown in Figures 4.23C-D. It could be that the amperometric signals resulted, not only in well-defined and separated peaks, but also no apparent interference from the sample matrix. The response, however, was slightly higher when comparing the GC electrode with the CNT modified GC electrode.

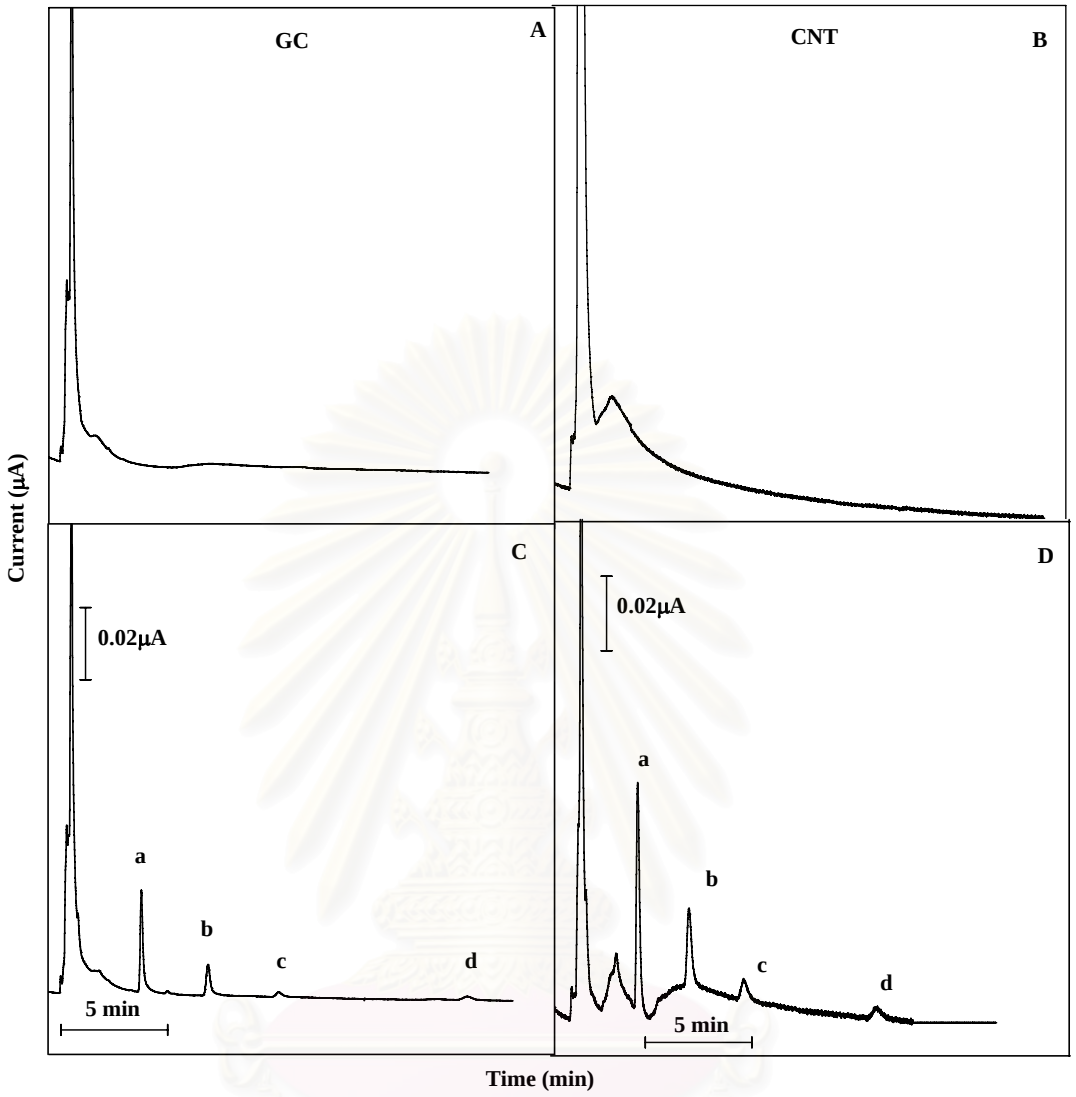


Figure 4.23 HPLC-EC chromatograms obtained from soft drink samples. (A) and (B) blank soft drink sample, and (C) and (D) soft drink samples spiked to 1 ppm concentrations of (a) sudan I, (b) sudan II, (c) sudan III, and (d) sudan IV at the GC electrode, (A and C) and CNT s modified GC electrode (B and D). The mobile phase was acetonitrile and 20 mM acetate buffer (90:10; v/v). The injection volume was 20 μL, and the flow rate was 1 mL min^{-1}.

4.4.1.4 Method accuracy and precision

The data for accuracy and precision are shown in Tables 4.21, 4.22, and 4.23 for F-EC, G-EC, and M-EC soft drink samples, respectively. The accuracy of the assay was determined by repetitive analysis of blank samples spiked with 0.5, 1.0, and 1.5 ppm of each sudan dyes. No significant improvement in the recovery was achieved by changing the electrode. The %RSD at 0.5, 1.0, and 1.5 ppm should range between 0.11 to 4.80, 0.17 to 2.66, and 0.16 to 4.39 % for GC electrode, and 0.44 to 2.42, 0.19 to 4.18, and 0.42 to 2.89 % for CNT modified GC electrode, respectively. It can be seen that the method presented a %RSD value less than the AOAC recommended value and also shows the method precision.

Table 4.20 Precision (relative standard deviation, RSD) and accuracy (percent recovery) from analysis of four sudan dyes in F-EC soft drink samples (n=3)

Analyte	Spike level (ppm)	GC-electrode (*n* = 3)		CNT-electrode (*n* = 3)	
		%Recovery (Mean ±S.D.)	%RSD	%Recovery (Mean ±S.D.)	%RSD
Sudan I	0.5	96.26±1.18	1.23	103.13±2.39	2.33
	1.0	99.36±4.68	4.70	103.05±2.18	2.12
	1.5	100.91±1.82	1.80	98.30±0.75	0.76
Sudan II	0.5	97.41±0.10	0.11	103.13±1.64	1.60
	1.0	98.75±1.53	1.53	98.18±0.91	0.93
	1.5	100.85±0.63	0.63	100.46±0.44	0.44
Sudan III	0.5	99.97±1.86	1.86	103.07±0.74	0.72
	1.0	97.07±4.80	4.80	104.09±2.51	2.42
	1.5	101.30±1.94	1.94	97.75±0.91	0.92
Sudan IV	0.5	104.56±4.16	4.16	104.00±0.64	0.61
	1.0	99.85±0.44	0.43	98.99±1.65	1.66
	1.5	99.47±0.53	0.53	100.00±0.85	0.85

Table 4.21 Precision (relative standard deviation, RSD) and accuracy (percent recovery) from analysis of four Sudan dyes in G-EC soft drink samples (n=3)

Analyte	Spike level (ppm)	GC-electrode (*n* = 3)		CNT-electrode (*n* = 3)	
		%Recovery (Mean ±S.D.)	%RSD	%Recovery (Mean ±S.D.)	%RSD
Sudan I	0.5	94.36±0.48	0.51	99.09±1.07	1.08
	1.0	97.94±0.86	0.87	99.39±4.15	4.18
	1.5	101.55±0.35	0.34	99.67±0.79	0.79
Sudan II	0.5	102.13±1.27	1.25	97.74±2.50	2.56
	1.0	97.17±1.80	1.85	103.40±1.62	1.57
	1.5	101.01±0.65	0.64	98.75±1.02	1.02
Sudan III	0.5	94.71±2.52	2.66	99.58±1.61	1.61
	1.0	100.87±0.17	0.17	95.68±2.12	2.22
	1.5	100.07±0.51	0.51	101.91±1.05	1.03
Sudan IV	0.5	98.86±0.95	0.95	93.98±2.38	2.53
	1.0	96.39±2.10	2.18	97.82±0.69	0.70
	1.5	101.12±0.81	0.80	101.62±0.19	0.19

Table 4.22 Precision (relative standard deviation, RSD) and accuracy percent recovery from analysis of four Sudan dyes in M-EC soft drink samples (n=3)

Analyte	Spike level (ppm)	GC-electrode (*n* = 3)		CNT-electrode (*n* = 3)	
		%Recovery (Mean ±S.D.)	%RSD	%Recovery (Mean ±S.D.)	%RSD
Sudan I	0.5	93.74±1.24	1.32	100.64±2.91	2.89
	1.0	93.91±0.58	0.62	98.47±2.78	2.82
	1.5	103.39±0.16	0.16	99.94±0.42	0.42
Sudan II	0.5	102.52±4.50	4.39	102.50±1.24	1.21
	1.0	100.22±3.68	3.67	101.14±0.94	0.93
	1.5	99.62±1.86	1.86	99.22±0.48	0.48
Sudan III	0.5	103.25±0.33	0.32	101.73±0.52	0.51
	1.0	101.94±1.36	1.33	99.45±1.46	1.47
	1.5	98.75±0.62	0.62	100.03±0.65	0.65
Sudan IV	0.5	104.00±0.33	0.32	99.70±0.75	0.76
	1.0	96.77±1.84	1.90	98.01±1.62	1.65
	1.5	100.98±0.70	0.70	100.67±0.62	0.62

CHAPTER V

CONCLUSION AND FURTHER WORK

A new method, HPLC-EC was developed for simultaneous analysis of sudan dyes in soft drink and chili sauce samples. The investigation for electroanalysis started with cyclic voltammetry, which showed the irreversible oxidation peak of four sudan dyes at GC and CNT modified GC electrodes.

The FIA-EC was set for analysis of each sudan dye at the GC electrode. The analysis was carried out with acetonitrile and 20 mM acetate buffer (90: 10 %v/v). From the hydrodynamic voltammetric investigation, the optimal potential was obtained at 0.80 volt for sudan I, 0.90 volt for sudan II and 0.95 volt for sudan III and sudan IV. The optimal potential at 0.95 volt was selected for preliminary HPLC-EC analysis because of high sensitivity for sudan III and sudan IV, as well as good sensitivity for sudan I and sudan II.

The HPLC-EC analysis was performed with various compositions of the mobile phase, the optimum mobile phase was determined to be acetonitrile and 20 mM acetate buffer (90:10 %v/v) at a flow rate 1.0 mL min^{-1}. Because, all sudan dyes have sharp, symmetric peaks and high resolution between them, the characterization and simultaneous analysis of sudan dyes was investigated with the above optimal conditions.

The HPLC-EC at the GC and CNT modified GC electrodes have been successfully applied to determine sudan dyes in three soft drink samples. The results from HPLC at GC and CNT modified GC electrode were compared for quantitative analysis of sudan I, sudan II, sudan III, and sudan IV in three soft drink samples. They were found that the GC electrode provided the linear quantitation at 0.01-10, 0.01-12.0, 0.025-120.0, and 0.10-150.0 ppm for four sudan dyes and the CNT modified GC electrode provided linear quantitation at 0.005-15.0, 0.005-20.0, 0.05-20.0, and 0.02-25.0 ppm for four sudan dyes, respectively with a correlation coefficient of R^2>0.99. The LOQ were in the range of 0.01-0.10 ppm at GC electrode and 0.005-0.05 ppm at CNT modified GC electrode. The LOD values of

GC electrode higher than these of the CNT modified electrode. These results indicated that HPLC-EC at the CNT modified GC electrode provides higher sensitivity than the GC electrode.

The developed HPLC-EC method can be applied for the determination of sudan dyes in soft drinks and as well as chili sauce sample. The recovery of four sudan dyes at the spiking level of 1.0 ppm was obtained in the range of 93.91-102.61% and 95.68 – 104.09% at GC and CNT modified GC electrode, respectively for soft drink samples with %RSD<10. It was found that the CNT modified GC electrode provided higher accuracy and precision than the GC electrode.

The optimization HPLC-EC especially CNT modified GC electrode was demonstrated to be a powerful method because of the high accuracy and precision of the results.

Further work

The HPLC-EC method was successfully applied for the determination of sudan dyes in soft drink and chili sauce samples. Thus, this method can be applied for analysis of sudan dyes in other sample. Moreover, the carbon nanotube modified on screen print carbon (SPC) can be used as the working electrode, replacing the GC electrode, which improves sensitivity for determination of sudan dyes in food samples.

REFERENCES

[1] Tripathi, M., Khanna, S. K., & Das, M. "Surveillance on use of synthetic colours in eatables vis a vis Prevention of Food Adulteration Act of India.Food." Food Control. In Press, Corrected Proof (2007).

[2] Ma, M., Luo, X., Chen, B., Su, S., & Yao, S. "Simultaneous determination of water-soluble and fat-soluble synthetic colorants in foodstuff by high performance liquid chromatography-diode array detection-electrospray mass spectrometry" J. Chromatogr. A 1103 (2006): 170-176.

[3] Ryan, A. J., & Welling, P. G. "Some observations on the metabolism and excretion of the bisazo dyes sudan III and sudan IV." Food and Cosmetics Toxicology 5 (1967): 755-761.

[4] Chung, K.-T. "The significance of azo-reduction in the mutagenesis and carcinogenesis of azo dyes." Mutation Research/Reviews in Genetic Toxicology 114 (1983): 269-281.

[5] Cornet, V., Govaert, Y., Moens, G., Van Loco, J., & Degroodt, J. M. "Development of a fast analytical method for the determination of sudan dyes in chili- and curry-containing foodstuffs by high-performance liquid chromatography-photodiode array detection." J. Agric. Food. Chem. 54 (2006): 639-644.

[6] Collier S. W., Storm J. E., & Bronaugh R. L. "Reduction of Azo Dyes During in Vitro Percutaneous Absorption." Toxicol. Appl. Pharmacol. 118 (1993): 73-79.

[7] Dixit, S., Pandey, RC., Das, M., Khanna, SK. "Food quality surveillance on colours in eatables sold in rural markets of Uttar Pradesh . J. Food Sci. Technol.-Mysore 32 (1995): 373-376.

[8] Waite, S., Hansen, D., & McGinley, M.. "Easy isocratic HPLC determination of Sudan dyes." LC GC North America. 46 (2006): 46-46.

[9] Stiborova, M., Asfaw, B., Frei, E., Schmeiser, HH., & Wiessler, M. "Benzenediazonium ion derived from Sudan I forms an 8-(Phenylazo) guanine adduct in DNA." Chem. Res. Toxicol. 8 (1995): 489-498.

[10] Stiborova, M., Asfaw, B., & Anzenbacher, P. "Activation of carcinogens by peroxidase Horseradish peroxidase-mediated formation of benzenediazonium ion from a non-aminoazo dye, 1-phenylazo-2-hydroxynaphthalene (Sudan I) and its binding to DNA." FEBS Lett. 232 (1988): 387-390.

[11] Stiborova, M., Frei, E., Schmeiser, H. H., Wiessler, M., & Hradec, J. "Detoxication products of the carcinogenic azodye Sudan I (Solvent Yellow 14) bind to nucleic acids after activation by peroxidase." Cancer Lett. 68 (1993): 43-47.

[12] Pielesz, A., Baranowska, I., Rybak, A., & Wlochowicz, A. "Detection and Determination of Aromatic Amines as Products of Reductive Splitting from Selected Azo Dyes." Ecotoxicology and Environmental Safety. 53 (2002): 42-47.

[13] Fujita, S., Peisach, J., Ohkawa, H., Yoshida, Y., Adachi, S., Uesugi, T., Suzuki, M., & Suzuki, T. "The effect of Sudan III on drug metabolizing enzymes." Chem. Biol. Interact. 48 (1984): 129-143.

[14] Mirceski, V., & Gulaboski, R. "A Theoretical and Experimental Study of a Two-step Quasireversible Surface Redox Reaction by Square-wave Voltammetry." Croat. Chem. Acta 76 (2003): 37-48.

[15] Mohammed Ali, Q., Palanisamy, P. K., Manickasundaram, S., & Kannan, P. "Sudan IV dye based poly(alkyloxymethacrylate) films for optical data storage." Opt. Commun. 267 (2006): 236-243.

[16] Zhang, Y., Wu, H.-L., Xia, A.-L., Han, Q.-J., Cui, H., & Yu, R.-Q. "Interference-free determination of Sudan dyes in chilli foods using second-order calibration algorithms coupled with HPLC-DAD." Talanta. In Press (2006): *Accepted Manuscript*.

[17] Puoci, F., Garreffa, C., Iemma, F., Muzzalupo, R., Spizzirri, U., & Picci, G. N. "Molecularly imprinted solid phase extraction for detection of Sudan I in food matrices." Food Chem. 93 (2005): 349-353.

[18] E Mejia, E., Ding, Y., Mora, M. F., & Garcia, C. D. "Determination of banned sudan dyes in chili powder by capillary electrophoresis." Food Chem. In Press (2006): Corrected Proof.

[19] Valencia, M. C., Uroz, F., Tafersiti, Y., & Capitan-Vallvey, L. F. "A flow-through sensor for the determination of the dyes Sunset Yellow and its subsidiary Sudan 1 in foods." Quim. Anal. 3 (2000): 129-134.

[20] Kumar, S., & Singh, H. N. "Competitive solubilization of Sudan IV and anthracene in micellar systems." Colloids Surf. 69 (1992): 1-4.

[21] Zhang, Y. P., Zhang, Y. J., Gong, W. J., Gopalan, A. I., & Lee, K.-P. "Rapid separation of Sudan dyes by reverse-phase high performance liquid chromatography through statistically designed experiments." J. Chromatogr. A 1098 (2005): 183-187.

[22] Daood, G, H., Biacs, & A, P. "Simultaneous Determination of Sudan Dyes and Carotenoids in Red Pepper and Tomato Products by HPLC." J. Chromatogr. Sci. 43 (2005): 461-465.

[23] Zhang, Y., Zhang, Z., & Sun, Y. "Development and optimization of an analytical method for the determination of Sudan dyes in hot chilli pepper by high-performance liquid chromatography with on-line electrogenerated BrO-luminol chemiluminescence detection." J. Chromatogr. A 1129 (2006): 34-40.

[24] Mazzetti M., Fascioli, R., Mazzoncini, I., Spinelli, G., Morelli, I., & Bertoli, A. "Determination of 1-phenylazo-2-naphthol (Sudan I) in chilli powder and in chilli-containing food products by GPC clean-up and HPLC with LC/MS confirmation." Food Addit. And Contam. 21 (2004): 935-941.

[25] Calbiani, F., Careri, M., Elviri, L., Mangia, A., Pistara, L., & Zagnoni, I. "Development and in-house validation of a liquid chromatography-electrospray-tandem mass spectrometry method for the simultaneous determination of Sudan I, Sudan II, Sudan III and Sudan IV in hot chilli products." J. Chromatogr. A 1042 (2004): 123-130.

[26] Calbiani, F., Careri, M., Elviri, L., Mangia, A., & Zagnoni, I. "Accurate mass measurements for the confirmation of Sudan azo-dyes in hot chilli products by capillary liquid chromatography-electrospray tandem quadrupole orthogonal-acceleration time of flight mass spectrometry." J. Chromatogr. A 1058 (2004): 127-135.

[27] Tateo, F., & Bononi, M. "Fast determination of sudan I by HPLC/APCI-MS in hot chilli, spices, and oven-baked foods." J.Agric. Food. Chem. 52 (2004): 655-658.

[28] L Di Donna, L., Maiuolo, L., Mazzotti, F., De Luca, D., & Sindona, G. "Assay of sudan I contamination of foodstuff by atmospheric pressure chemical ionization tandem mass spectrometry and isotope dilution." Anal. Chem. 76 (2004): 5104-5108.

[29] Chen, H. W., Zhang, X., & Luo, M. B. "Desorption electrospray ionization mass spectrometry for fast detection of Sudan dyes in foods without sample pretreatment." . Chin. J. Anal. Chem. 34 (2006): 464-468.

[30] [Anon]. "The determination of sudan dyes in food products by HPLC." LC GC North America. 47 (47):

[31] Shi, M., Xu, J., Zhang, S., Liu, B., & Kong, J. "A mediator-free screen-printed amperometric biosensor for screening of organophosphorus pesticides with flow-injection analysis (FIA) system." Talanta. 68 (2006): 1089-1095.

[32] Fernamdez-Abedul, M. T., & Costa-Garcia, A. "Flow injection analysis with amperometric detection of naltrexone in pharmaceuticals." J. Pharm. Biomed. Anal. 16 (1997): 15-19.

[33] Agrafiotou, P., & Sotiropoulos, S. "Characterisation of a simple electrochemical detector for high-performance liquid chromatography and flow-injection analysis based on carbon microcylinder electrodes." Anal. Chim. Acta 497 (2003): 175-189.

[34] Bergamini, M. F., Santos, A. L., Stradiotto, N. R., & Zanoni, M. V. B. "Flow injection amperometric determination of procaine in pharmaceutical formulation using a screen-printed carbon electrode." J. Pharm. Biomed. Anal. In Press, Corrected Proof.

[35] Jakmunee, J., & Grudpan, K. "Flow injection amperometry for the determination of iodate in iodized table salt." Anal. Chim. Acta 438 (2001): 299-304.

[36] Harris, P. J. F. "Solid state growth mechanisms for carbon nanotubes." Carbon 45 (2007): 229-239.

[37] Chen, C., Liu, L., Lu, Y., Kong, E. S.-W., Zhang, Y., Sheng, X., & Ding, H. "A method for creating reliable and low-resistance contacts between carbon nanotubes and microelectrodes." Carbon 45 (2007): 436-442.

[38] Li, Y., Shi, X., & Hao, J. "Electrochemical behavior of glassy carbon electrodes modified by multi-walled carbon nanotube/surfactant films in a buffer solution and an ionic liquid." Carbon. 44 (2006): 2664-2670.

[39] Huang, R., Zheng, X., & Qu, Y. "Highly selective electrogenerated chemiluminescence (ECL) for sulfide ion determination at multi-wall carbon nanotubes-modified graphite electrode." Anal. Chim. Acta 582 (2007): 267-274.

[40] Zare, H. R., & Nasirizadeh, N. "Hematoxylin multi-wall carbon nanotubes modified glassy carbon electrode for electrocatalytic oxidation of hydrazine." Electrochim. Acta 52 (2007): 4153-4160.

[41] Liu, Y., Zou, X., & Dong, S. "Electrochemical characteristics of facile prepared carbon nanotubes-ionic liquid gel modified microelectrode and application in bioelectrochemistry." Electrochem. Commun. 8 (2006): 1429-1434.

[42] Skoog, D. A., Holler, F. J., & Nieman, T. A. 5 th ed. Principles of Instrumental Analysis. United States of America: Saunder College Publishing, 1998.

[43] Rouessac, F., & Rouessac, Chemical analysis:modern instrumental methods and techniques. England: John Wiley & Sons, Ltd., 2000.

[44] Bard, A. J., & Faulkner, L. R. Electrochemical Methods Fundamentals and Applications. The United States of america: John Wiley & Sons,Inc., 1980.

[45] Skoog, D. A., West, D. M., Holler, F. J., & Crouch, S. R. Fundamentals of Analytical Chemistry. 8 th ed. The United States of America: a division of Thomson Learning Thomson Learning, Inc., 2004.

[46] Wang, J. Analytical electrochemistry. The United States of America: Wiley-VCH, 2000.

[47] Kinoshita, K. Carbon electrochemical and physicochemical properties. The United States of America: Jonh Wiley & Sons, Inc., 1988.

[48] Iriyama, K., Iwamoto, a., & Yoshiura, M. "Electrochemically treated glassy carbon electrode for amperometric detection in high-performance liquid chromatography." J. Chromatogr. A 400 (1987): 263-269.

[49] Hoogvliet, J. C., Beld, C. M. B. v. d., Poel, C. J. v. d., & Bennekom, W. P. v. "Influence of polishing and of electrochemical pretreatment on the performance of glassy-carbon electrodes in electrochemical detection." J. Electroanal. Chem. 201 (1986) 11-21.

[50] Wolf, E. L. Nanophysics and Nanotechnology. Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 2004.

[51] Xiao, P., Zhao, F., & Zeng, B. "Voltammetric determination of quercetin at a multi-walled carbon nanotubes paste electrode." Microchem. J. 85 (2007): 244-249.

[52] Ruzicka, J., & Hansen, E. H. Flow injection Analysis. The United States of America: Jonh Wiley & Sons Inc., 1988.

[53] J Corujo-Antuna, J. L., Abad-Villar, E. M., Fernandez-Abedul, M. T., & Costa-Garcia, A. "Voltammetric and flow amperometric methods for the determination of melatonin in pharmaceuticals." J. Pharm. Biomed. Anal. 31 (2003): 421-429.

[54] Daniel, D., & Gutz, I. G. R. "Flow injection spectroelectroanalytical method for the determination of promethazine hydrochloride in pharmaceutical preparations." Anal. Chim. Acta 494 (2003): 215-224.

[55] Snyder, L. R., Kirkland, J. J., & Glajch, J. L. Practical HPLC Method Development. 2 nd ed. The United States of America: Jonh Wiley & Sons Inc.,

[56] Smith, R. M. Gas and Liquid Chromatography in Analytical Chemistry. Great Britain: John Will & Sons, Ltd., 1988.

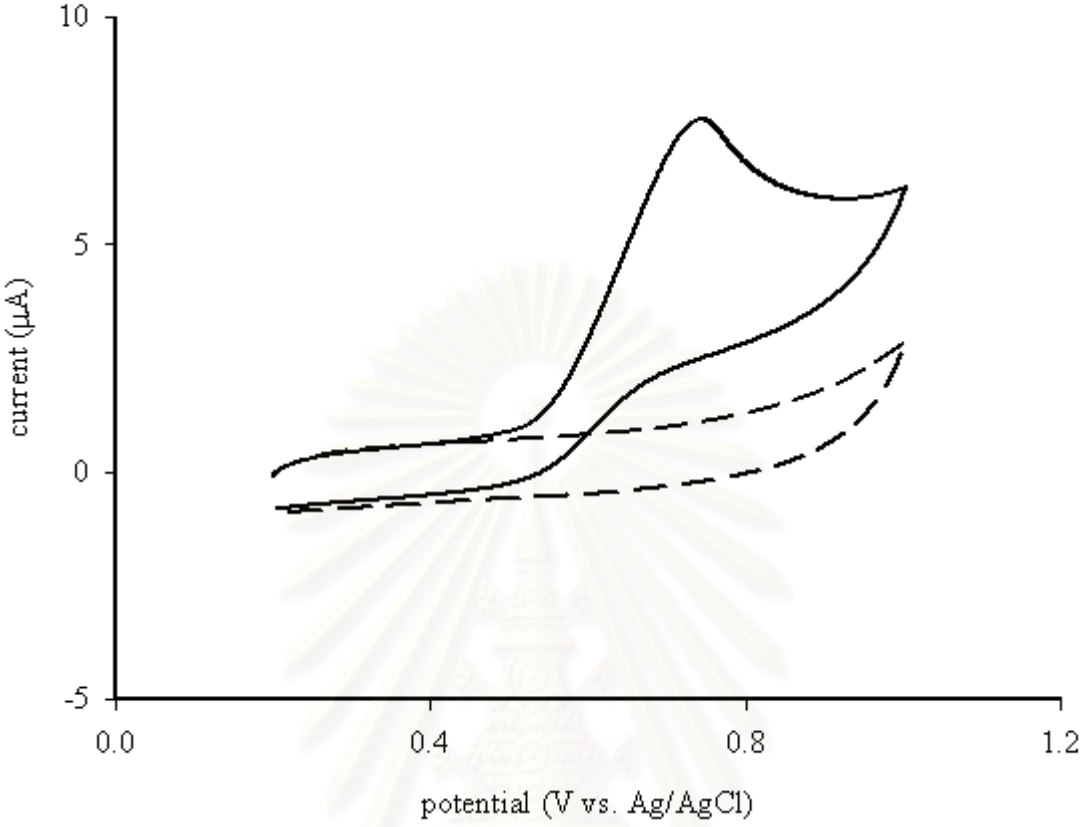
[57] H.A. Laitinen, W.E. Harris, Chemical Analysis. (2000).

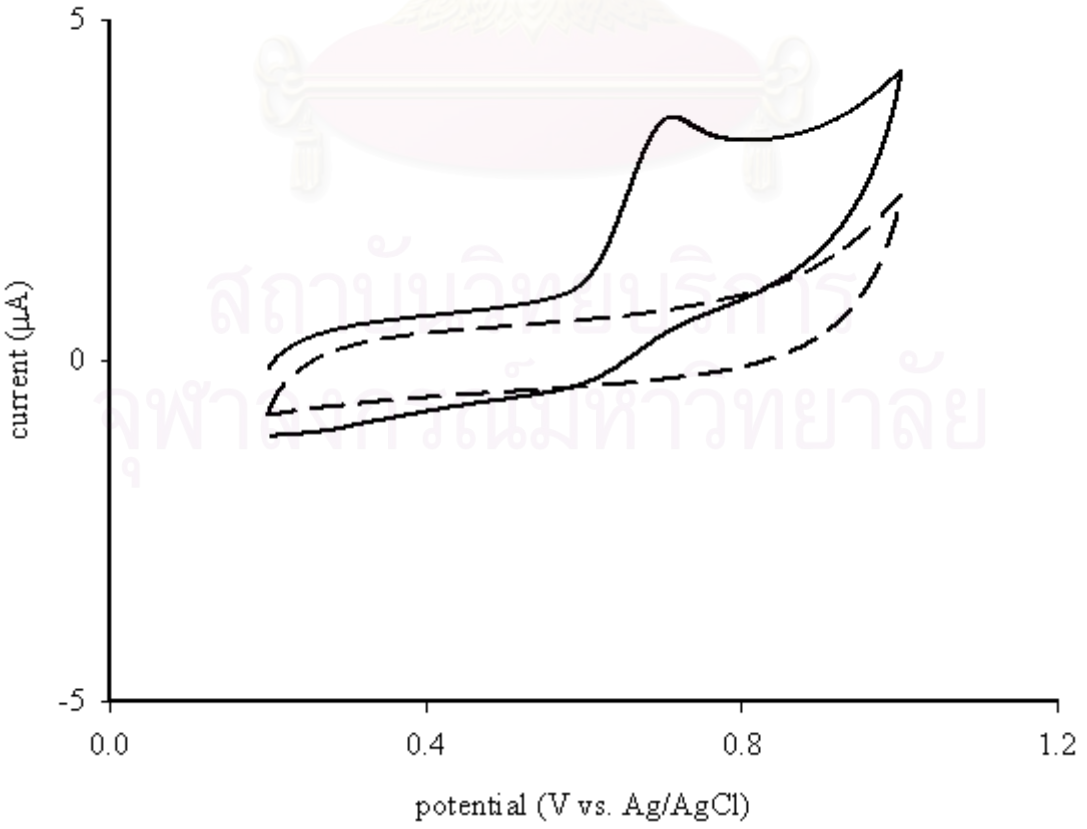
[58] Fukushima, T., Kosaka, A., Ishimura, Y., Yamamoto, T., Takigawa, T., Ishii, N., & Aida, T. Molecular Ordering of Organic Molten Salts Triggered by Single-Walled Carbon Nanotubes. Science. 300 (n.d.). 2072-2074.

[59] AOAC Peer Verified methods Program, Manual on policies and producedures, arlinglon, VA, (1993).

APPENDICES

APPENDIX A: Voltammetric Investigation





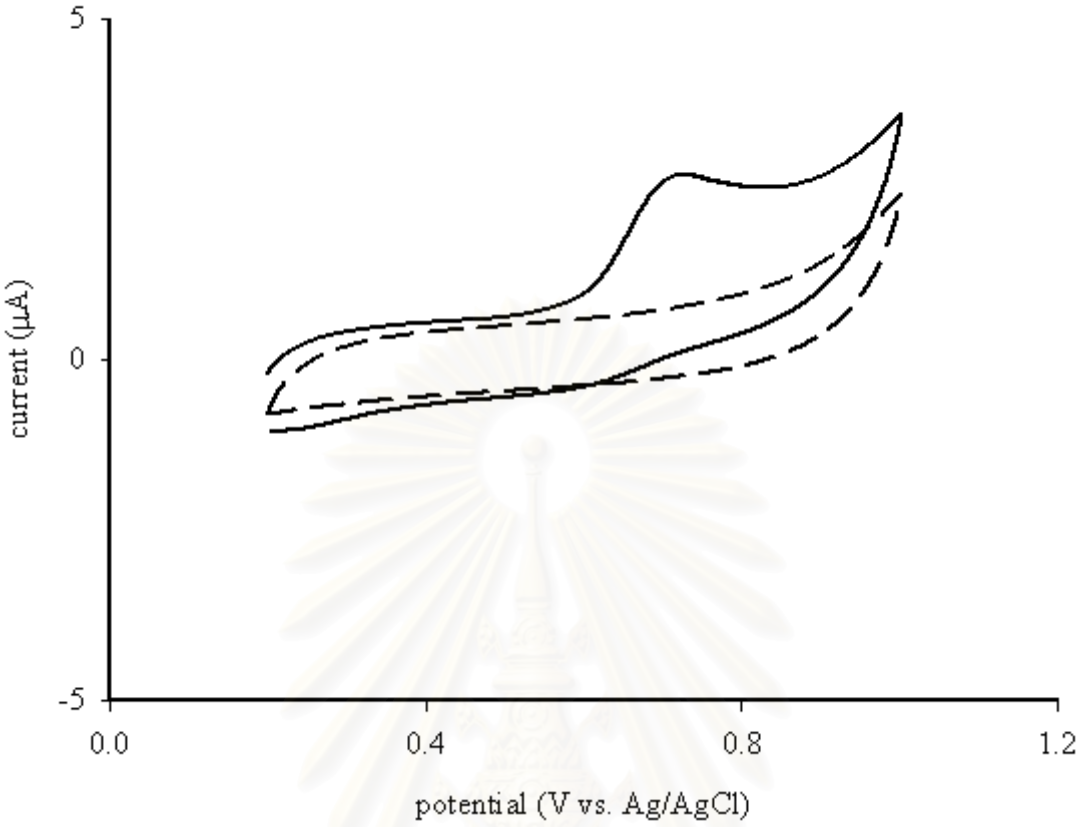


Figure A-1 Cyclic voltammograms for 100 μm a.) sudan II, b.) sudan III and, c) sudan IV in acetonitrile and 20 mM acetate buffer (90:10; v/v) together with the corresponding background current (dash line) at GC electrode. The sweep rate was 50 mV/s; area electrode, 0.07 cm^2.

APPENDIX B

Flow injection analysis with amperometric detection results and calibration curve

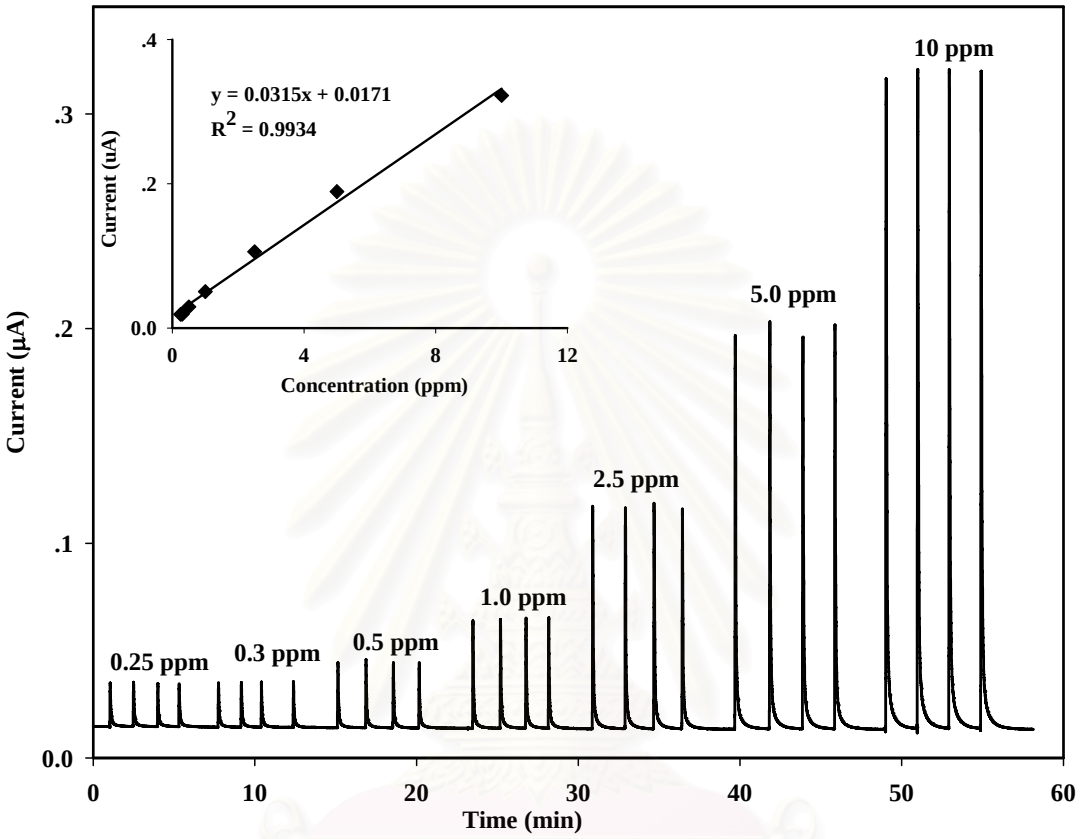


Figure B1 Flow injection analysis with amperometric detection results of sudan II in acetonitrile and 20 mM acetate buffer (90:10; v/v) at GC electrode. The sweep rate was 50 mV/s; area electrode, 0.07 cm^2. The corresponding calibration curve is also shown (inset Figure).

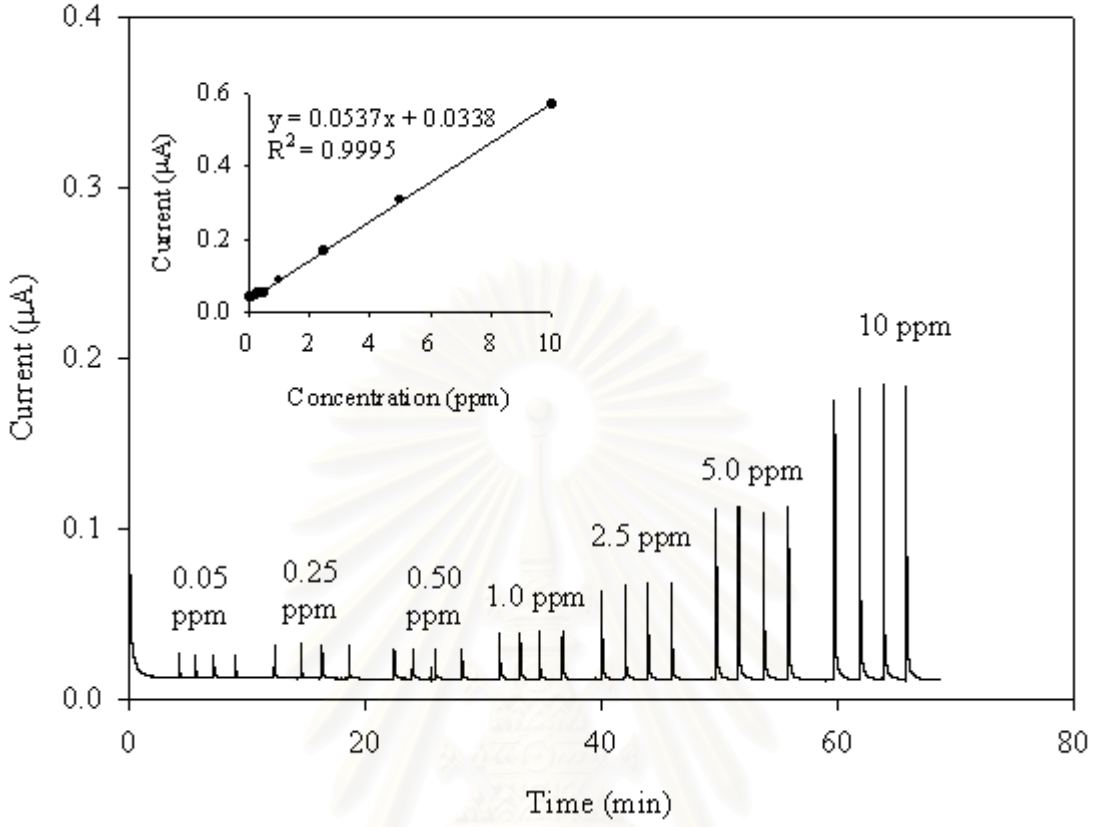


Figure B2 Flow injection analysis with amperometric detection results of sudan III in acetonitrile and 20 mM acetate buffer (90:10; v/v) at GC electrode. The sweep rate was 50 mV/s; area electrode, 0.07 cm^2. The corresponding calibration curve is also shown (inset Figure).

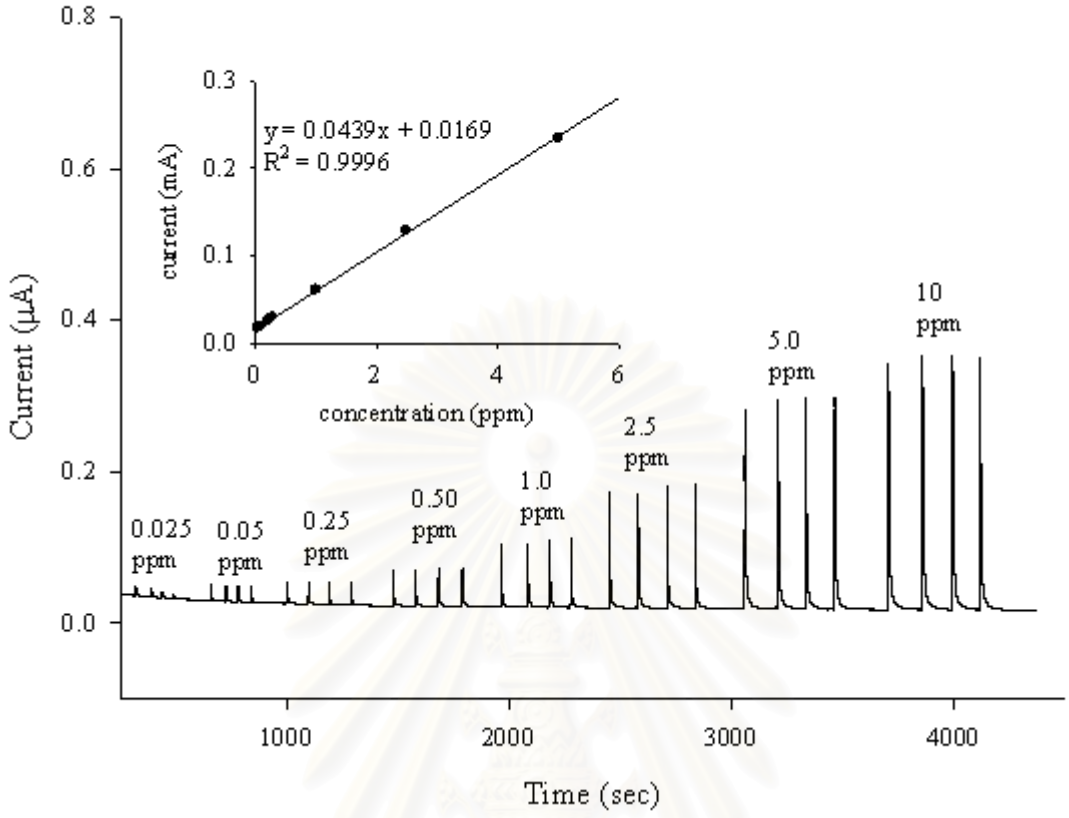


Figure B3 Flow injection analysis with amperometric detection results of sudan IV in acetonitrile and 20 mM acetate buffer (90:10; v/v) at GC electrode. The sweep rate was 50 mV/s; area electrode, 0.07 cm^2. The corresponding calibration curve is also shown (inset Figure).

APPENDIX C

Retention time and resolution of sudan I, sudan II, sudan III and sudan IV from the mobile phase investigated: Flow rate 1 mL min^{-1}, Column temperature 25°C and elution mode was isocratic.

Investigation conditions	Analytes	Retention time (min)	Resolution
Condition A	Sudan I	-	-
	Sudan II	-	-
	Sudan III	-	-
	Sudan IV	-	-
Condition B	Sudan I	1.6	3.12
	Sudan II	8.6	2.83
	Sudan III	16.6	-
	Sudan IV	28.2	-
Condition C	Sudan I	8.8	16.12
	Sudan II	17.2	14.92
	Sudan III	28.5	19.48
	Sudan IV	58.2	-
Condition D	Sudan I	5.6	9.93
	Sudan II	9.5	8.54
	Sudan III	13.7	13.65
	Sudan IV	25.3	-

APPENDIX D

Table 1-D Percent relative recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in F-soft drink sample in the second day (n=3)

Analytes	Sample Levels (ppm)	% Recovery			Mean	%RSD
		1	2	3		
Sudan I	0.5	98.58	97.33	90.72	95.54±4.23	4.42
	1.0	102.87	103.69	101.27	102.61±1.23	1.20
	1.5	98.86	98.68	100.45	99.33±0.97	0.98
Sudan II	0.5	91.14	86.55	81.99	86.56±4.58	5.29
	1.0	97.69	101.53	101.60	100.28±2.24	2.23
	1.5	102.01	100.81	101.32	101.38±0.61	0.60
Sudan III	0.5	99.62	97.96	94.60	97.39±2.56	2.63
	1.0	101.84	101.14	93.19	98.72±4.80	4.86
	1.5	99.21	99.62	103.20	100.68±2.20	2.18
Sudan IV	0.5	92.37	96.81	101.79	96.99±4.72	4.86
	1.0	99.88	98.07	97.78	98.58±1.14	1.15
	1.5	100.84	101.25	100.66	100.91±0.30	0.30

Table2-D Percent relative recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in F-soft drink sample in the third day (n=3)

Analytes	Sample Levels (ppm)	% Recovery			Mean	%RSD
		1	2	3		
Sudan I	0.5	101.30	95.10	95.58	97.66±3.24	3.32
	1.0	105.93	100.86	98.23	101.67±3.91	3.85
	1.5	97.22	100.16	101.18	99.52±2.06	2.07
Sudan II	0.5	99.59	95.53	90.99	95.37±4.30	4.51
	1.0	100.06	100.17	99.57	99.93±0.32	0.32
	1.5	100.04	100.41	101.15	100.53±0.57	0.56
Sudan III	0.5	93.43	98.46	85.78	92.55±6.38	6.90
	1.0	100.23	94.46	106.00	100.23±5.77	5.76
	1.5	100.40	102.55	98.88	100.61±1.84	1.83
Sudan IV	0.5	95.10	92.41	85.93	91.15±4.72	5.17
	1.0	107.68	105.18	100.10	104.32±3.86	3.70
	1.5	97.24	98.55	101.53	99.10±2.20	2.22

Table 3-D Percent relative recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in G-soft drink sample in the second day (n=3)

Analytes	Sample Levels (ppm)	% Recovery			Mean	%RSD
		1	2	3		
Sudan I	0.5	101.83	102.16	104.26	102.75±1.32	1.28
	1.0	99.23	99.74	100.30	99.76±0.53	0.53
	1.5	100.15	99.89	99.40	99.81±0.38	0.38
Sudan II	0.5	102.49	102.12	100.06	101.56±1.31	1.29
	1.0	99.23	98.89	98.36	98.83±0.44	0.44
	1.5	100.03	100.32	100.65	100.33±0.31	0.31
Sudan III	0.5	101.94	101.58	97.95	100.49±2.21	2.20
	1.0	103.09	102.30	100.04	101.81±1.59	1.56
	1.5	98.52	98.54	100.45	99.17±1.11	1.12
Sudan IV	0.5	103.91	103.60	99.34	102.29±2.56	2.50
	1.0	99.97	98.41	100.14	99.51±0.96	0.96
	1.5	99.79	100.40	100.09	100.10±0.30	0.30

Table 4-D Percent relative recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in G-soft drink sample in the third day (n=3)

Analytes	Sample Levels (ppm)	% Recovery			Mean	%RSD
		1	2	3		
Sudan I	0.5	92.93	94.99	98.54	95.49±2.84	2.97
	1.0	103.78	102.77	104.03	103.53±0.67	0.64
	1.5	99.10	99.35	99.14	99.20±0.14	0.14
Sudan II	0.5	93.79	92.97	101.00	95.92±4.42	4.61
	1.0	106.80	107.29	103.55	105.88±2.03	1.92
	1.5	97.59	97.48	99.70	98.26±1.25	1.28
Sudan III	0.5	93.44	97.14	97.30	95.96±2.18	2.27
	1.0	104.03	103.26	103.84	103.71±0.40	0.38
	1.5	98.81	98.89	98.73	98.81±0.08	0.08
Sudan IV	0.5	93.00	97.60	98.40	96.34±2.91	3.02
	1.0	100.22	100.91	101.49	100.87±0.63	0.63
	1.5	100.62	99.28	99.36	99.75±0.75	0.75

Table 5-D Percent relative recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in M-soft drink sample in the second day (n=3)

Analytes	Sample Levels (ppm)	% Recovery			Mean	%RSD
		1	2	3		
Sudan I	0.5	98.44	99.33	97.83	98.54±0.76	0.77
	1.0	100.10	101.67	99.28	100.35±1.21	1.21
	1.5	100.13	99.30	100.53	99.99±0.62	0.63
Sudan II	0.5	98.14	99.94	98.40	98.83±0.97	0.98
	1.0	100.69	101.11	101.41	101.07±0.36	0.36
	1.5	99.52	99.45	99.52	99.50±0.03	0.04
Sudan III	0.5	102.54	102.27	102.50	102.44±0.15	0.14
	1.0	101.45	100.41	100.78	100.88±0.53	0.52
	1.5	99.43	99.33	99.14	99.30±0.15	0.15
Sudan IV	0.5	102.63	102.37	99.71	101.57±1.62	1.59
	1.0	101.29	100.14	100.97	100.80±0.59	0.59
	1.5	99.08	99.85	99.82	99.59±0.43	0.44

Table 6-D Percent relative recoveries and percent RSD of sudan I, sudan II, sudan III and sudan IV at 0.5, 1.0 and 1.5 ppm spiking levels in M-soft drink sample in the third day (n=3)

Analytes	Sample Levels (ppm)	% Recovery			Mean	%RSD
		1	2	3		
Sudan I	0.5	92.68	94.79	98.08	95.18±2.72	2.86
	1.0	107.58	105.91	105.35	106.28±1.16	1.09
	1.5	97.44	97.95	97.82	97.74±0.26	0.27
Sudan II	0.5	97.29	97.70	99.24	98.08±1.03	1.05
	1.0	102.66	104.91	105.48	104.35±1.49	1.43
	1.5	99.09	98.05	97.61	98.25±0.76	0.78
Sudan III	0.5	96.01	100.42	102.65	99.69±3.38	3.39
	1.0	100.85	105.43	105.24	103.84±2.59	2.50
	1.5	99.90	97.56	97.52	98.33±1.36	1.39
Sudan IV	0.5	96.07	96.17	97.42	96.55±0.75	0.78
	1.0	107.48	106.43	102.86	105.59±2.42	2.30
	1.5	97.10	95.24	99.01	97.12±1.89	1.94

APPENDIX E

Description of analytical performance characteristics

Accuracy

Accuracy denotes that closeness of a measurement or set of measurements to the accepted value. Accuracy is normally reported in terms of error. Error is the difference between the accepted and measured values. There are several ways and units in which the accuracy can be expressed. Recovery is a term often used to describe accuracy, the equation for recovery is:

$$\%\,\mathrm{Recovery} = \frac{Measured\ value}{True\ value} x100$$

Relative error is another term that can be expressing the accuracy. The equation is shown below:

$$\%Error = \frac{(Measured\ value - True\ value}{True\ value} x100$$

Precision

Precision refers to the agreement between values in a set of data that have been carried out in exactly the same mode. It is a measure of the reproducibility of the analysis. Precision of the results can be ascertained through the use of replicate measurements. There are several popular ways to express the precision of data. Multiple injections of a homogeneous sample and calculation of the relative standard deviation (%RSD) do it. The equation for %RSD is shown below:

$$\%RSD = \frac{\mathrm{Standard\ deviation}}{\mathrm{Mean}} x100$$

Linearity (Linear range)

Linearity is the range where the analyte response is linearly proportional to concentration. The working sample concentration and samples tested for accuracy should be in the linear range.

Sensitivity

Sensitivity is the change in the analytical response divided by the corresponding change in the concentration of a standard (calibration) curve, i.e. the slope of the analytical calibration.

Limit of detection (LOD)

The detection limit of a method is the lowest analyte concentration that can be determined to be different from an analyte blank. There are numerous way that detection limit have been defined. An example is the lowest analyte concentration that is above the noise level of the system, typically, three time the noise level (S/N = 3). For high analyte concentrations, the detection limit is defined as the lowest concentration that provides a signal to background ratio S/B of three. The equation of S/B ratio is shown below:

$$S/B\ ratio = \frac{(total\ signal - blank\ signal)}{blank\ signal}$$

APPENDIX F

The AOAC manual for the Peer Verified Methods program includes a table with estimated precision and recovery data as a function of analyte concentration [59]

Precision and Reproducibility

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. The acceptance criteria for precision depend very much on the type of analysis. For environmental and food samples, the precision is very much dependent on the sample matrix, the concentration of the analyte and on the analysis technique. It can vary between 2% and more than 20%.

Table 1. Analyte concentration versus precision within or between days

Analyte %	Analyte ratio	Unit	RSD (%)
100	1	100%	1.3
10	10-1	10%	2.8
1	10-2	1%	2.7
0.1	10-3	0.1 %	3.7
0.01	10-4	100 ppm	5.3
0.001	10-5	10 ppm	7.3
0.0001	10-6	1 ppm	11
0.00001	10-7	100 ppb	15
0.000001	10-8	10 ppb	21
0.0000001	10-9	1 ppb	30

Accuracy and recovery

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. The expected recovery depends on the sample matrix, the sample processing procedure and on the analyte concentration.

Table 4. Analyte recovery at different concentrations

Active Ingred (%)	Analyte ratio	Unit	Mean recovery (%)
100	1	100%	98-102
>=10	10-1	10%	98-102
>=1	10-2	1%	97-103
>=0.1	10-3	0.1 %	95-105
0.01	10-4	100 ppm	90-107
0.001	10-5	10 ppm	80-110
0.0001	10-6	1 ppm	80-110
0.00001	10-7	100 ppb	80-110
0.000001	10-8	10 ppb	60-115
0.0000001	10-9	1 ppb	40-120

CURRICULUM VITAE

Name Wanida Wonsawat

Date of birth 16 June 1976

Place of Birth Chonburi, Thailand

Institutions attend

2004- 2006 Chulalongkorn University,
Master of Science (Chemistry)

1995-1999 Burapha University,
Bachelor of Education
(Chemistry Teaching) B.Ed (Chemistry Teaching)

Research scholarships

The 90 th Anniversary of Chulalongkorn University
(Ratchadphiseksomphot Endowment Fund)