การตรวจวัดสีซูดาน I-IV ในผลิตภัณฑ์พริกโดยใช้การสกัดด้วยเฟสของแข็งแบบอัตโนมัติออนไลน์ร่วมกับลิควิดโครมาโทกราฟีแมสสเปกโทรเมตรี

นางสาว กิตติมา โสนะมิตร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2552

DETERMINATION OF SUDAN I-IV IN CHILLI PRODUCTS USING AUTOMATED ON-LINE SOLID PHASE EXTRACTION COUPLED WITH LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	Determination of Sudan I-IV in chilli products using automated on-line solid phase extraction coupled with liquid chromatography-mass spectrometry
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กิตติมา โสนะมิตร์ : การตรวจวัดสีซูดาน I-IV ในผลิตภัณฑ์พริกโดยใช้การสกัดด้วยเฟสของแข็งแบบอัตโนมัติออนไลน์ร่วมกับลิควิดโครมาโทกราฟี-แมสสเปกโทรเมตรี. (DETERMINATION OF SUDAN I-IV IN CHILLI PRODUCTS USING AUTOMATED ON-LINE SOLID PHASE EXTRACTION COUPLED WITH LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.อรรวรรณ ชัยลภากุล, 109 หน้า.

งานวิจัยนี้ได้พัฒนาวิธีวิเคราะห์สีซูดาน I-IV ในผลิตภัณฑ์พริกโดยใช้เทคนิคการสกัดด้วยเฟสของแข็งแบบออนไลน์ร่วมกับลิควิดโครมาโทกราฟี-แมสสเปกโทรเมตรี ซึ่งให้สามารถปฏิบัติงานได้รวดเร็ว มีความเที่ยง และความแม่นสูง โดยใช้อะซีโตนเป็นตัวทำละลายที่เหมาะสมในการสกัดสีซูดาน I-IV จากผลิตภัณฑ์พริก จากนั้นกำจัดตัวรบกวน และเพิ่มความเข้มข้นของสารโดยการสกัดด้วยเฟสของแข็งชนิด C_{18} (ขนาดอนุภาค 15-40 ไมโครเมตร) ซึ่งต่อออนไลน์กับระบบ LC-MS สีซูดาน I-IV ถูกชะออกมา โดยใช้เวลาน้อยกว่า 8 นาที จากคอลัมน์ชนิด C_{18} (2.1x150 มิลลิเมตร, ขนาดอนุภาค 3 ไมโครเมตร) ด้วยระบบเกรเดียนท์ระหว่าง 0.1% กรดฟอร์มิกในน้ำกับ 0.1% กรดฟอร์มิกในอะซิโตไนไตรล์ ผลที่ได้จากการทดสอบความถูกต้องของวิธี ได้ความเป็นเส้นตรงของกราฟมาตรฐานที่มีค่าสัมประสิทธิ์สหสัมพันธ์ (R^2) มากกว่า 0.9997 ขีดจำกัดการตรวจพบ (LOD) และขีดจำกัดการวิเคราะห์ปริมาณ (LOQ) สำหรับสีซูดาน I, II และ IV มีค่า 0.03 และ 0.05 มิลลิกรัมต่อกิโลกรัม ตามลำดับ ส่วนสีซูดาน III มีค่า 0.04 และ 0.1 มิลลิกรัมต่อกิโลกรัม ตามลำดับ ค่าการคืนกลับของสีซูดาน I-IV ในพริกป่นโดยวิเคราะห์ซ้ำภายในวันเดียวกันอยู่ในช่วง 90.14 ถึง 101.65% พร้อมด้วยค่าเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ในช่วง 0.014 ถึง 0.164% ส่วนการวิเคราะห์ซ้ำระหว่างวัน ได้ค่าการคืนกลับอยู่ในช่วง 90.22 ถึง 102.02% และค่าเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ในช่วง 0.011 ถึง 0.202% การตรวจวิเคราะห์ปริมาณการปนเปื้อนของสีซูดาน I-IV ในผลิตภัณฑ์พริกต่างๆ ได้แก่ ซอสพริก น้ำพริกเผา และพริกแห้ง ตรวจวิเคราะห์ไม่พบทุกตัวอย่าง แต่ยืนยันความใช้ได้ของวิธีด้วยการหาค่าการคืนกลับโดยการเติมสีซูดานที่ระดับ 3 ความเข้มข้น ได้ค่ามากกว่า 90% ดังนั้นวิธีที่เสนอนี้สามารถใช้หาปริมาณสีซูดาน I-IV ที่ปนเปื้อนในผลิตภัณฑ์พริกได้ ซึ่งเป็นวิธีที่รวดเร็ว มีการตอบสนองได้ดี และใช้ปริมาณสารตัวอย่างและตัวทำละลายน้อย

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KITTIMA SONAMIT: DETERMINATION OF SUDAN I-IV IN CHILLI PRODUCTS USING AUTOMATED ON-LINE SOLID PHASE EXTRACTION COUPLED WITH LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY. THESIS ADVISOR: ASSOC. PROF. ORAWON CHAILAPAKUL, Ph.D., 109 pp.

A rapid, accurate and precise method has been developed for the determination of Sudan I-IV in chili products using on-line solid phase extraction and LC-MS. Chili products were extracted with acetone and the analytes were cleaned up and enriched on an SPE column (C_{18}, 15-40 μm) through on-line SPE. Chromatographic separation was performed on a C_{18} analytical column (2.1x150 mm, 3 μm) with gradient elution programming of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. All four Sudan dyes were separated in less than 8 min. Using in-house validation data, a linearity coefficient of determination (R^2) of more than 0.9997 was obtained. The limits of detection (LOD) and limits of quantitation (LOQ) were the same for Sudan I, II and IV and those values were 0.03 and 0.05 mg kg^{-1}, respectively, and 0.04 and 0.1 mg kg^{-1} for Sudan III. The intra- and inter-day recoveries of the four Sudan dyes in chili powder were between 90.14-101.65% and 90.22-102.02%, respectively, with relative standard deviation (RSD) between 0.014-0.164% and 0.011-0.202%, respectively. The proposed method was used to detect Sudan I-IV in food samples such as chili sauce, roasted chili paste and dried chili. The results showed that Sudan I-IV were not found in real samples. To verify, real samples were spiked at 3 levels of standard Sudan dyes. The recoveries were found to be more than 90%. Therefore, the proposed method which is rapid, sensitive, less sample and solvent consumption could be applicable for the determination of Sudan I-IV in chili products.

Department : Chemistry Student's Signature Kittima Sonamit

Field of Study : Chemistry Advisor's Signature

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LIST OF ABBREVIATIONS

ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
CF-FAB	continuous flow- fast atom bombardment
CI	chemical ionization
DLI	direct liquid introduction
DSSBME	dual solvent stir bars microextraction
EC	electrochemical
EI	electron ionization
ESI	electrospray ionization
EU	European Union
FAB	fast atom bombardment
GPC	gel permeation chromatography
HED	high energy dynode detector
HF-LPME	hollow fiber-liquid phase microextraction
HPLC	high performance liquid chromatography
ICC	ion charge control
LC	liquid chromatography
LC-ESI-MS	liquid chromatography-electrospray-mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
MALDI	matrix assisted laser desorption ionization
MeOH	methanol

MS	mass spectrometry
PDA	photodiode array
PB	particle beam
RSD	relative standard deviation
SPE	solid phase extraction
TOF	time of flight
TSP	thermospray
UV	ultraviolet
MW	molecular weight
$^{\circ}C$	degree Celsius
cm	centimeter
g	gram
i.d.	internal diameter
L	liter
mL	milliliter
mm	millimeter
min	minute
m/z	mass per charge ratio
ppb	part per billion
ppm	part per million
psi	pound per square inch
R^2	correlation coefficient
sec	second
v/v	volume by volume
µg	microgram
µL	microliter
µm	micrometer

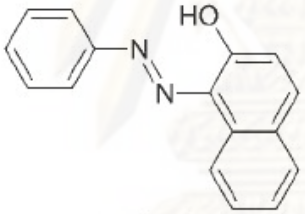
CHAPTER I

INTRODUCTION

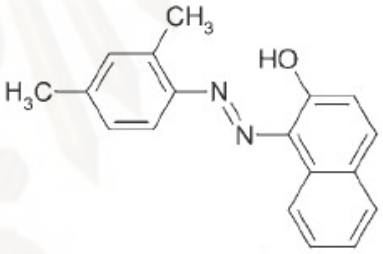
1.1 Introduction and Literature Reviews

The food market has changed rapidly with increasing of processed foods. The uses of colors to make foodstuffs more aesthetically and psychologically attractive have been known for many centuries. Synthetic organic colors added in the foods to replace the natural color that lost in the processing are of consumer interest.

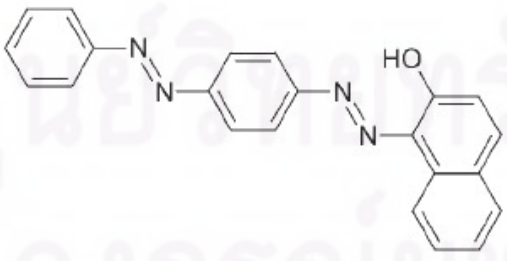
Sudan azo-dyes are synthetic colorants, known also as Sudan I-IV (Figure 1.1), widely used as coloring agents in oils, waxes, petrol, shoe and floor polishes [1].



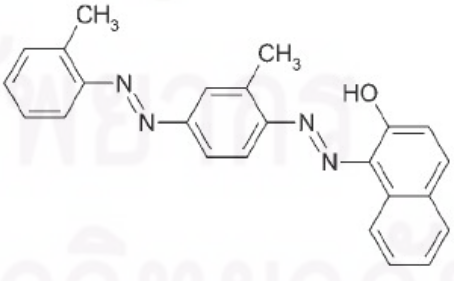
Sudan I (MW 248.28)



Sudan II (MW 276.34)



Sudan III (MW 352.40)



Sudan IV (MW 380.45)

Figure 1.1 Chemical structures of four Sudan dyes

In 2003, the Food Standard Agency (FSA) alarmed on the contamination of Sudan dyes in hot chili and hot chili products originating from India [1]. Furthermore, these dyes are classified by the International Agency for Research on Cancer (IARC) as category 3 carcinogens [2]. Therefore, Sudan dyes may cause cancer and hence Sudan dyes at any level are considered unsafe for human consumption.

Because of these problems the EU and the US do not permit the use of these colorants as food additives. The EU has set the detection limit in analyses of Sudan dyes at 0.5-1 mg kg^{-1}. Any food material containing more than this limits must be withdrawn from the market [3].

As the illegal use of dyes has the big problem for world-wide food industries and economic section in south-east Asia, especially Thailand, as well as an impact on public health. 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 chili products and the levels of Sudan dyes residues may be unacceptable for the international markets. For this reason, a sensitive, simple, accurate, reliable and rapid analytical method for the determination of Sudan dyes in food is needed for the assurance of consumer health.

As reported in the literature, a wide variety of analytical methods have been developed for the determination of Sudan dyes in food stuffs, with the most common based on liquid chromatography (LC) coupled with ultraviolet (UV), photodiode array (PDA), mass spectrometry (MS), electrochemical (EC) and chemiluminescence detection [4-10]. Other reports have included sample preparation methods such as gel permeation chromatography (GPC) [11], dual solvent-stir bars microextraction (DSSBME) and hollow fiber-liquid phase microextraction (HF-LPME) [12]. Although these methods have been successfully applied to analysis of Sudan dyes at trace levels, they have tedious sample preparation, require long analysis times and use large

amounts of organic solvent. Therefore, the development of simple, rapid and efficient sample preparation technique for the trace analysis of Sudan dyes is still needed.

Solid-phase extraction (SPE) is a method of sample preparation that concentrates and purifies analytes from complex samples [13]. This extraction technique has been developed in the on-line mode with column switching to improve sample throughput. On-line SPE offers an exceptional opportunity to reduce the analysis time associated with manual off-line SPE while minimizing reagent and sample consumption. This method allows the samples to be directly injected onto a fully automated HPLC system. Because of these reasons, the on-line SPE was applied for many fields such as pharmaceuticals [14-16], environmental samples [17-19], biological samples [20, 21] and food samples [22]. The purpose of the present study was to develop a simple, rapid and reliable the on-line SPE-LC-MS method for the determination of Sudan I-IV in chili products without additional pre-purification steps. The method provides high recovery and short analysis time as compared to existing methods. Finally, the method was used to analyze Sudan dyes in various chili samples.

1.2 Research Objective

The target of this research is to develop a rapid and accurate sample preparation and analytical method for the determination of four Sudan dyes in chili products using the on-line solid phase extraction coupled with liquid chromatography-mass spectrometry.

1.3 Scope of Research

The on-line solid phase extraction coupled with liquid chromatography-mass spectrometry was studied for separation and detection of four Sudan dyes in chili products. The effect of loading sample, switching valve time, types of solid phase, limit of detection, limit of quantitation, linearity and matrix effect, precision and accuracy were studied in detail.

CHAPTER II

THEORY

2.1 High Performance Liquid Chromatography (HPLC) [23, 24]

High performance liquid chromatography is the most versatile and widely used type of elution chromatography. The technique is used by chemists for separating and determining species in a variety of organic, inorganic, and biological materials. In liquid chromatography, the mobile phase is a liquid solvent containing the sample as a mixture of solutes. There are several types of high performance liquid chromatography, depending on the nature of the stationary phase. Figure 2.1 showed the four most widely used types of HPLC. These methods include: (1) partition or liquid-liquid chromatography; (2) adsorption or liquid-solid chromatography; (3) ion exchange chromatography; and (4) two types of size exclusion chromatography; including gel permeation chromatography and gel filtration chromatography.

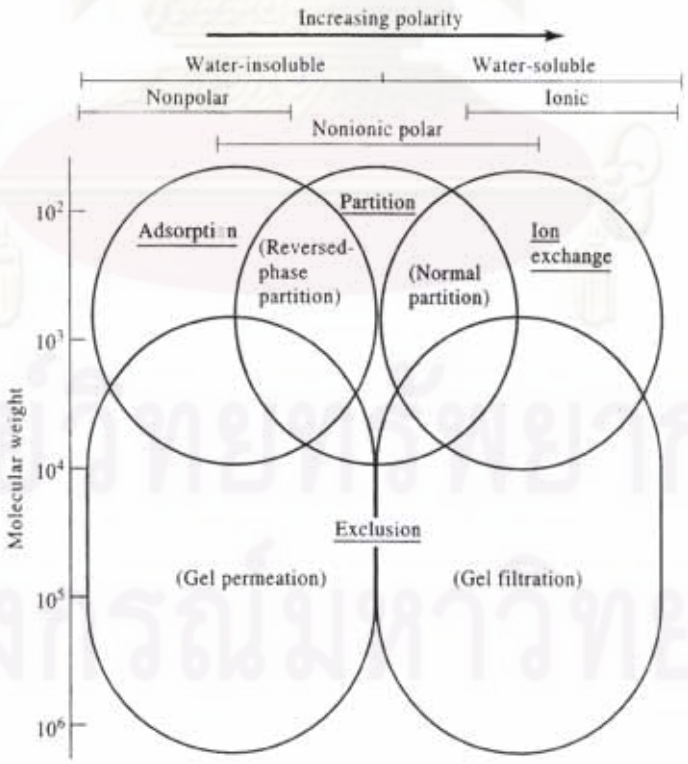


Figure 2.1 Types of liquid chromatography

2.1.1 Types of HPLC [23, 25]

The schematic of the four modes of liquid chromatography are demonstrated in Figure 2.2.

2.1.1.1 High Performance Adsorption Chromatography

Adsorption chromatography (Figure 2.2 (B)), often referred to as liquid-solid chromatography, is based on adsorption of analyte species on a solid surface. Here, the stationary phase is the surface of a finely divided polar solid. With such a packing, the analyte competes with the mobile phase for sites on the surface of the packing, and retention is the result of adsorption forces. The adsorbent is generally an active, porous solid with a large surface area, such as silica gel, alumina or charcoal. The active sites, such as silanol groups of silica gel generally interacted with the polar functional groups of compounds to be separated. The nonpolar portion of a molecule showed only a minor influence on the separation.

2.1.1.2 High Performance Partition Chromatography

Partition chromatography (Figure 2.2 (C)), also referred to as liquid-liquid chromatography, the solute molecules distribute themselves between two immiscible liquid phases, the stationary phase and the mobile phase, according to their relative solubilities. The stationary phase is uniformly spread on an inert support-a porous or nonporous particulate solid or porous paper. To avoid mixing of the two phases, the two partitioning liquids must differ greatly in polarity. There are two typed based on the relative polarities of the mobile phase and stationary phase. Firstly, they were based on highly polar stationary phases and a relatively nonpolar solvent was used as mobile phase. For historic reasons, this type of chromatography is so called normal-phase chromatography. Secondly, they were reverse-phase chromatography, which consisted of nonpolar stationary phase and polar solvent mobile phase.

2.1.1.3 High Performance Ion Exchange Chromatography

Ion exchange chromatography, depicted in Figure 2.2 (D), is based on the affinity of ions in solution to the oppositely charged ions on the stationary phase. Ion exchange packing consisted of a porous solid phase, usually a resin, onto which ionic groups are chemically bonded. The mobile phase is usually a buffered aqueous solution containing a counter ion whose charge is opposite to that of the surface groups on stationary phase. Anyhow, they had same charge as the solute which is in charge equilibrium with the resin in the form of an ion pair. Competition between the solute and the counter ion for the ionic site governs the chromatographic retention. Ion-exchange chromatography has found wide application in inorganic chemistry for separating metallic ions, and in biological systems for separating water-soluble ionic compounds such as proteins, nucleotides, and amino acids.

2.1.1.4 High Performance Size Exclusion Chromatography

The mechanism of size exclusion chromatography, also referred to as gel-permeation or gel-filtration chromatography is shown in Figure 2.2 (E). Here, the stationary phase should be chemically inert. Size exclusion chromatography involves the selective diffusion of solute molecules into and out of mobile phase filled pores in a three dimensional network, which may be a gel or porous inorganic solid. The degree of retention depends on the size of the solvated solute molecule relative to the size of the pore. Small molecules will be permeated into the smaller pores, intermediate sized molecules will be permeated only part of the pores and be excluded from other, and the very large molecules will be completely excluded. The larger molecules will be traveled faster through the stationary phase and eluted from the column first. Thus, size exclusion chromatography is especially useful in separation of high molecular weight organic compound and biopolymers from smaller molecules.

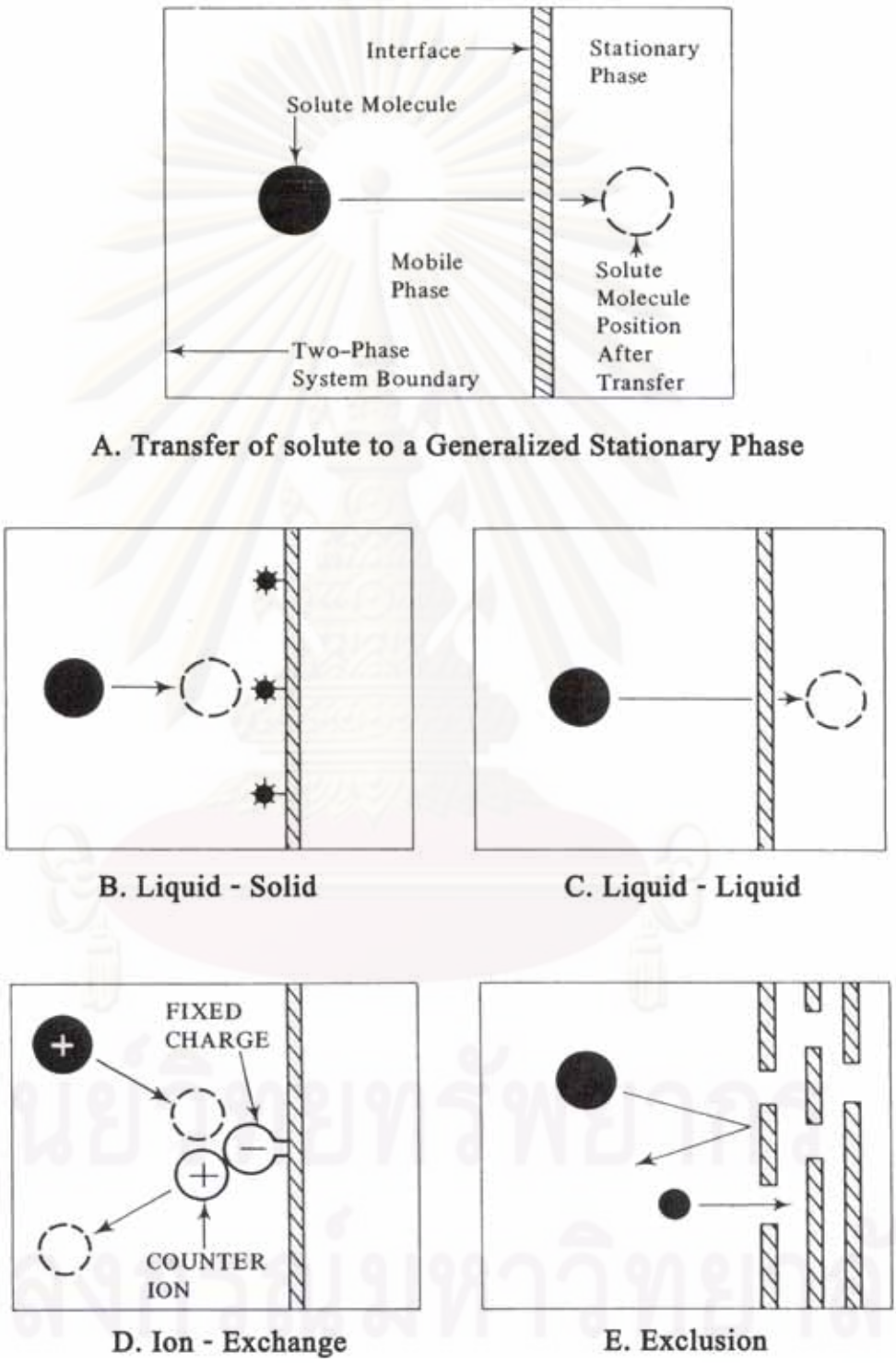


Figure 2.2 Schematic representations of the four modes of liquid chromatography

2.1.2 Instrumentation [24, 26]

HPLC is a widely accepted separation technique for both sample analysis and purification in a variety of areas including the pharmaceutical, biotechnological, environmental, polymer and food industries. Figure 2.3 is a diagram showing the important components of a typical HPLC instrument.

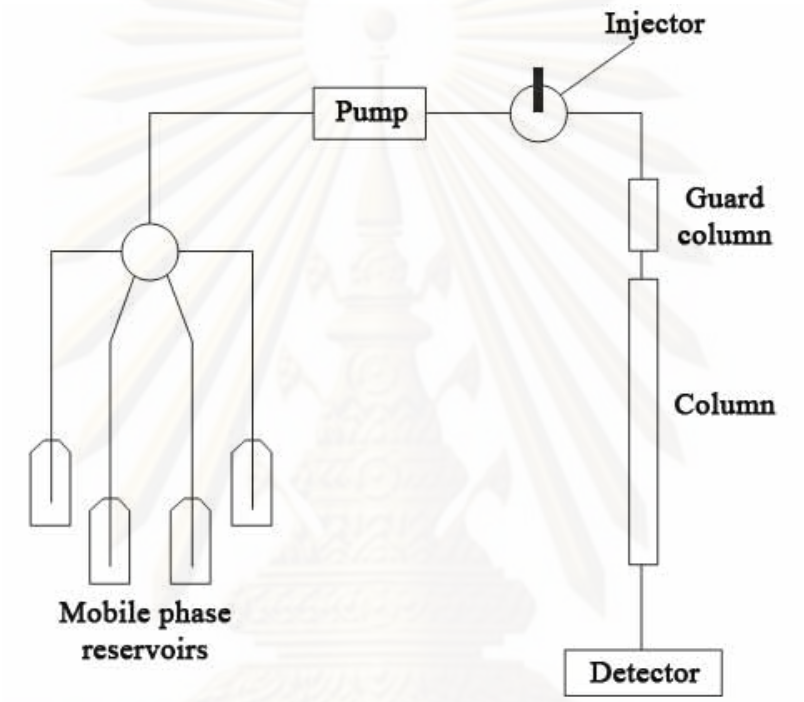


Figure 2.3 Components of a typical HPLC instrument

2.1.2.1 Mobile Phase Reservoirs

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, each of which contains 500 mL or more of a solvent. Provisions are often included to remove dissolved gases and dust from the liquids. The former produce bubbles in the column and thereby causes band spreading; in addition, both bubbles and dust interfere with the performance of detectors. Degassers may consists of a vacuum pumping system, a distillation system, a device for heating and stirring, or a system for sparging, in which the dissolved gases are swept out of solution by fine bubbles of an inert gas that is not soluble in the mobile phase.

An elution with a single solvent of constant composition is termed isocratic. In gradient elution, two (and sometimes more) solvent systems that differ significantly in polarity are employed. The ratio of the two solvents is varied in a preprogrammed way, sometimes continuously and sometimes in a series of steps.

2.1.2.2 Pump

The requirements for liquid chromatographic pumps are severe and include (1) the generation of pressures of up to 6,000 psi, (2) pulse-free output, (3) flow rates ranging from 0.1 to 10 mL min^{-1}, (4) flow reproducibility of 0.5% relative or better, and (5) resistance to corrosion by a variety of solvents.

Reciprocating pumps, which are the most widely used, usually consist of a small cylindrical chamber that is filled and then emptied by the back-and forth motion of a piston. The pumping motion produces a pulsed flow that must be subsequently damped. Advantages of reciprocating pumps include small internal volume, high output pressure (up to 10,000 psi), ready adaptability to gradient elution, and constant flow rates, which are largely independent of column back-pressure and solvent viscosity.

2.1.2.3 Injector

The most widely used method of sample introduction in HPLC is based on sampling loops. From a mass spectrometry perspective, the injector is of little concern other than the fact that any bubbles introduced into the injector may interrupt the liquid flow, resulting in an unstable response from the mass spectrometer.

2.1.2.4 Columns

The majority of HPLC analyses employ reversed phase systems where the columns contain chemically modified silica stationary phases. Generally, the column has an internal diameter of 4.6 mm and operates at high flow rate which is too high to

be directly introduced to MS. Because of high selectivity and sensitivity of the LC-MS, good results can only be obtained at low flow rate. One way to reduce flow is to reduce the internal diameter of the LC column, for example, microbore column has 2.1 mm internal diameter and compatible with low flow rate. Further miniaturization of the column dimension is widely accepted when limited sample quantities are available.

The column thermostat, close control of column temperature, for many applications is not necessary and columns are operated at room temperature. Often, however, better chromatograms are obtained by maintaining column temperatures constant to a few tenths of a degree Celsius. Most modern commercial instruments are now equipped with column heaters that control column temperatures to a few tenths of a degree from near ambient to 150 ºC. Columns may also be fitted with water jackets fed from a constant temperature bath to give precise temperature control.

2.1.2.5 Detectors [28, 29]

The detector controls the sensitivity with which compounds can be measured once separated on the column. To be effective, the detector must be capable of responding to concentration changes in all of the compounds of interest, with sensitivity sufficient to measure the component present in the smallest concentration. Not all detectors will see every component separated by the column. Generally, the more sensitive the detector, the more specific it is and the more compounds it will miss. Detectors can be used in series to gain more information while maintaining sensitivity for the detection of minor components.

Spectroscopic detector, the most popular HPLC detectors are based on spectroscopic measurements, including UV/Vis absorption, and fluorescence. Electrochemical detector, another common group of HPLC detectors are those based on electrochemical measurements such as amperometry, voltammetry, coulometry, and conductivity. A refractive index detector is nearly universal; responding to almost all

compounds, but has a poor detection limit. Furthermore, a refractive index detector is not useful for a gradient elution unless the mobile phase components have identical refractive indexes.

Another useful detector is a mass spectrometer, detection limits are quite good. In addition, a mass spectrometer provides qualitative, structural information that can help identify the analytes.

2.2 Mass Spectrometry (MS) [27, 30-32]

Mass spectrometry (MS) is an analytical method concerned with the separation of molecular (and atomic) species according to their mass. MS can be used for the establishment of the molecular weight and structure of compounds, or the identification and determination of the components. The instrument used to carry out this measurement is called a mass spectrometer. The basic compartments of a mass spectrometer are shown in Figure 2.4.

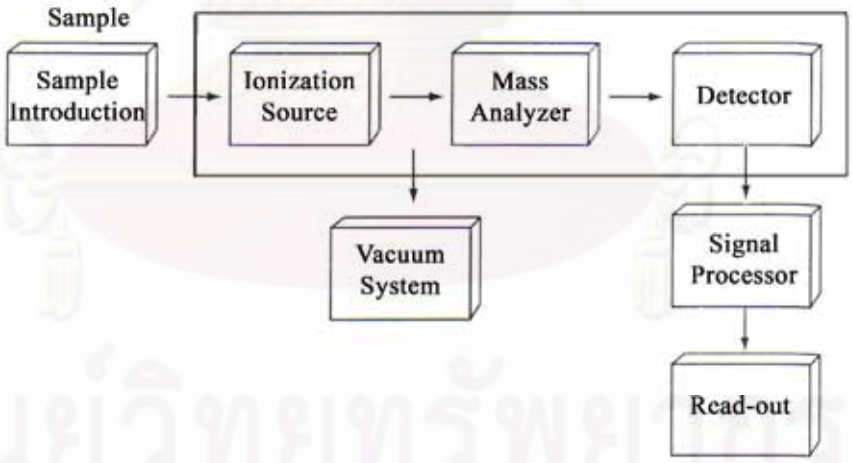


Figure 2.4 The components of a mass spectrometer

2.2.1 Sample Introduction

The purpose of a sample introduction is to introduce a very small amount of a sample into an ionization source that depending on the ionization method being used,

as well as the type and complexity of the sample. Direct insertion probe is a common sample introduction, in which a sample (solid or liquid) is placed on a probe and inserted, usually through a vacuum lock into the ionization region of the mass spectrometer.

2.2.2 Ionization Source

In the ionization sources, the analysed samples are ionized prior to analysis in the mass spectrometer. A variety of ionization techniques are used for mass spectrometry. The most important considerations are the internal energy transferred during the ionization process and the physico-chemical properties of the analyte that can be ionized. Some ionization techniques are very energetic and cause extensive fragmentation.

2.2.2.1 Electron Ionization (EI)

EI source is formerly called electron impact. Sample molecules in the vapor state are bombarded by fast moving electrons which produce 70 eV by passing the current through a filament.

$$M + e^- (70\ eV) \longrightarrow M^{+\bullet} + 2e^-$$

The electron beam gives excess energy. EI mass spectra contain fragment ion peaks and much smaller molecular ion peak. Molecular can also lose an electron. The sample can be introduced to the EI source via a gas chromatography device or directly via a solids probe device. Consequently, the EI ionization method is suitable for non-thermally labile compounds.

2.2.2.2 Chemical Ionization (CI)

CI is a technique that has been developed to reduce the fragmentation associated with ionization. It is term a “soft ionization technique”.

In CI, the vapor phases of analyte molecules are introduced into the mass spectrometer source containing a reagent gas. The mixture is then bombarded with electrons, initiating the ionization processes. Ion-molecule interactions then take place between the reagent gas ions and the neutral analyte molecules in the high-pressure region of the mass spectrometer source. For example, the process of chemical ionization mass spectrometry using methane as the reagent gas described as follows:

$$CH_4 + e^- \longrightarrow CH_4^{+\bullet} + 2e^-$$

$$CH_4^{+\bullet} \longrightarrow CH_3^+ + H^\bullet$$

$$CH_4^{+\bullet} + CH_4 \longrightarrow CH_5^+ + CH_3^\bullet$$

$$CH_3^+ + CH_4 \longrightarrow C_2H_5^+ + H^2$$

$$CH_5^+ + M \longrightarrow MH^+ + CH_4$$

CI leads to the formation of adducts of reagent ions with analyte molecules in relatively low energy processes that lead to little fragmentation. The m/z of the ion observed in the molecular ion form does not give the molecular weight directly but arises from the combination of the analyte with adduct.

2.2.2.3 Fast-Atom Bombardment (FAB)

FAB is one of the soft ionization techniques which utilize a high energy beam of Xe atom or CS^+ ions to sputter the analyte and matrix from the probe surface that placed in the source of the mass spectrometer. When the beam of fast-moving ions is directed to the solution on the probe, the matrix will absorb the incident energy and transfer to the analyte molecules to facilitate ionization.

FAB matrix (*m*-nitrobenzylic alcohol or glycerol) is a non-volatile liquid material that the analyte is dissolved. It serves to replenish the probe surface with new sample and minimize sample damage from the high-energy particle beam.

2.2.2.4 Matrix-Assisted Laser Desorption Ionization (MALDI)

MALDI is an ionization method that permits the analysis of polar, thermally labile, and high molecular weight compounds with high sensitivity.

Ion formation in MALDI is achieved by directing a pulsed laser beam onto a sample suspended in a matrix that dried on the laser target. The matrix absorbs the laser light energy causing the matrix material to vaporize. Then the vaporized matrix reacts with analyte molecules and transfers excess energy to facilitate the ionization.

2.2.3 Mass Analyzer

The mass analyzer is designed to separate and resolve the ions from the ionization source prior to their mass-to-charge (m/z) ratios. There are many mass analyzers currently available, the better known being magnetic sector, quadrupole, time-of-flight (TOF) analyzer and fourier transform and quadrupole ion traps.

2.2.3.1 Magnetic Sector

A magnetic sector mass spectrometer (Figure 2.5) separates ions in a magnetic field according to the momentum and charge of the ion. Ions are accelerated from the source region into the magnetic sector by a 1 to 10 kV electric field.

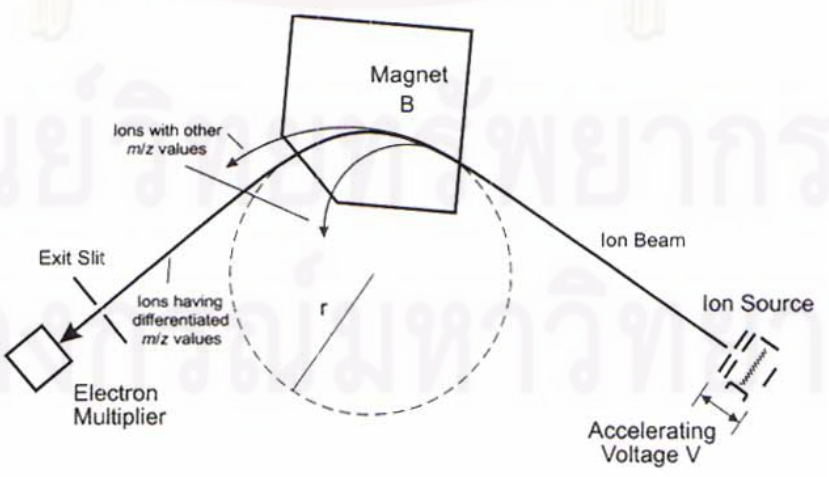


Figure 2.5 Diagram of a magnetic sector mass spectrometer

Since the ions are charged, they move through the magnetic sector. This separates ions according to their momentum; so magnetic sectors are often called momentum analyzers. Analysis of equal mass and charge can follow the same path through the fixed magnetic field because the momentum (for a given mass the velocity or kinetic energy is constant). Double-focusing magnetic sector uses a magnetic and an electrostatic sector to focus and accelerate ions. The combination of two analyzers improves resolution and accuracy of the mass spectrometer and mass ranger. However, double-focusing magnetic sector has some tiny drawback such as reduced of sensitivity due to use of a narrow slit and decreased voltage.

2.2.3.2 Quadrupole

Quadrupole analyzers are the most widely used mass spectrometers in many types of organic analytical laboratories. As seen in Figure 2.6, the quadrupole analyzer consists of four electrical poles (usually called rods) that are held in strict alignment with one another. Indeed, it is crucial that these poles remain parallel to and at a fixed distance from one another. Opposing poles are connected in pairs to both radio frequency (RF) and direct current (DC) generators, bathing ions in a combined electric and RF field during their passage through the analyzer.

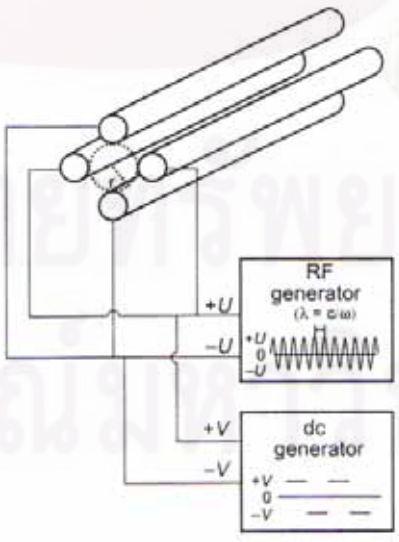


Figure 2.6 Diagram of a quadrupole mass analyzer

2.2.3.3 Time-of-Flight (TOF)

The TOF analyzer is the simplest of the mass separation devices. This system relies on the fact that if all of the ions produce in the source of a mass spectrometer, by whatever technique, are given the same kinetic energy then the velocity of each will be inversely proportional to the square root of its mass. As a consequence, the time taken for them to traverse a field-free region (the flight tube of the mass spectrometer) will be related in the same way to the m/z of the ion. A complete mass spectrum is obtained simply by allowing sufficient time for all of the ions of interest to reach the detector. A schematic of the TOF is shown in Figure 2.7

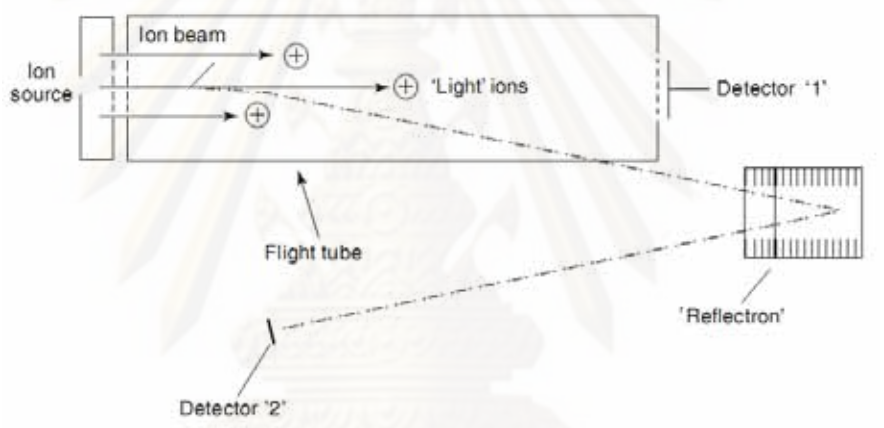


Figure 2.7 Diagram of a time-of-flight mass analyzer

2.2.3.4 Quadrupole Ion Trap

The quadrupole ion trap is shown in Figure 2.8. The dome-shaped end caps and toroidal (roughly doughnut-shaped) ring electrode bathe the interior cavity with RF and/or electric field. Although ions may be formed prior to their entering the ion trap, ionization is most often provided by electrons emitted from a filament imbedded in the upper end cap and focused through an aperture in the end-cap surface. A similar opening in the lower end cap allows ions to reach the detector.

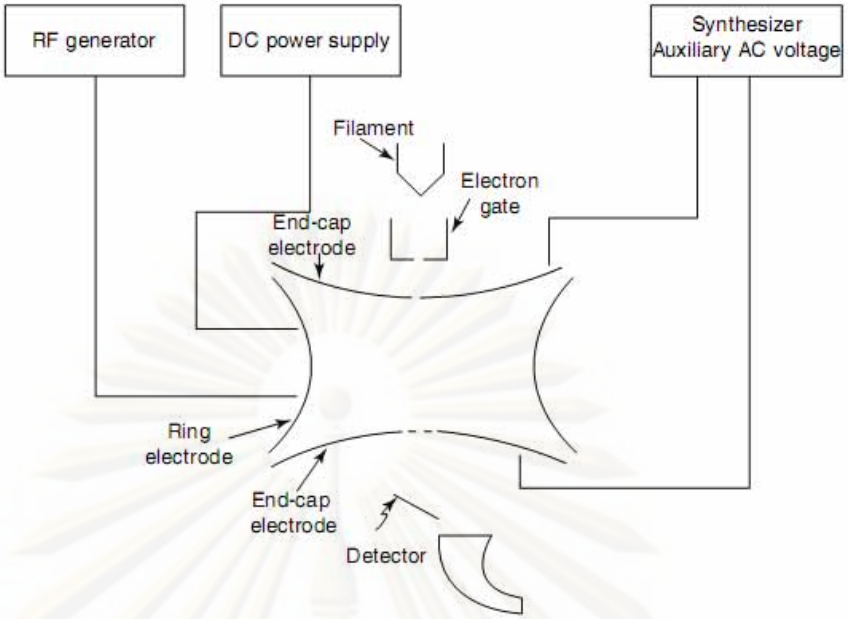


Figure 2.8 Diagram of a quadrupole ion trap

2.2.4 Detector

The mass spectrometry detector monitors the ion current, amplifies it and the signal is then transmitted to the data system where it is the signal recorded in the form of mass spectra. The type of detector is supplied to suit the type of analyzer. The mass spectrum was plotted between the intensities and the m/z value to present the information such as the molecular weight of each component and the relative abundance of the various components in the sample.

2.2.4.1 Faraday Cup

A faraday cup operates based on the change in charge on a metal plate, which results in a flow of electrons which creates an electric current. When an ion strikes the dynode surface of the faraday cup, which is connected to a resistor, several secondary electrons are induced, ejected and temporarily displaced. The resulting neutralization of the charge on the ions (temporary emission of electrons) leads to flow of current and provides a small amplification of signal.

2.2.4.2 Electron Multiplier

Electron multiplier is the most common type of detector. This detector consists of a layer of metal oxides (lead and tin oxide) on an anode glass tube, or coat on a channel with a glass construction. The principle of this detector is the amplification of electrons by producing secondary electrons when the ions from the analyzer hit a cascade of accelerated electrodes (dynode). These electrons are forced by a proper electric field to collide with the wall and these electrons act like ions, causing electrons to be emitted. A series of biased dynodes eject secondary electrons into the vacuum space. These secondary electrons travel down the channel and repeatedly collide with the next dynode to produce more secondary electrons. This process will continue until the resulting cloud of electrons exits the channel and was collected by the anode. Typical amplification or ion gain of an electron multiplier is 10^6 with a lifetime of 1 to 2 years due to surface contamination from incident ions or from poor vacuum.

The electron multiplier can also be made from continuous dynode material such as a channeltron, which is horn-shaped. It can improve the signal sensitivity.

2.2.4.3 Photomultiplier Tube

Photomultiplier tubes have been in use for a long time as detectors in radiation-based spectrometry. Magnification of the signal in a photomultiplier tube is based on the same principle as that governing the electron multiplier, except that the inner surface of the photomultiplier tube is sensitive to photons rather than to charged particles.

2.2.4.4 High-Energy Dynode Detector (HED)

The HED uses an electrostatic field prior to the electron multiplier. Once an ion passes through the field, it is accelerated to strike a conversion dynode, resulting in the emission of electrons. The secondary electrons are attached into the electron multiplier,

thus the producing the cascade of electrons. HED serve to increase ion energy and therefore the signal intensity, which results in greater sensitivity.

2.3 Liquid Chromatography-Mass Spectrometry (LC-MS) [33]

Mass spectrometers are typically not standalone instruments. Most often they are connected physically and electronically to a chromatography as well as a computer. The chromatograph separates mixtures and introduces the sample into the mass spectrometer. The mass spectrometer ionizes analyte molecules, then separates and detects the resulting ions. The computer system controls the operation of the chromatograph and the MS, and provides data manipulation and storage during and after data collection.

A combination of LC and MS offers the possibility to take advantage of both LC as a powerful and versatile separation technique and MS as a powerful and sensitive detection and identification technique. Fully the exploiting intrinsic properties of these two techniques results in an extremely powerful analytical tool. The initial objectives in developing LC-MS research are MS is a universal detector for LC, analysis of nonvolatile analytes, avoid analyte derivatization, MS affords a low detection limit, identification of the analytes and assessment of peak purity. In order to realize these objectives, a variety of LC-MS interfaces was developed.

2.3.1 Interface Technology [34]

The major problems were met in developing a combination of LC and MS are the large volume formation and the vacuum requirement. Several interfaces have been developed for solving this problem. However, the combination of two powerful analytical techniques is not easy; there are three major difficulties: the incompatibility of flow rate (from conventional LC column; 1 mL min^{-1} into the high vacuum MS),

the compatibility of solvent consumption by using a non-volatile mobile phase; salt and the ionization of non-volatile and/or thermally labile analytes.

Coupling methods are being developed to overcome these difficulties. This includes a moving-belt, a direct-liquid-introduction (DLI), a thermospray (TSP), a continuous flow fast atom bombardment (CF-FAB), an atmospheric-pressure chemical ionization (APCI), a particle-beam (PB) and an electrospray (ESI).

2.3.1.1 Moving-Belt Interface

The first interface to be made available commercially was the moving-belt interface, shown schematically in Figure 2.9

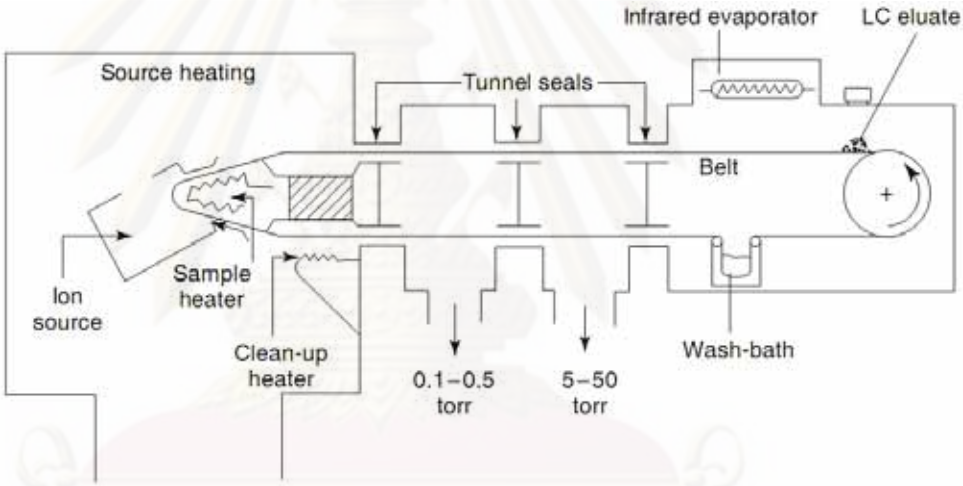


Figure 2.9 Schematic of a moving-belt LC-MS interface

The operation of the interface may be divided conveniently into four stages are as follows:

1) application of mobile phase and analyte(s) to a continuously moving belt
2) removal of the mobile phase by passage of the belt under an infrared heater
3) flash desorption/vaporization of the analyte into the source of the mass spectrometer

4) cleaning of the belt with a heater and/or a wash-bath to remove any involatile materials or excess sample prior to the application of further mobile phase and analyte(s) and a repeat of step 2-4

2.3.1.2 Direct-Liquid-Introduction (DLI) Interface

The direct-liquid-introduction interface is shown schematically in Figure 2.10. This system is effectively a probe, at the end of which is a pinhole of approximately 5 μm diameter, which abuts a desolvation chamber attached to the ionization source of the mass spectrometer. The eluate from an HPLC column is circulated through the probe and as it reaches the pinhole. The vacuum in the mass spectrometer draws a proportion of it into the desolvation chamber, and subsequently the source of the mass spectrometer, where analytes are ionized by solvent-mediated chemical ionization processes.

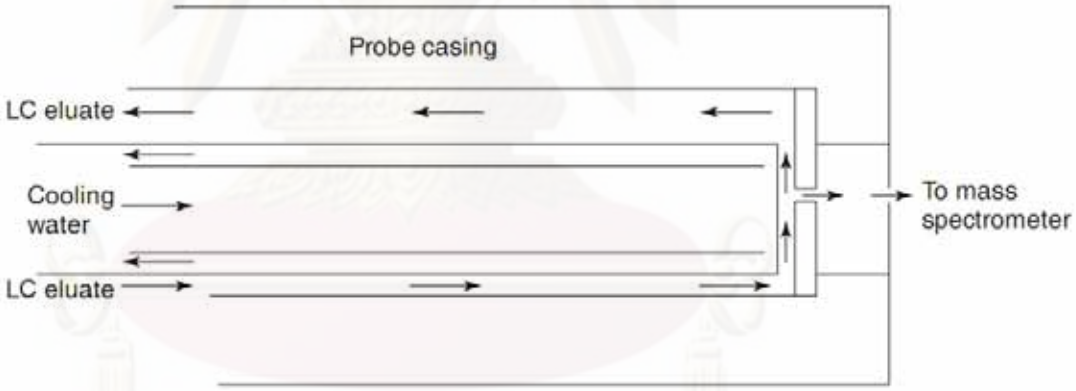


Figure 2.10 Schematic of a direct-liquid-introduction LC-MS interface

The maximum flow rate that can be accommodated while still allowing the mass spectrometer to operate is in the range of 10-20 μL min^{-1}. Typical flow rates used in conventional HPLC separations are between 500 and 1000 μL min^{-1} and therefore only between 1 and 4% of the column eluate, and therefore analyte(s), enter the mass spectrometer source. The sensitivity, or more accurately the lack of sensitivity, of the DLI interface is one of its major limitations.

2.3.1.3 Continuous-Flow Fast Atom Bombardment (CF-FAB) Interface

The CF-FAB interface or dynamic FAB was developed to study non-volatile and thermally labile compounds. This interface is a modification of the FAB ionization technique that allows continuous on-line refreshing of the HPLC eluate on the FAB target. The schematic of a continuous-flow FAB LC-MS interface as shown in Figure 2.11.

When linking HPLC to a FAB system, FAB matrix will be mixed with the LC effluent and transported to the probe tip. Then, fast-atoms bombard the sample and ions are sputtered out of the solution into the gas phase and drawn to the high vacuum of the MS. Excess mobile phase has to be removed from the probe tip by an absorbent pad situated adjacent to the area subjected to atoms or ions bombardment, thus the stability of the ion current can be a problem.

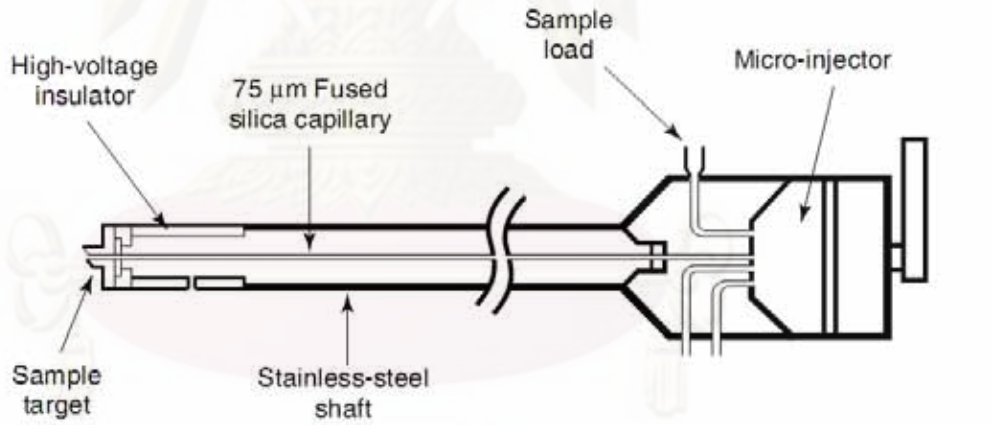


Figure 2.11 Schematic of a continuous-flow FAB LC-MS interface

2.3.1.4 Particle-Beam Interface

A general schematic of a particle-beam interface is shown in Figure 2.12. In each of the designs, droplets are formed from the HPLC eluent by passage through a nebulizer. The droplets are them carried, by a high-velocity gas stream, into a desolvation chamber. It is maintained virtually at ambient temperature by providing

sufficient heat to overcome the latent heat of vaporization of the mobile phase. While the volatile components vaporize, the less volatile components, such as the analyte, condense to form sub-micron diameter particles or liquid droplets, with the smaller the particle size the lower the temperature required for their subsequent vaporization prior to ionization.

The mixture of sample, vapour and carrier gas finally passes through a two-stage momentum (jet) separator. As the mixture emerges from the first orifice, those components with lower momentum, i.e. the carrier gas and eluent, tend to diverge and are pumped away, while those with higher momentum, e.g. the higher-molecular-weight analytes, pass into the second orifice which forms the inlet to a second similarly configured region where an identical process occurs. Transfer of the higher-momentum particles into the mass spectrometer therefore takes place. The region between the first set of orifices is maintained at between 2 and 10 torr, and that between the second set between 0.1 and 1 torr.

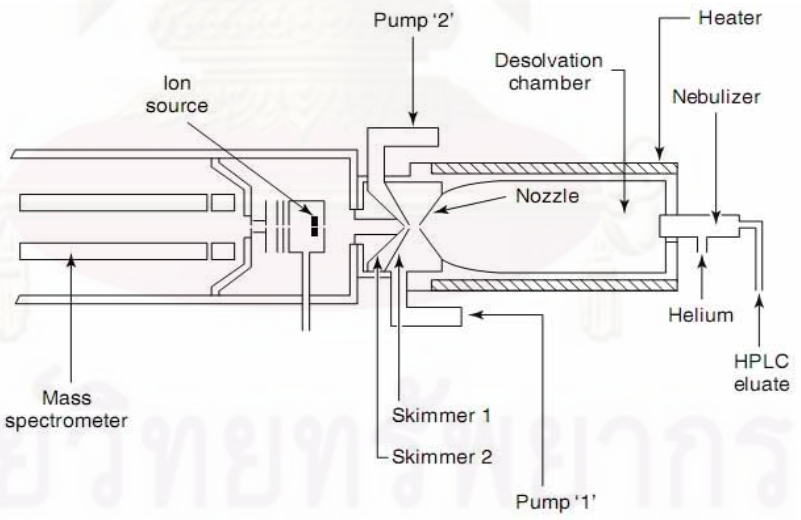


Figure 2.12 Schematic of a particle-beam LC-MS interface

2.3.1.5 Thermospray Interface

This interface may be considered to comprise the following four stages, can be described as follow:

1) the formation of droplets from the HPLC eluate
2) charging of these droplets
3) desolvation of the droplets
4) the formation of ions from the analyte

A thermospray interface system is shown schematically in Figure 2.13. The HPLC eluate flows through a heated capillary, which creates a spray of superheated mist. The temperature of this capillary is controlled to completely vaporize the liquid. The vapor so produced acts as a nebulizing gas which aids the breaking-up of the liquid stream into droplets. When the droplets travel through a heated region, the desolvation takes place and analyte ions are formed by means of ion-molecule reactions or ion-evaporation. Subsequently, these ions are directed through the entrance of MS.

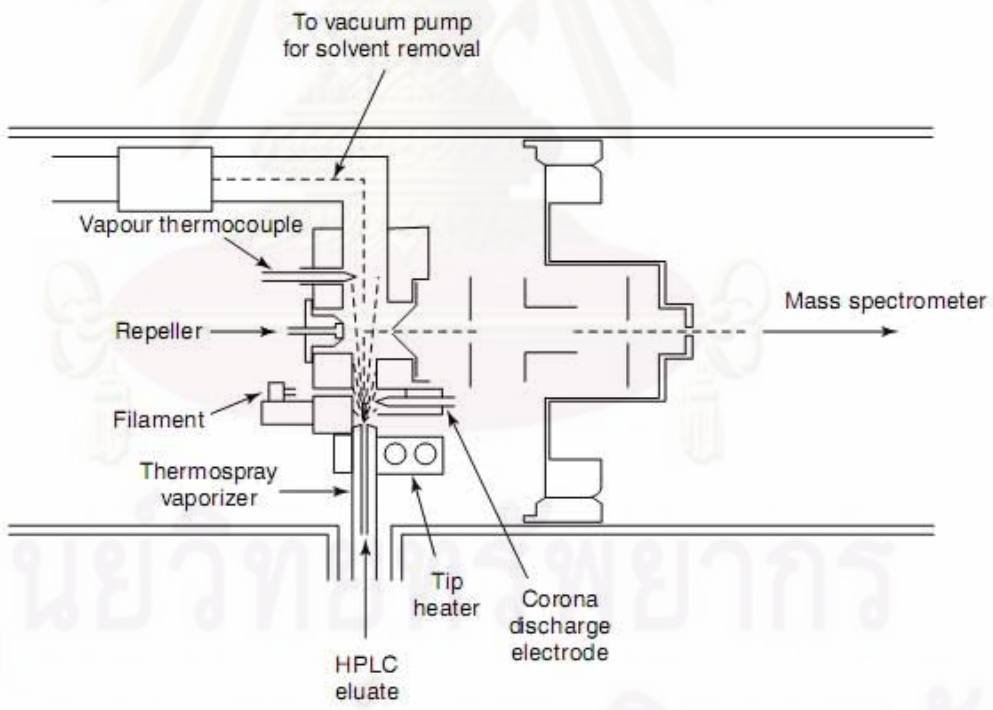


Figure 2.13 Schematic of a thermospray LC-MS interface

2.3.1.6 Electrospray Interface

HPLC is an effective technique for the separation of compounds of high molecular weight. There are the major problems with the use of MS for the study of this type of molecule and these have severely limited the application of LC-MS.

Electrospray is an ionization method that overcomes the problems. A liquid from HPLC, in which the analyte(s) of interest have been dissolved, is passed through a capillary, at atmospheric pressure, maintained at high voltage. The liquid stream breaks up with the formation of highly charged droplets which are desolvated as they pass through the atmospheric-pressure region of the source towards a counter electrode. Desolvation is assisted by a stream of a drying gas, usually nitrogen, being continually passed into the spraying region. Analyte ions are obtained from these droplets which then pass through two differentially pumped regions into the source of the mass spectrometer. A schematic of an electrospray interface system is shown in Figure 2.14.

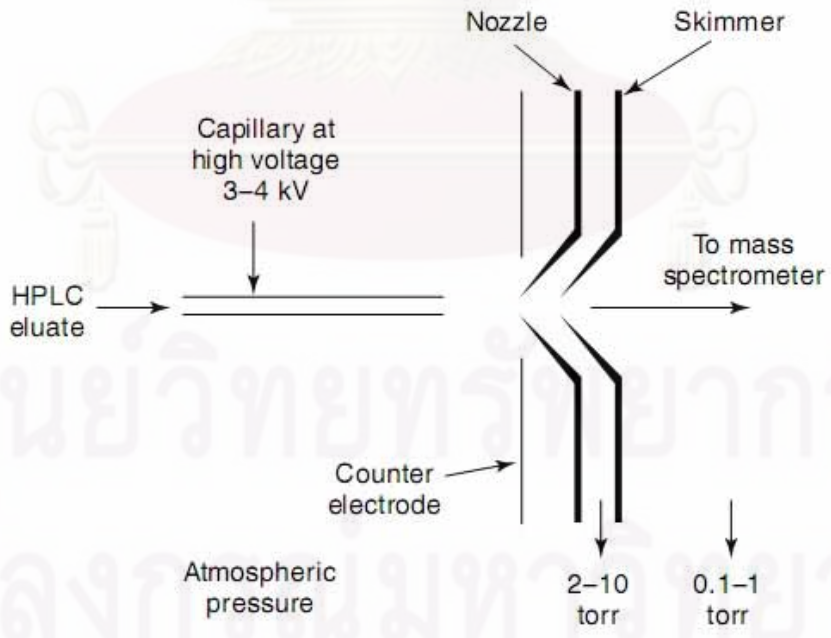


Figure 2.14 Schematic of an electrospray LC-MS interface

1. The mechanism of electrospray ionization

ESI usually takes place at atmospheric pressure and the electrospray process occurs by the same four steps as in thermospray. The ESI source comprises of two electrodes, the electrospray capillary and the counter electrode. Electrospray spectra are produced by passing a HPLC eluate through a high voltage metal capillary (typically 3-4 kV). The applied potential difference between both electrodes causes the liquid stream to break into fine threads that disintegrate to small droplets, so called electrospray. Under the influence of the applied electric field, ions of the same polarity migrate toward the liquid at the capillary tip. The liquid surface is drawn out of the capillary forming a "Taylor cone" as illustrated in Figure 2.15. When an excess of ions of one polarity at the surface of the liquid reaches the point that repulsive forces between charges are sufficient to overcome the surface tension of the liquid, one ion polarity is emitted from the capillary, so called "coulombic explosion" as shown in Figure 2.16. These droplets shrink by solvent evaporation followed by a number of coulombic explosions, leading to very small charged droplets. A series of such explosions then take place until a point is reached at which an appropriated amount of analyte ions dissolved in these droplets are produced. The ions are transferred through a set of focusing devices into the mass analyzer.

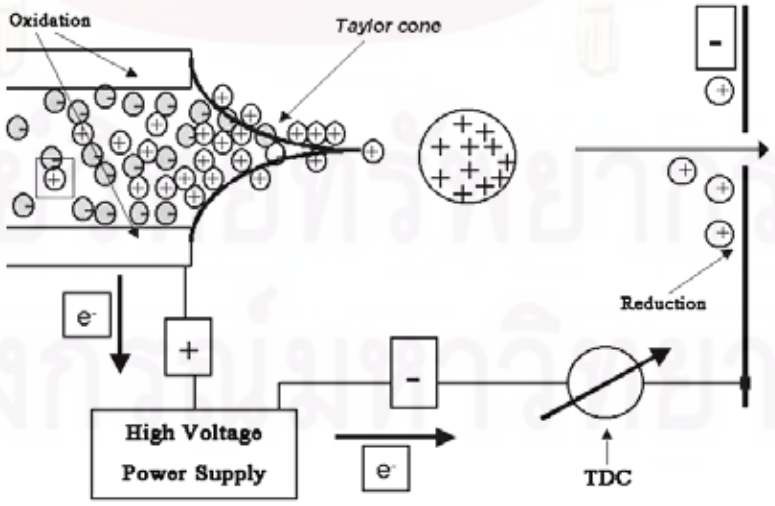


Figure 2.15 Schematic of a Taylor cone in ESI

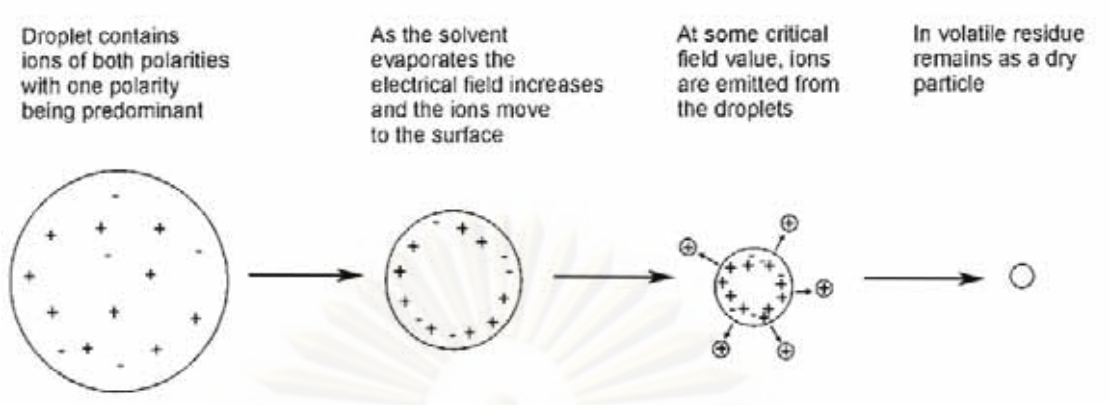


Figure 2.16 Schematic of coulombic explosion

The actual mechanism of ion production from the very small charged droplets can be explained in to models:

1) Ion-evaporation model, the droplets become smaller until a point is reached at which the surface charge is sufficiently high for direct ion evaporation into the gas phase.
2) Charge-residue model, repeated coulombic explosions take place until droplets of a single ion are formed. Evaporation of solvent continues until ions are formed in the vapour phase.

An important factor that will affect the production of ions by the electrospray process and the mass spectra is the HPLC characteristics. Because the ions are being generated directly from the mobile phase by electrospray, therefore, the identity and the concentration of any buffer and the flow rate of mobile phase are important considerations.

Desolvation of the droplets formed and ionization of analytes is favoured by the initial production of small droplets. Therefore, the mobile phase with high surface tension and high viscosity should be avoided. Morever, the small droplets are observed when a high buffer concentration is used.

The flow rate of HPLC affects the size of the droplets formed that resulting in the number of charged on each droplet. In general, the small diameter of the spraying capillary, the narrower droplet size distribution is obtained leading to higher efficiency in transferring of sample to the mass spectrometer.

Electrospray is most efficiency when operating at flow rate between 5-10 μL min^{-1}. Microbore columns are now available.

The use of electrospray in conjunction with pneumatically assisted nebulization at high liquid flow rates, also known as "Ion spray". Ion spray using a probe that provides a flow of nitrogen gas concentrically to the mobile phase stream, which aids the formation of droplets sprayed from the bulk liquid and will allow a flow rate of around 200 μL min^{-1} to be used.

The alternative is to employ a heated source inlet as shown in Figure 2.17. A heated capillary is located directly in line with the electrospray probe so that droplets produced by the electrospray process enter through this capillary to the lens system and mass spectrometer.

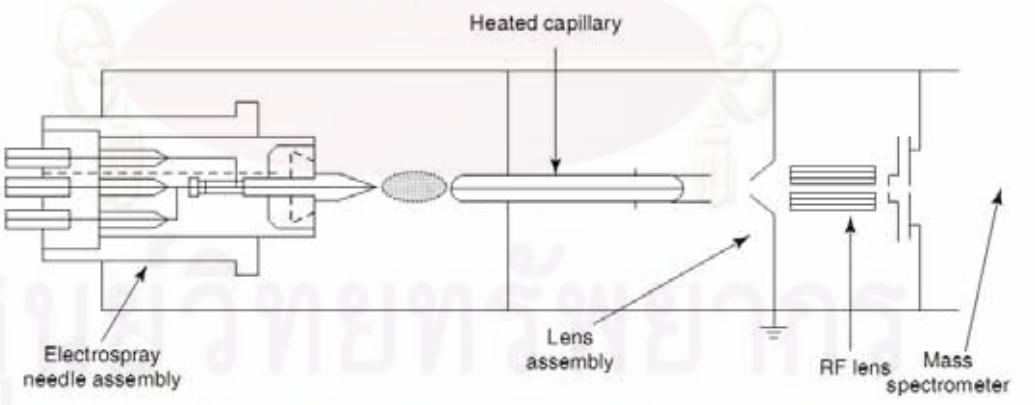


Figure 2.17 Schematics of a heated capillary electrospray LC-MS interface

2. Sample types

The ability to produce ions using electrospray ionization is dependent on the solution chemistry of the analyte. The electrospray system can be considered as an electrochemical cell, in positive ion mode, an oxidation reaction occurs at the capillary tip and a reduction reaction occurs at the counter electrode (the production of negative ions). For this reason, the compounds that can be studied are as follow:

1) ionic compounds that are intrinsically charged in solution
2) neutral/polar compounds that may be protonated (for positive-ion mass spectra) or deprotonated (for negative-ion mass spectra) under the solution conditions employed
3) non-polar compounds that undergo oxidation or reduction at the electrospray capillary tip

Moreover, the properties of the solvent, such as its viscosity, conductivity, surface tension and polarity all affect the electrospray process. However, a crucial important parameter that has a direct effect on the ionization is the pH of the solution. The production of positive ion is favored at acidic pH.

The electrospray process is susceptible to competition effects. All polar or ionic species in solution are not only derived from the analytes, but it derived from buffers or additives as well. The best analytical sensitivity will be obtained from a solution with a single analyte, thus competition is not possible, at low flow rate and with the narrowest diameter electrospray capillary. If excess electrolyte materials are present, competition of each species become present and the efficiency of analyte ionization depend upon the concentration of each species present and the relative efficiency of the conversion of each to the gas phase.

The electrospray does not provide only ions from molecular species, but it also consists of a number of fragment ions depending on molecular weight of the analyte. Unlike most ionization techniques, if the analyte contains more than one site of protonation or deprotonation, multiple charged ions are usually observed. Thus, the most striking application is that high molecular weight, thermalabile, polar biomolecules such as peptides, proteins, oligonucleotides, etc. The production of multiple charged ions using electrospray ionization effectively extends the mass range of the mass spectrometer by a factor directly related to the number of charges attached to the analyte molecule.

3. Structural information from electrospray ionization

The great strength of MS is its ability to generate structural information from the analyte. The electrospray is the "softest" ionization process; the transfer of ions to the gas phase is a low energy process that does not disrupt their structures.

Advantages

- Ionization occurs directly from solution and consequently allows ionic and thermally labile compounds to be studied.
- Mobile phase flow rates from nL min^{-1} to in excess of 1 mL min^{-1} can be used with appropriate hardware, thus allowing conventional and microbore columns to be employed.
- Electrospray ionization produces multiply charged ions of the intact solute molecule. This allows the study of molecules with molecular weights well outside its normal range.
- For high molecular weight materials, an electrospray spectrum provides a number of independent molecular weight determinations from a single spectrum and thus increased precision.

Disadvantages

- Electrospray is not applicable to non-polar or low polarity compounds.
- The mass spectrum produced from an analyte, in terms of the m/z range of the ions observed and their relative intensities, depends upon the experimental conditions.
- Suppression effects may be observed and the direct analysis of mixtures is not always possible.
- Electrospray is a soft ionization method producing intact molecular species and structural information from cone-voltage fragmentation but these spectra are not always easily interpretable.

2.3.1.7 Atmospheric Pressure Chemical Ionization (APCI) Interface

The APCI interface (Figure 2.18) is another technique which transports and ionizes samples at atmospheric pressure region; a corona discharge to mass spectrometer. The stream of liquid emerging from an HPLC column dispersed into small droplets by a coaxial nebulizing gas, its pass through a heated nebulizer (350-500ºC) where the droplets are both generated and desolvated. The spray formed then passes through a heated region where the vopor is dried. The neutral species produced are then ionized by a corona discharge electrode (3-6 kV). The electric field at the tip of electrode ionizes the gas surrounding it and these solvent ions interact with the analytes in the gas phase at atmospheric pressure. The analyte ions formed are extracted into the MS with a curtain of drying gas to reduce the background cluster ions from the solvent.

The reagent species in the positive ion mode are protonated solvent ions or proton transfer. While deprotonated solvent ions; O_2^-, its hydrates and clusters may be observed in negative ion mode. APCI is a soft ionization leading to molecular species with little or no fragmentation, $[M+H]^+$ and $[M-H]^-$. However, APCI leads to the formation of ion cluster involving solvent molecules.

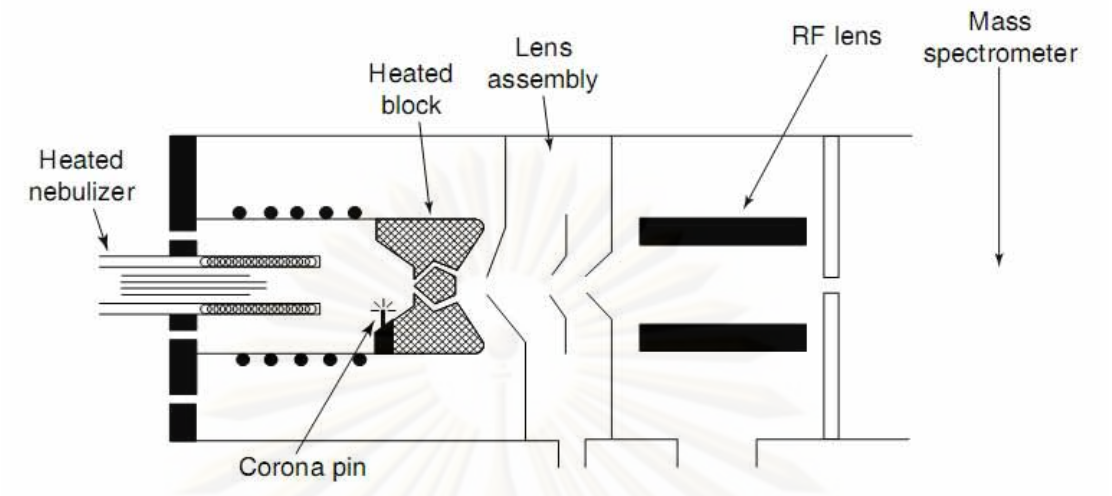


Figure 2.18 Schematic of an APCI LC-MS interface

2.4 Sample Preparation [27]

A major problem in chromatography is the analysis of analytes in complicated sample matrices such as food, biological matrices, and water. Some matrices contain interfering substances which can affect chromatographic performance by masking the peaks of interest. To eliminate these problems, sample must be treated before injection. Therefore, the quality of the result often depends on sample preparation. This preliminary step can have a more important influence on the end result than the measurement itself or the precision of the instrument used. Sample preparation, which follows the so-called sampling procedure, can often be tedious, delicate and time-consuming. Nonetheless, it has become an active area of study that benefits from the recent progress in chemistry and robotics. Currently used instruments that allow fast and selective measurements on very small amounts of sample have encouraged the development of new, rapid sample preparation methods.

2.4.1 Solid-Phase Extraction (SPE) [13, 35]

Solid-phase extraction is a method of sample preparation that concentrates and purifies analytes from solution by sorption onto a disposable solid-phase cartridge, followed by elution of the analyte with a solvent appropriate for instrumental analysis. The disadvantages with liquid-liquid extraction including the use of large volumes of organic solvent, cumbersome glassware, creates emulsion with aqueous samples that are difficult to extract and liquid-liquid extraction is not easily automated. As a result of these difficulties, SPE techniques have been developed to replace the traditional liquid-liquid extraction method. SPE is a simple method and safe to use, provides high recoveries of analytes, purified extracts, ease of automation, compatibility with chromatographic analysis, and reduction in the consumption of organic solvents. The formats of SPE are shown in Figure 2.19

(a) (b)

Figure 2.19 The formats of SPE: (a) cartridge, (b) syringe barrel

2.4.1.1 Mode of Solid-Phase Extraction

2.4.1.1.1 Reversed Phase

The interested analytes are usually moderate to non-polar. The hydrophobic interactions are non-polar interactions, van der Waals or dispersion forces. The secondary interaction between silica-based and analytes present. The endcapping is useful to reduce these interactions. However, secondary interaction may be useful in the extraction of highly polar compounds or matrices. Reversed-phase sorbents are packed with more hydrophobic material. The aqueous sample is commonly analyzed

by reversed phase SPE. The reversed phase sorbents are non-polar functionalized such as C-18, C-8, C-2, cyclohexyl and phenyl functional groups and bonded to the silica or polymeric sorbent.

2.4.1.1.2 Normal Phase

Normal phase SPE refers to the sorption of an analyte by a polar surface. It is a standard type of separation. The mechanism is polar interaction such as hydrogen bonding, dipole-dipole interaction, π-π interaction and induced dipole-dipole interaction. Polar-functionalized bonded silica (LC-CN, LC-NH_2, and LC-Alumina) are typically used in normal phase conditions. For example, silica base is extremely hydrophilic. This material adsorbs polar compounds from non-polar matrix and elutes compounds with a more polar organic solvent than the original sample matrix.

2.4.1.1.3 Ion Exchange

Ion exchange can be used for compounds that are changed in solution. The hydrophobic ion exchange is capable of exchanging both a cations and anions with free cation or anion functional groups. Strong cation-exchange sorbent consists of interaction sites like sulfonic acid groups and weak cation-exchange sites like carboxylic acidic groups. Strong anion-exchange sorbents would be quaternary amine, primary, and secondary. Tertiary amines refer to weak ion exchange. The secondary non-polar interaction with non-polar portions can be provided. A decrease in the balance of pH, ionic strength, and organic content may be necessary for elution of interested analyte from these sorbent. The strong sites are always shown as an exchange site at any pH. Weak sites present are only at pH levels greater or less than the pKa. It has found many applications, for example, it is used for natural products, protein, cellulose, and trace enrichment.

2.4.1.1.4 Mixed Mode

The deliberate use of two different function groups on the same sorbent is called “mixed-mode SPE”. This sorbents are useful for complex samples that differ in polarity and ionization. Mixed-mode sorbent contains co-bonded ion exchange and alkyl group cartridge. Two different functional groups eliminate the complex sample matrix. The initial hydrophobic interaction is a function of the chain length, with shorter chain (C-4) being retained less than longer chains (C-18).

2.4.1.2 Step of Solid-Phase Extraction

There are four common steps of SPE process, which comprised of conditioning, loading, washing, and eluting.

Conditioning step is the first of the solid-phase sorbent. This simply means that a solvent is passed through the sorbent to wet the packing material and to solvate the functional groups of the sorbent. Furthermore, the air present in the column is removed and the void spaces are filled with solvent. Typically the conditioning solvent is methanol, which is then followed by water or an aqueous buffer. The methanol followed by water or buffer activates the column in order for the sorption mechanism to work properly for aqueous samples. Care must be taken not allow the bonded-silica packing or the polymeric sorbent to go dry. In fact, if the sorbent dries for more than several minutes under vacuum, the sorbent must be reconditioned. If it is not reconditioned, the mechanism of sorption will not work effectively and recoveries will be poor for the analyte.

In the next step, the sample and analyte are applied to the column. This is the retention or loading step. Many sample cases are in solid form, therefore, sampling needs to be homogenized and dissolved in an appropriate solvent before loading. Sample sizes must be scaled to suit the capacity of the column. Also, the flow rate of

a sample through the column should be controlled. Morever, the column should not be allowed to dry out. Some matrix that has similar properties as anayltes may retain the sorbent.

In the washing step, this step is to rinse the column of interferences and to retain the analyte. This rinse will remove the sample matrix from the interstitial spaces of the column, while retaining the analyte.

In the last step, the analyte is eluted from the sorbent with an appropriate solvent that is specifically chosen to disrupt the analyte-sorbent interaction, resulting in elution of the analyte.

2.4.2 Integration of SPE with the Analytical Technique

The illustration of how the two techniques of SPE and LC blend together is provided by column switching. It is called an on-line technique. The benefits of the on-line SPE, notably analysis of the entire eluent and 0% anayte loss from evaporative or other effects, makes it possible to analyze much smaller samples than are required for conventional off-line SPE extraction.

The SPE columns are back-flushed (eluted in the opposite direction to the flow of the sample at the loading step) by switching valve, this can reduce the volume to be loaded onto the analytical column and minimize band broadening. Furthermore, it can also enhance recovery and improve precision. The basic instrument of on-line SPE is shown in Figure 2.20.

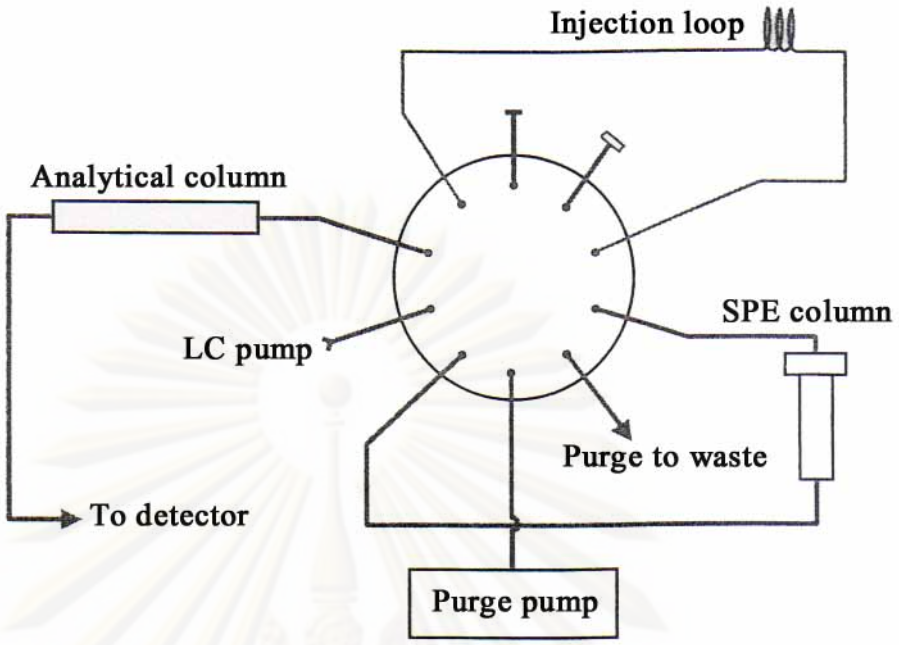


Figure 2.20 The basic instrument of on-line SPE

CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1 High Performance Liquid Chromatography (HPLC): A module 1100™ consists of automatic degasser, quaternary pump, auto sampler and column thermostat compartment with a 6-port 2-position switching valve (Agilent Technologies, U.S.A)

3.1.2 Mass spectrometry detector (MSD-Trap-XCT): A module 1100™ with electrospray ionization interface (Agilent Technologies, U.S.A)

3.1.3 LC/MS grade nitrogen generation system: Model 75-72-K727 (Agilent Technologies, U.S.A.)

3.1.4 Air pump for nitrogen generator: Model NCS-20 (PUMA Industries Co., Ltd.)

3.1.5 Refrigeration compressed air dryer: Model J2K-08G (JIA MENG ELECTRIC Co., Ltd., TAIWAN)

3.1.6 Helium gas: 99.999% purity (World Siam Group, Thailand)

3.1.7 Ultrapure water system: model ULTRA ANALYTIC (Elga Labwater, U.K.)

3.1.8 HPLC column: Fortis C_{18}, 150 x 2.1 mm I.D., 3 µm (Fortis, U.K.)

3.1.9 SPE column: direct-connect refillable column, 22.50 mm x 1.85 mm id. (Alltech, U.S.A.)

3.1.10 Mobile phase filter set: 300 mL glass reservoir, 1 L flask, glass base with tube cap, and metal spring clip (Millipore, U.S.A.)

3.1.11 Autopipette 0.1-10, 10-100, 100-1,000 µL and tips (Eppendorf, Germany)

3.1.12 Analytical balance: model ED 2248 (Satorius, Germany)

3.1.13 Ultrasonic bath: model 950D (CHEST ULTRASONICS, Malaysia)

3.1.14 Nylon filter membrane: 0.2 µm, 47 mm (Vertical, Thailand)

3.1.15 Cellulose acetate filter membrane: 0.2 µm, 47 mm (Vertical, Thailand)

3.1.16 Nylon syringe filter: 0.2 µm, 13 mm (Vertical, Thailand)

3.1.17 HPLC amber vial 2 mL with PTFE caps (Agilent Technologies, U.S.A.)

3.1.18 Pipette 1, 2, 3, 4, 5 and 10 mL

3.1.19 Beaker 10, 25, 50, 100, 250, 500 and 1,000 mL

3.1.20 Volumetric flask 10, 25, 50 and 100 mL

3.1.21 Graduated cylinders 1,000 mL

3.1.22 SPE cartridge

- VertiPak™ C_{18}, 50 mg, 3 mL (Vertical, Thailand)
- VertiPak™ HCP-SC, 60 mg, 3 mL (Vertical, Thailand)
- VertiPak™ silica, 50 mg, 3 mL (Vertical, Thailand)

3.2 Chemicals and Reagents

3.2.1 Standard Compounds

3.2.1.1 Sudan I: CAS no. 842-07-9, purity 97% (Sigma-Aldrich)

3.2.1.2 Sudan II: CAS no. 3118-97-6, purity 90% (Sigma-Aldrich)

3.2.1.3 Sudan III: CAS no. 85-86-9, purity 90% (Sigma-Aldrich)

3.2.1.4 Sudan IV: CAS no. 85-83-6, purity 90% (Sigma-Aldrich)

3.2.2 Organic Solvent

3.2.2.1 Acetonitrile: HPLC grade (Fisher)

3.2.2.2 Methanol: HPLC grade (Fisher)

3.2.2.3 Ethyl acetate: AR grade (Fisher)

3.2.2.4 Acetone: AR grade (Fisher)

3.2.3 Other Chemicals

3.2.3.1 Formic acid 99% (Fisher)

3.2.3.2 Glacial acetic acid (Fisher)

3.3 Preparation of Standard Solutions

3.3.1 The Standard Stock Solutions of Sudan I-IV

Individual stock standard solutions containing 100 μg mL^{-1} of Sudan I-IV were prepared by weighing 0.0100 g of each standard and dissolving with acetonitrile: water (90:10, v/v) in 100.00 mL volumetric flasks, except Sudan III and IV were dissolved in a small amount of ethyl acetate and subsequently diluting them with acetonitrile: water (90:10, v/v). Then, each standard stock solution was transferred to an amber glass bottle with Teflon screw cap and stored at 4 ºC in the refrigerator for maximum period of 2 months.

3.3.2 The Diluted Standard Solutions of Sudan I-IV

Individual standard solution containing 1 μg mL^{-1} of Sudan I-IV was prepared by diluting 1 mL of each standard stock solution (section 3.3.1) to 100.00 mL with acetonitrile in volumetric flask. Then, each diluted standard stock solution was transferred to an amber glass bottle with Teflon screw cap and stored at 4 ºC in the refrigerator for maximum period of 5 days.

3.3.3 The Standard Mixture Solutions of Sudan I-IV

Working standard mixture solutions of Sudan I-IV used for preparation of calibration curves were freshly prepared by suitable dilution of the stock standard solutions with acetonitrile.

3.4 Preparation of Solution for HPLC-MS

3.4.1 Mobile Phase

The mobile phase for HPLC condition consisted of individual 0.1% formic acid in acetonitrile and 0.1% formic acid in water. The 0.1% formic acid in acetonitrile was prepared by pipetting 1 mL of formic acid into a 1,000 mL volumetric flask, diluting to

the mark with acetonitrile and then filtering through 0.2 μm nylon membrane filter into a glass bottle. The 0.1% formic acid in water was prepared by pipetting 1 mL of formic acid into a 1,000 mL volumetric flask, diluting to the mark with water and then filtering through 0.2 μm cellulose acetate membrane filter into a glass bottle. Both bottles were degassed in ultrasonic bath.

3.5 Procedure

3.5.1 The Optimum Instrumental Analysis Conditions

In this research, the LC-MS instrument was supported from Agilent Technologies; U.S.A. Liquid chromatograph is an Agilent 1100^{TM} series, with a solvent degassing unit, a quaternary pump, an automatic sample injection, and column thermostat compartment with a 6-port 2-position switching valve. Mass spectrometry detector is an Agilent 1100^{TM} XCT equipped with electrospray ionization source, quadrupole ion trap, and electron multiplier detector.

3.5.1.1 Optimization of ESI Parameters

Mass spectrometry detector using electrospray ionization as interface was optimized for the analysis of Sudan I-IV. The electrospray ionization parameters such as nebulizer pressure, drying gas flow rate and drying gas temperature under positive ion mode were studied for high sensitivity of detection. Only solution of 0.5 μg mL^{-1} standard Sudan III was preliminary used to optimize ESI conditions. Nebulizer pressure in a range 30-50 psi was firstly studied while other parameters were fixed as shown in Table 3.1. The solution was injected into LC-ESI-MS in full scan mode (100-400 m/z) by using 0.1% formic acid in water and 0.1% formic acid in acetonitrile with gradient elution as mobile phase and a flow rate of 0.4 mL min^{-1}. After obtaining the optimum nebulizer pressure, the other ESI parameters, drying gas flow rate and drying gas temperature were studied as follows.

Table 3.1 Optimization of ESI conditions

ESI parameter	Optimization of ESI condition		
	Nebulizer pressure (psi)	Drying gas flow rate (L min^{-1})	Drying gas temperature (ºC)
Nebulizer pressure	30-50	10	325
Drying gas flow rate	optimum	8-10	325
Drying gas temperature	optimum	optimum	300-350

3.5.1.2 Optimization of Quadrupole Ion Trap Mass Analyzer

In quadrupole ion trap mass analyzer, the parameter influencing the sensitivity of the analyte solutions, ion charge control (ICC) was studied. The ICC target controls the number of ions stored in the ion trap on a scan-to-scan basis. If there are too many ions stored in the ion trap, they interfere with each other's motions and are ejected from the ion trap at the wrong instant during the scan. The ICC target in a range of 30,000-120,000 ions was studied while other parameters were fixed.

3.5.1.3 Optimization of Mobile Phase Type for LC-ESI-MS System

The optimization of mobile phase type for separation a mixture of 4 standard solutions was developed by use two acids and two solvent. To achieve the optimum HPLC conditions, the preliminary chromatographic conditions used in this study are shown in Table 3.2. During this test 0.5 µg mL^{-1} of 4 standard solutions was injected and mobile phase was composed of 50% constituent in A and 50% B. The MS conditions were investigated under same static conditions that are presented in Table 3.3.

Table 3.2 Chromatographic conditions used for the HPLC optimization

Parameters	Conditions
Analytical column	Fortis C_{18} (2.1x150 mm, i.d. 3.0 µm)
Mobile phase*	1. A = 0.1% formic acid in water B = MeOH 2. A = 0.1% formic acid in water B = ACN 3. A = 0.1% acetic acid in water B = MeOH 4. A = 0.1% acetic acid in water B = ACN 5. A = 0.1% formic acid in water B = 0.1% formic acid in MeOH 6. A = 0.1% formic acid in water B = 0.1% formic acid in ACN
Flow rate	0.4 mL min^{-1}
Analyte concentration	0.5 µg mL^{-1}
Injection volume	5 µL
Column temperature	40 ºC
Detector	Mass spectrometer (ESI/positive)

* refer to the varied parameters

Table 3.3 MS static parameters

Parameters	MS condition
Ionization source	ESI/positive
Nebulizer pressure	45 psi
Drying gas flow	10.0 L min^{-1}
Drying gas temperature	325 ºC
ICC target	100,000 ions
Scan mass range	100-400 *m/z*

3.5.1.4 Optimization of Mobile Phase Composition of LC System

The gradient program of mobile phase from LC optimized condition (0.1% formic acid in water and 0.1% formic acid in ACN) was developed by varying percentage of mobile phase type. A flow rate of 0.4 mL min^{-1} with an oven temperature 40 ºC was applied. A standard mixture solution at 0.5 µg mL^{-1} was injected at 5 µL. The gradient program was adjusted until it reached a baseline resolution for all Sudan dyes. The operated condition followed as Table 3.4.

Table 3.4 The LC-MS operating conditions for the separation of four Sudan dyes

LC-MS parameters	Condition
Analytical column	Fortis C_{18} (2.1x150 mm, i.d. 3.0 μm)
Mobile phase	A: 0.1% formic acid in water B: 0.1% formic acid in ACN
Flow rate	0.4 mL min^{-1}
Injection volume	5 μL
Column temperature	40 ºC
Ionization source	ESI/positive
Nebulizer pressure	45 psi
Drying gas flow	10.0 L min^{-1}
Drying gas temperature	325 ºC
ICC target	100,000 ions
Scan mass range	100-400 *m/z*

3.5.2 Sample Preparation

3.5.2.1 Comparison of Extracting Solvents

In this work, three extracting solvents (acetonitrile, acetone and ethyl acetate) were used to extract Sudan I-IV from spiked chili powder sample at a level of 5 ppm. The extracted solutions were injected into LC-MS and analyzed under the optimum LC-ESI-MS conditions. The results of each extraction solvent were compared and reported as percent recoveries of each analyte.

3.5.2.2 Comparison of SPE Sorbent

The comparison between the C_{18}, silica and HCP-SC cartridges was investigated. The SPE cartridge was conditioned with 5 mL methanol and 5 mL of water. Then, 1 mL of a 0.5 μg mL^{-1} Sudan I-IV standard mixture solution, prepared in acetone was loaded onto the cartridge. Next, 5 mL of MeOH: water (50:50, v/v) was used to wash the column. Finally the Sudan dyes were eluted from the SPE cartridge with 0.1% formic acid in acetonitrile. The eluted fraction was filtered with 0.2 μm nylon syringe filter and injected into LC-MS system under the optimum LC-ESI-MS conditions. The results of each SPE sorbent were compared and reported as percent recoveries of each analyte.

3.5.2.3 The Effect of the Ratio of Washing Solvent

The effect of SPE washing solvent to provide the cleaned extract was studied to determine the optimal SPE conditions. In this work, methanol and water were monitored under a gradient of 0% methanol to 100% methanol while other parameters for SPE were fixed. C_{18} SPE cartridge was used and the experimental SPE procedures were the same as described in section 3.5.2.2. The percent recoveries of each analyte were used for comparison.

3.5.3 On-line SPE-LC-MS

The instrument set up is shown in Figure 3.1. The HPLC-MS system consists of a binary pump system with separately operated pump A and B with degasser, an autosampler, a 6-port 2-position switching valve and MSD detector.

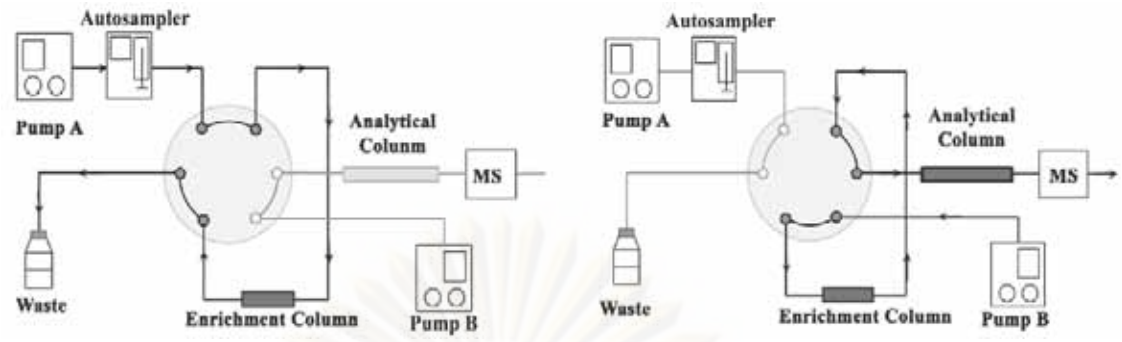


Figure 3.1 Schematic view of the on-line SPE-LC-MS system (left side: sample enrichment and rinsing step, right side: analyte elution and transfer to analytical column and analysis by MS)

The analytical column was a reversed phase C_{18} column (Fortis C_{18}, 150 mm x 2.1 mm, i.d. 3 µm), and the column temperature was maintained at 40 ºC throughout the experiments. The SPE cartridge used for an on-line enrichment column was contained with C_{18} sorbent (15-40 µm particle size).

3.5.3.1 Loading Volume Dependence

The influence of loading volume on the on-line SPE was investigated to determine by the optimal LC-MS conditions. In this work, 0.05 µg mL^{-1} of standard mixture Sudan I-IV was injected by loading volumes of 5, 10, 20, 50 and 100 µL while other parameters for on-line SPE were fixed.

3.5.3.2 Switching Time Dependence

The effect of the switching time on the on-line SPE of Sudan I-IV was studied to determine under the optimal LC-MS conditions. The standard mixture solution of Sudan I-IV 0.05 µg mL^{-1} was injected by optimal loading volume. The switching time was varied at 5, 15, 30, 45 second and 1 minute and other parameters for on-line SPE were fixed.

3.6 Method Validation

The purpose of method validation is to study the method performance parameter and demonstrate a particular method for quantitative measurement of analytes in matrix (chili powder). There were many parameters to study such as standard calibration curves and linear range, matrix effect, LOD, LOQ, accuracy and precision.

The method performance parameters were studied under the optimization conditions and on-line SPE-LC-MS procedures as follows:

In Figure 3.1, for enrichment, pump A supplied eluent A (MeOH/water, 50/50 v/v) and is required for sample introduction and rinsing step at a flow rate 1.0 mL min^{-1}. 20 µL of sample supernatant was injected onto the enrichment column. When loading was complete, the valve was switched as shown in Figure 3.1 (right side) and pump B began the solvent composition program at flow rate 0.4 mL min^{-1}, back flushing the analytes to the analytical column and mass spectrometer. After the analytes were eluted, the valve was returned to the load position (left side in Figure 3.1) and initial solvent conditions for re-equilibration in the next sample began. The solvent composition program of pump B and the switching valve program are shown in the Table 3.5.

Table 3.5 The HPLC gradient program of pump B and valve position

Time (min.)	Switching valve position	Pump B	
		%A	%B
0-0.5	Enrichment column	8	92
0.5-4	Analytical column	2	98
4-5	Analytical column	0	100
5-7	Analytical column	0	100
7-8	Analytical column	8	92
8-12	Enrichment column	8	92

A: 0.1% formic acid in H_2O, B: 0.1% formic acid in acetonitrile

The mass spectrometer was operated in positive ion mode. The ESI interface conditions for all target analytes were as follow: drying gas temperature 325 ºC, drying gas flow rate 10 L min^{-1} and nebulizer pressure of 45 psi. The MS analysis was divided into four segments, each containing one of the analytes, and used manual MS^2 mode.

3.6.1 The Study of Standard Calibration Curves and Linear Range

The experimental procedures to study the calibration curves of standard Sudan I-IV can be described as follows:

1. The concentration of standards mixture in acetone solution: 0.005, 0.01, 0.02, 0.04, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 4.0, 8.0, 10.0, and 20.0 µg mL^{-1} were injected respectively into on-line SPE-LC-MS under the optimum conditions.
2. The relationship between concentrations and peak area of each compound were plotted.
3. The results of this study were reported in form of slope, intercept, and coefficient of determination (R^2) values of each compound.

3.6.2 The Study of Matrix Effect

The matrix effect was studied by the comparison between standard mixtures (acetone used as a diluting solvent) and matrix standard mixtures (chili powder extract used as a diluting solvent). The experimental procedures to study the matrix effect can be described as follows:

1. The mixture of standard Sudan dyes solution at concentration level 0.005, 0.01, 0.02, 0.04, 0.1, 0.2, 0.4, 0.8 and 1.0 µg mL^{-1} except standard Sudan III solution at concentration level 0.01, 0.04, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0 and 4.0 µg mL^{-1} were prepared using the blank chili powder extract as diluting solvent instead of acetone.

2. Each concentration level was injected respectively into on-line SPE-LC-MS under the optimum conditions and analyzed in triplicate.
3. The relationship between concentrations and peak area of each compound were plotted.
4. The results of this study were reported in form of slope, intercept, and coefficient of determination (R^2) values of each compound.
5. A paired t-test at 95% confidence level was used to study the standard calibration curve and matrix calibration curve.

3.6.3 The Study of Limit of Detection (LOD)

The limit of detection was determined by analyzing samples containing low concentration of analytes that provided peaks at signal-to-noise ratio equal to 3. The LOD value was obtained from 10 replicate analyses. The experimental procedure can be described as follows:

1. 1 g of blank chili powder sample was extracted by acetone and then adjusted to 10 mL with acetone. The sample solution was filtered using 0.2 μm nylon syringe filter.
2. The blank chili powder sample solution was analyzed under the optimum on-line SPE-LC-MS conditions. The peak signals of each compound were measured from the chromatograms.
3. The LOD of each compound was obtained from the concentration that gave peak height at 3 times over the baseline.

3.6.4 The Study of Limit of Quantitation (LOQ)

The limit of quantitation was determined by analyzing a sample containing low concentration of analytes that provided peaks at signal-to-noise ratio equal to 10. The analyses were repeated 10 times and the average result was reported. The experimental procedure can be described as follows:

1. 1 g of blank chili powder sample was extracted by acetone and then adjusted to 10 mL with acetone. The sample solution was filtered using 0.2 μm nylon syringe filter.
2. The blank chili powder sample solution was analyzed under the optimum on-line SPE-LC-MS conditions. The peak signals of each compound were measured from the chromatograms.
3. The LOQ of each compound was obtained from the concentration that gave peak height at 10 times over the baseline.

3.6.5 The Study of Method Precision

In this study, precision of the method was divided into two categories according to the AOAC Peer Verified Methods Program: repeatability or intra-day precision, and intermediate precision (inter-day precision). Intra-day precision was obtained by repeatedly analyzing aliquots of a homogeneous sample in the same day. Intermediate precision referred to the results from the same laboratory and equipment, but performed on different days.

The study of method precision was carried out by extraction of spiked chili powder sample at three concentration levels, LOQ, medium and high, as shown in Table 3.6.

For intra-day precision study, the extraction at each concentration level was consecutively repeated five times within the same day and the extraction at each concentration level was repeated on two different days for intermediate precision study.

Table 3.6 The concentration of standard solutions at LOQ, medium and high levels

No.	Compounds	Concentration level (ppm)		
		LOQ	Medium	High
1	Sudan I	0.05	1.0	10.0
2	Sudan II	0.05	1.0	10.0
3	Sudan III	0.1	4.0	40.0
4	Sudan IV	0.05	1.0	10.0

The experimental procedures for the study of method precision can be described as follows:

3.6.5.1 Intra-Day Precision

1. At LOQ level, five spiked samples were prepared by pipetting a standard mixture solution at LOQ concentration level into 1.0 g of chili powder sample and then were extracted and filtered before injected into on-line SPE-LC-MS under the optimum conditions.
2. The final concentration of each compound was calculated using the linear equation and showed the recovery as a percentage of the original spiked concentration.
3. The intra-day precision of this method was calculated and reported in form of percent relative standard deviation (%RSD) of each compound, using the following formula:

$$\%RSD = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

4. At medium concentration level, five spiked samples were prepared by spiking a standard mixture solution at medium concentration level to 1.0 g of chili powder sample.
5. The extraction procedures and calculation were similar to the step 1-3.
6. At high concentration level, five spiked samples were prepared by spiking a standard mixture solution at high concentration level to 1.0 g of chili powder sample.
7. The extraction procedures and calculation were similar to the step 1-3.

3.6.5.2 Intermediate Precision (Inter-day Precision)

Similar to the procedure described in section 3.6.5.1, the extraction at LOQ, medium, and high concentration levels were repeated on another different day. The summary of the results are reported as percent recoveries and percent relative standard deviations (%RSD) of each compound.

3.6.6 The Study of Method Accuracy

The accuracy was performed by spiking the standard mixture solution into chili powder sample at three concentration levels; LOQ, medium and high on two different days. The accuracy of the method was determined by the mean of the percentage recovery of each compound.

3.7 Real Samples Analysis

Food samples including chili sauce, roasted chili paste and dried chili were purchased from local supermarkets. One gram of a homogeneous chili product sample was extracted by acetone and diluted to 10 mL in a volumetric flask. The mixture was well mixed by shaking. Then, the mixture was filtered by 0.2 μm nylon syringe filter and analyzed by on-line SPE-LC-MS under the optimum conditions.

CHAPTER IV

RESULTS AND DISCUSSION

The results of the optimization studies and applications for real sample analysis using on-line SPE followed by LC-ESI-MS are discussed below.

4.1 Optimum Instrumental Analysis Conditions

Various parameters of mass spectrometry (MS) techniques affect the efficiency of analysis of the four Sudan dyes. The ESI parameters such as nebulizer pressure, drying gas flow rate and drying gas temperature were studied. In the quadrupole ion trap mass analyzer, the ICC target ion is the parameter that influences the sensitivity of the analyte solutions. The results obtained are summarized below.

4.1.1 Optimization of ESI Parameters

4.1.1.1 Nebulizer Pressure

The nebulizer pressure setting was adjusted to match the optimum flow rate of the mobile phase. From the manufacturer's recommendations, an instrument having a mobile phase flow rate in the range of 0.2-0.5 mL min^{-1} should be operated at a nebulizer pressure of not more than 50 psi. In this experiment, the flow rate was fixed at 0.4 mL min^{-1}, whereas the nebulizer pressure was varied from 30 to 50 psi. The relationship between the peak area of Sudan III and the nebulizer pressure is shown in Table 4.1 and Figure 4.1. It was found that the peak areas of Sudan III were different at nebulizer pressure over a range of 30 to 50 psi. In this case, a nebulizer pressure of 45 psi gave the highest peak area; therefore, a nebulizer pressure of 45 psi was chosen for further study.

Table 4.1 Peak areas of Sudan III obtained at different nebulizer pressures (n=3)

Nebulizer pressure (psi)	Peak area (mean ± SD)
30	3992411±125870
35	4204801±203121
40	4619701±201990
45	5018258±139239
50	4535008±260282

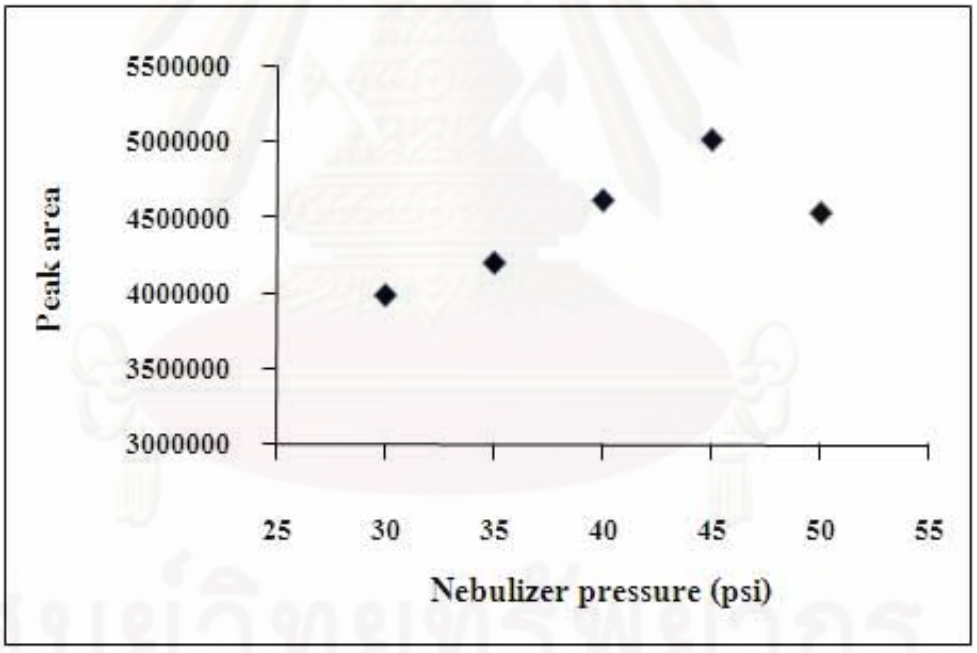


Figure 4.1 Peak areas of Sudan III obtained at different nebulizer pressures (n=3)

4.1.1.2 Drying Gas Flow Rate

By varying the drying gas flow rate from 8 to 10 L min^{-1}, as shown in Table 3.1, the relationship between the peak area of Sudan III and drying gas flow rate is shown in Table 4.2.

Table 4.2 Peak areas of Sudan III obtained at different drying gas flow rates (n=3)

Drying gas flow rate (L min^{-1})	Peak area (mean ± SD)
8	4089667±124799
9	4467703±205047
10	4928946±85271

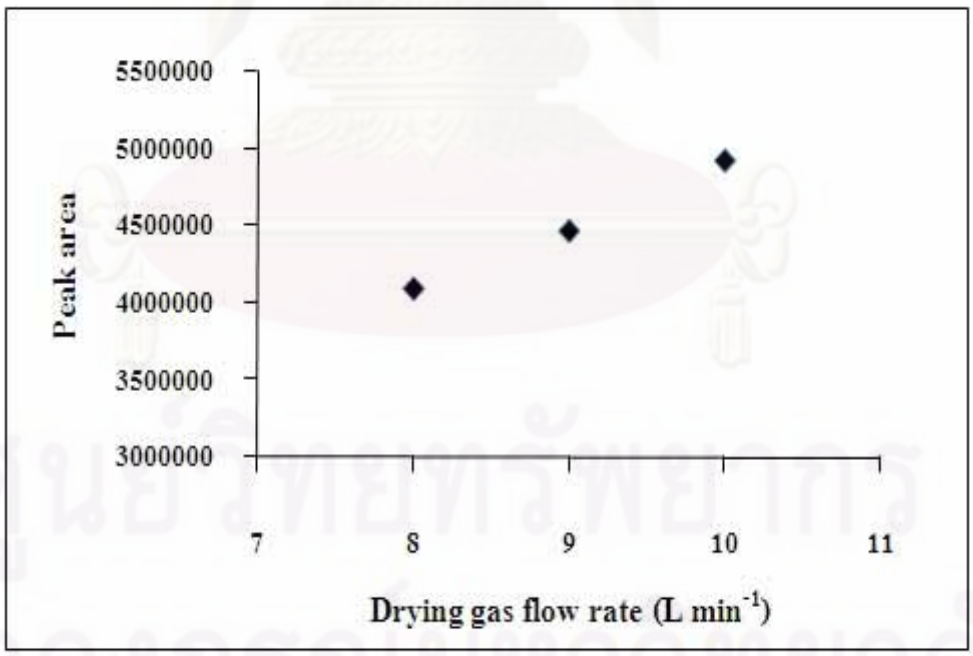


Figure 4.2 Peak areas of Sudan III obtained at different drying gas flow rates (n=3)

The drying gas flow rate is dependent on the mobile phase composition and flow rate. For this instrument, the recommended values of the drying gas flow rate were in the range of 8-10 L min^{-1}. We found that the peak areas obtained were slightly different at drying gas flow rates in the range of 8-10 L min^{-1}. However, we observed that a maximum peak area of Sudan III was achieved at a drying gas flow rate of 10 L min^{-1}. Therefore, a drying gas flow rate of 10 L min^{-1} was selected for further study.

4.1.1.3 Drying Gas Temperature

The drying gas temperature was varied from 300-350 ºC, as listed in Table 3.1. Because of the capability of the instrument, the maximum value of the drying gas temperature was 350 ºC. The relationship between peak area of Sudan III and drying gas temperature is shown in Table 4.3 and Figure 4.3.

Table 4.3 Peak areas of Sudan III obtained at different drying gas temperatures (n=3)

Drying gas temperature (ºC)	Peak area (mean ± SD)
300	4366519±60316
325	5048356±60236
350	4661702±141398

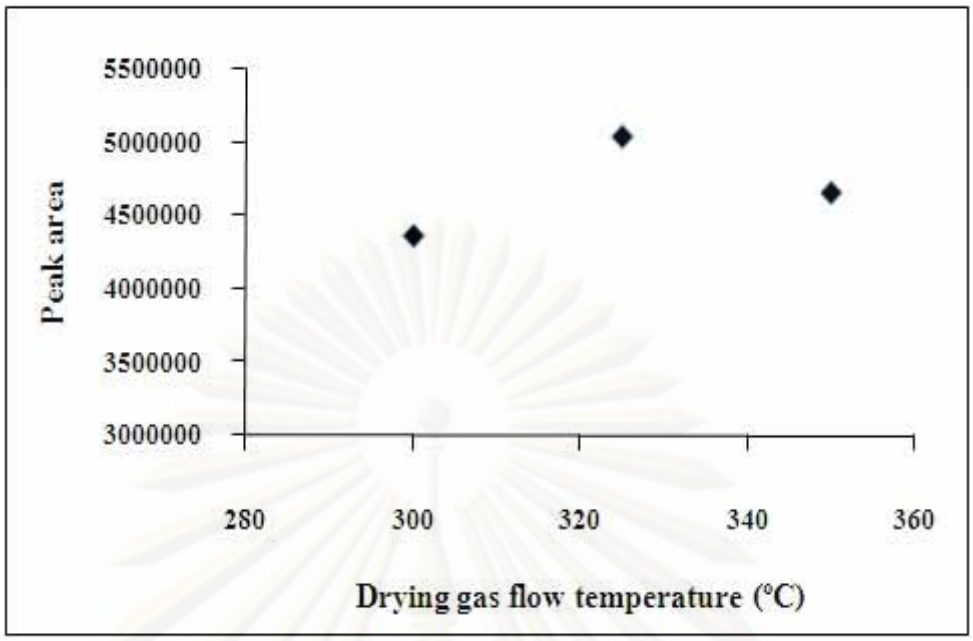


Figure 4.3 Peak areas of Sudan III obtained at different drying gas temperatures (n=3)

Heated nitrogen gas produced across the capillary at the entrance will cause desolvation of sample aerosols. The optimum drying gas temperature is dependent on the mobile phase composition. For instance, the higher water composition in a mobile phase the more drying gas temperature will be needed. It was found that the peak areas of Sudan III obtained were slightly different at drying gas temperatures in a range of 300-350 ºC. In this study, a solvent mixture consisting of 0.1% formic acid in water and acetonitrile was used as a mobile phase at flow rate of 0.4 mL min^{-1}. For complete desolvation, a drying gas temperature of 325 ºC was employed for subsequent tasks.

4.1.2 Optimization of Quadrupole Ion Trap Mass Analyzer

By varying the ICC target from 30,000 to 120,000 ions, the relationship between peak area of Sudan I-IV and ICC target was determined and is shown in Table 4.4.

Table 4.4 Peak areas of Sudan I-IV obtained at different ICC targets

ICC target (ions)	Peak area			
	Sudan I	Sudan II	Sudan III	Sudan IV
30,000	5660224	4497215	2501472	4118263
50,000	5777977	4542904	2593540	4173016
100,000	5930160	4639929	2618676	4307604
120,000	5537801	4589234	2534446	4057813

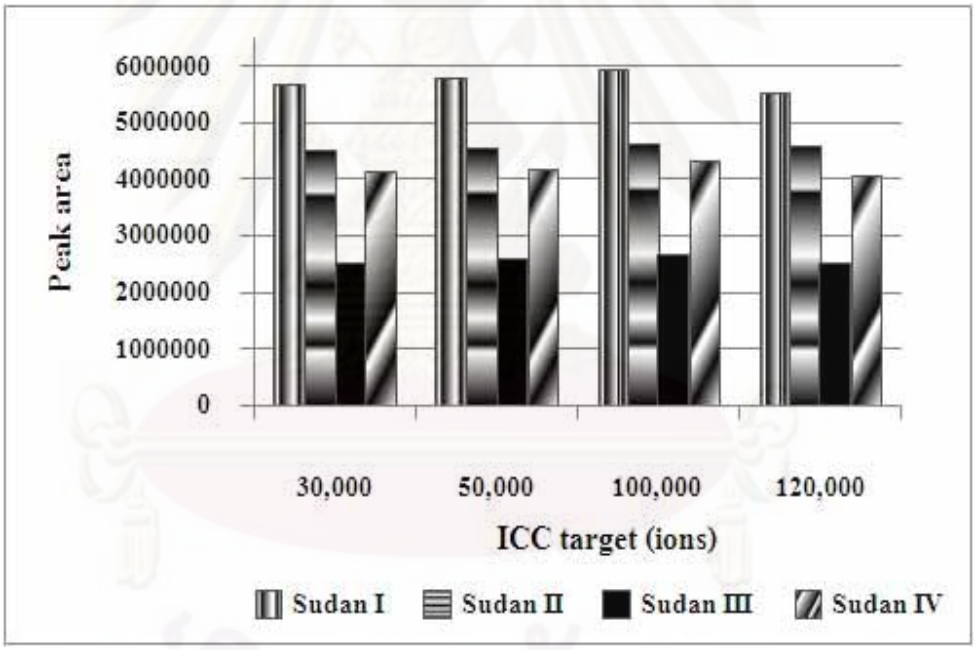


Figure 4.4 Peak areas of Sudan I-IV obtained at different ICC targets (n=3)

The ICC target is the parameter that influences the sensitivity of the analyses. It was found that the peak areas obtained for each Sudan dye were slightly different in the range of 30,000 to 120,000 ions. However, it was observed that the maximum peak areas of Sudan I-IV were achieved at an ICC target of 100,000 ions. Therefore, an ICC target at 100,000 ions was chosen for this study.

The overall optimum ESI-MS conditions are summarized in Table 4.5.

Table 4.5 The optimum ESI-MS conditions

ESI-MS parameter	Optimum condition
Ionization mode	Positive ion mode
Data acquisition mode	Scan mode (100-400 m/z)
Nebulizer pressure	45 psi
Drying gas flow rate	10 L min^{-1}
Drying gas temperature	325 ºC
ICC target	100,000 ions

4.2 Optimization of Mobile Phase Type for LC-ESI-MS

The mobile phase is important for LC-MS analysis and usually consists of buffers that improve ionization efficiency. The important properties of buffers for use in LC-MS are easy protonation and volatilization. Because the ability to produce ions by ESI depends on solution chemistry, the ionization process occurs directly from solution. Therefore, the choice of solvent has an effect on the ESI response. A mixed standard of Sudan I-IV was injected with different mobile phases, and the retention times and peak heights of each Sudan dye were determined. The results are shown in Table 4.6.

Table 4.6 Retention time and peak height of all four Sudan dyes at different mobile phase type

Mobile phase type		Compound			
		Sudan I	Sudan II	Sudan III	Sudan IV
Mobile phase 1 A: 0.1% formic acid in water B: MeOH	*t_R	15.39	21.75	25.18	29.66
	**P_H	2.21	2.83	1.32	0.80
Mobile phase 2 A: 0.1% formic acid in water B: ACN	*t_R	7.51	10.07	11.79	18.10
	**P_H	2.32	3.14	1.53	1.81
Mobile phase 3 A: 0.1% acetic acid in water B: MeOH	*t_R	17.79	20.52	23.33	29.75
	**P_H	1.53	2.04	0.65	0.51
Mobile phase 4 A: 0.1% acetic acid in water B: ACN	*t_R	8.25	11.43	14.12	19.35
	**P_H	1.81	2.22	1.04	1.25
Mobile phase 5 A: 0.1% formic acid in water B: 0.1% formic acid in MeOH	*t_R	12.15	17.23	20.78	24.18
	**P_H	2.32	3.01	1.43	1.02
Mobile phase 6 A: 0.1% formic acid in water B: 0.1% formic acid in ACN	*t_R	4.01	5.72	7.56	11.18
	**P_H	2.52	3.43	1.62	2.04

* Retention time (min)

** Peak height ($x10^6$)

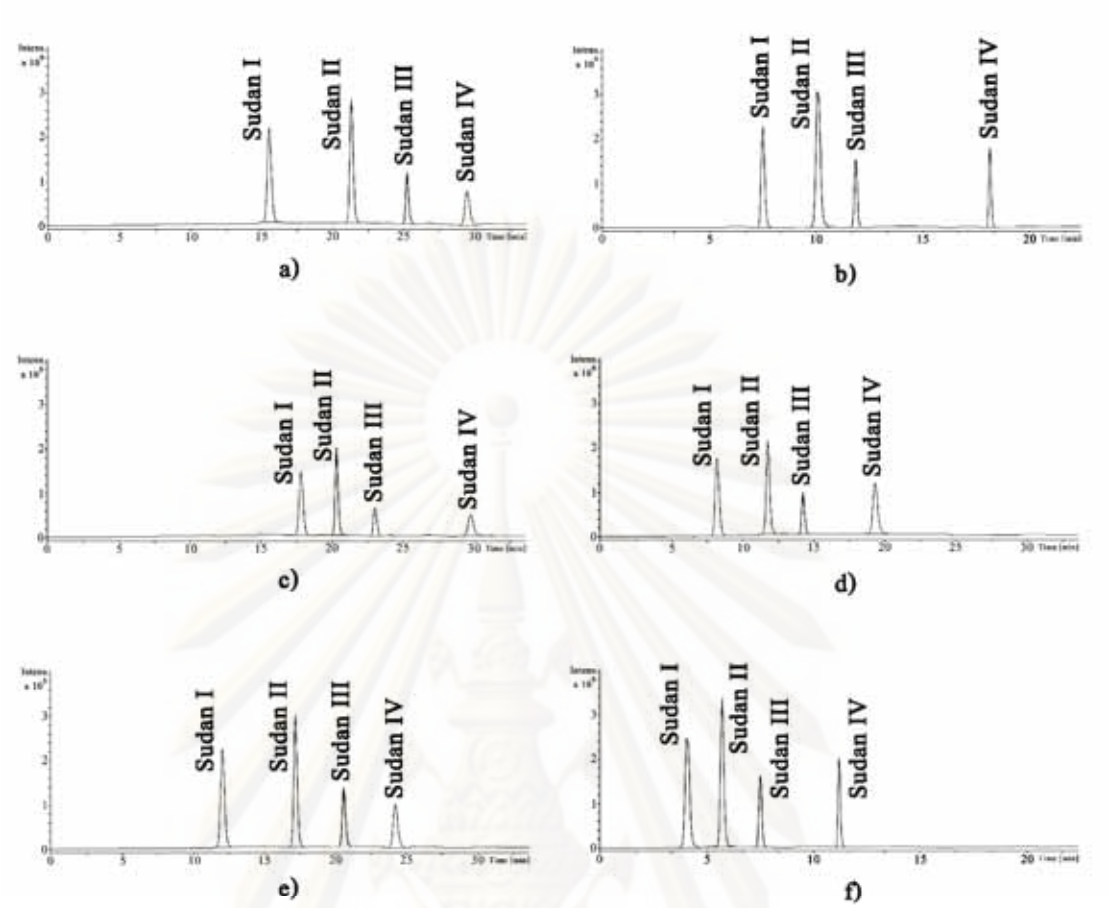


Figure 4.5 Chromatograms of all four Sudan dyes at different mobile phase type a) mobile phase 1, b) mobile phase 2, c) mobile phase 3, d) mobile phase 4, e) mobile phase 5 and f) mobile phase 6

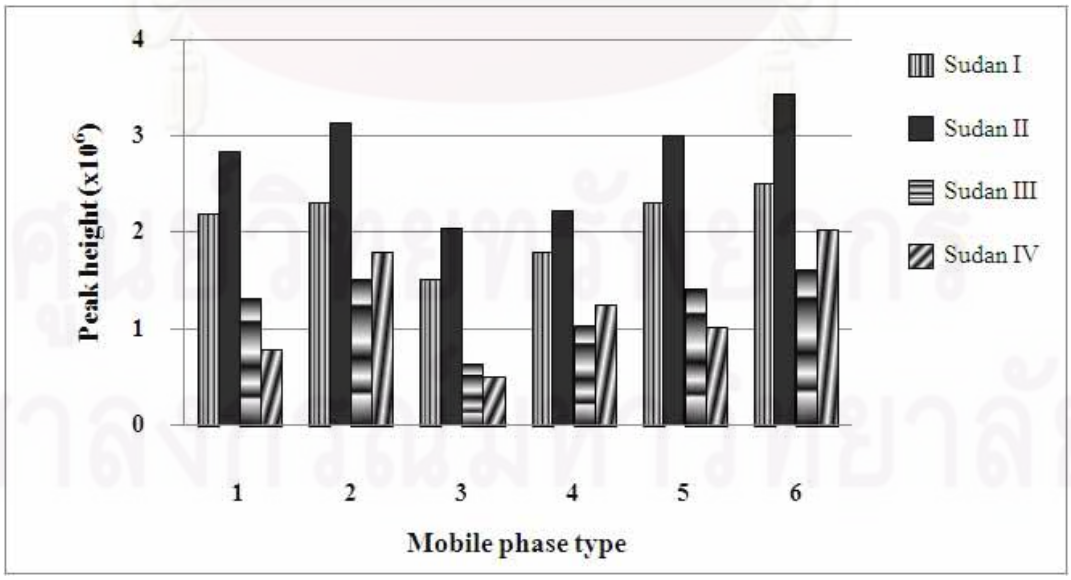


Figure 4.6 Peak height of all four Sudan dyes at different mobile phase type

From Figure 4.5, mobile phase type 6 gave a good separation of the four Sudan dyes within the shortest analysis time. In Figure 4.6, mobile phase type 6 gave the highest peak heights and achieved the best signal responses for most of the Sudan dyes. The maximum sensitivities and fastest analysis time of all compounds was observed when using 0.1% formic acid in water: 0.1% formic acid in ACN as the mobile phase.

4.3 Optimization of Mobile Phase Composition of the LC System

To optimize the mobile phase composition for the separation of all of the studied compounds, a mixture standard solution was injected into the HPLC with an ESI-MS detector. The optimum mobile phase from the LC optimized conditions (0.1% formic acid in water: 0.1% formic acid in ACN) was used. The gradient elution was developed to reach baseline resolution. After testing many conditions for separation of the four Sudan dyes, the preferable gradient program was discovered and is described in Table 4.7. Additionally, the chromatogram with the optimized conditions in Table 4.7 is presented in Figure 4.7.

Table 4.7 The HPLC optimization conditions

HPLC parameter	Condition		
Analytical column	Fortis C_{18} (2.1x150 mm, i.d. 3.0 µm)		
Mobile phase	A: 0.1% formic acid in water B: 0.1% formic acid in ACN		
Gradient program	Time	%A	%B
	0.5	8	92
	4	2	98
	5	0	100
	7	0	100
	8	8	92
Flow rate	0.4 mL min^{-1}		
Injection volume	5 µL		
Column temperature	40 ºC		
Detector	Mass spectrometer (ESI/positive)		

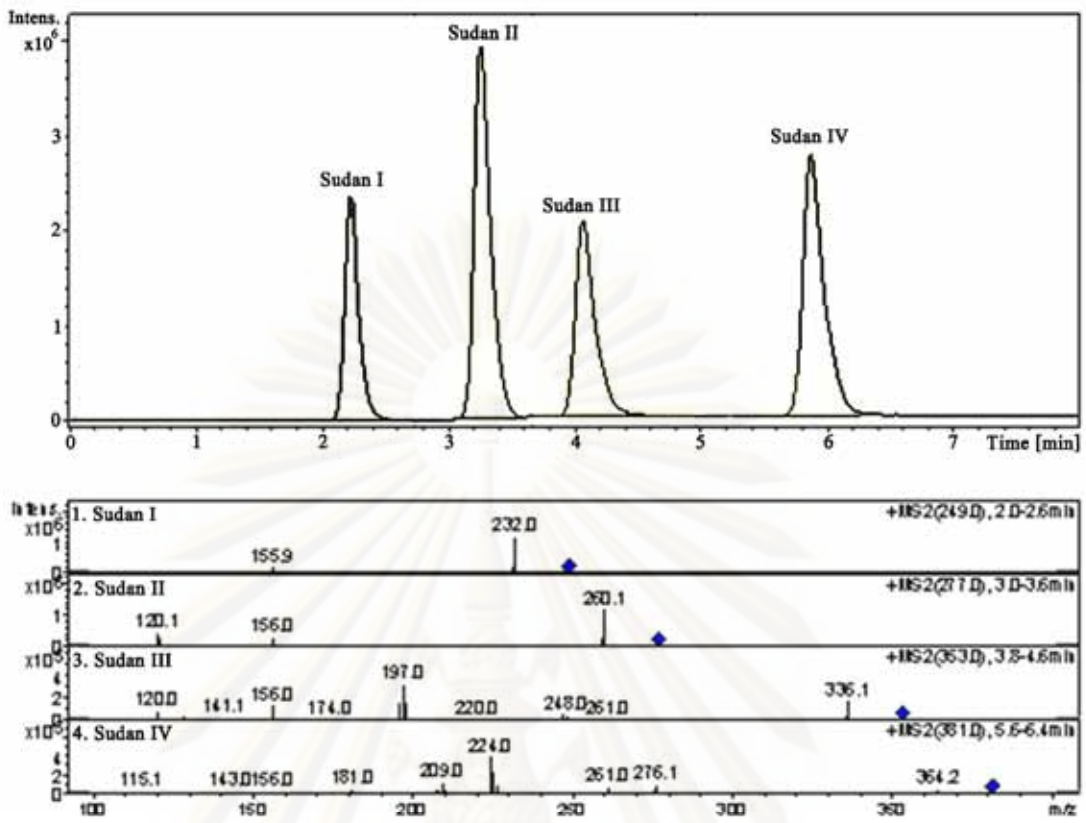


Figure 4.7 HPLC chromatogram and mass spectrum of a 0.5 µg mL^{-1} mixture of standard Sudan I-IV

4.4 Comparison of Extracting Solvents

The organic solvent for extraction significantly affected the extraction efficiency of the Sudan dyes in the samples. All four standard Sudan I-IV solutions were extracted from spiked sample with various extracting solvent, and the results are shown in Table 4.8 and Figure 4.8. Ethyl acetate provided the lowest recoveries (less than 65%), while acetone and acetonitrile provided high recoveries (above 85%) that were nearly equal. However, acetone was chosen because it is less expensive and less toxic than acetonitrile. Thus, in subsequent experiments, acetone was applied as the extracting solvent.

Table 4.8 The recovery of the extraction of four Sudan dyes from chili powder sample with different organic solvents

Organic solvent	% Recovery			
	Sudan I	Sudan II	Sudan III	Sudan IV
Acetone	86.59	97.41	85.79	88.91
Acetonitrile	85.21	97.95	85.11	88.12
Ethyl acetate	61.69	62.78	60.23	64.95

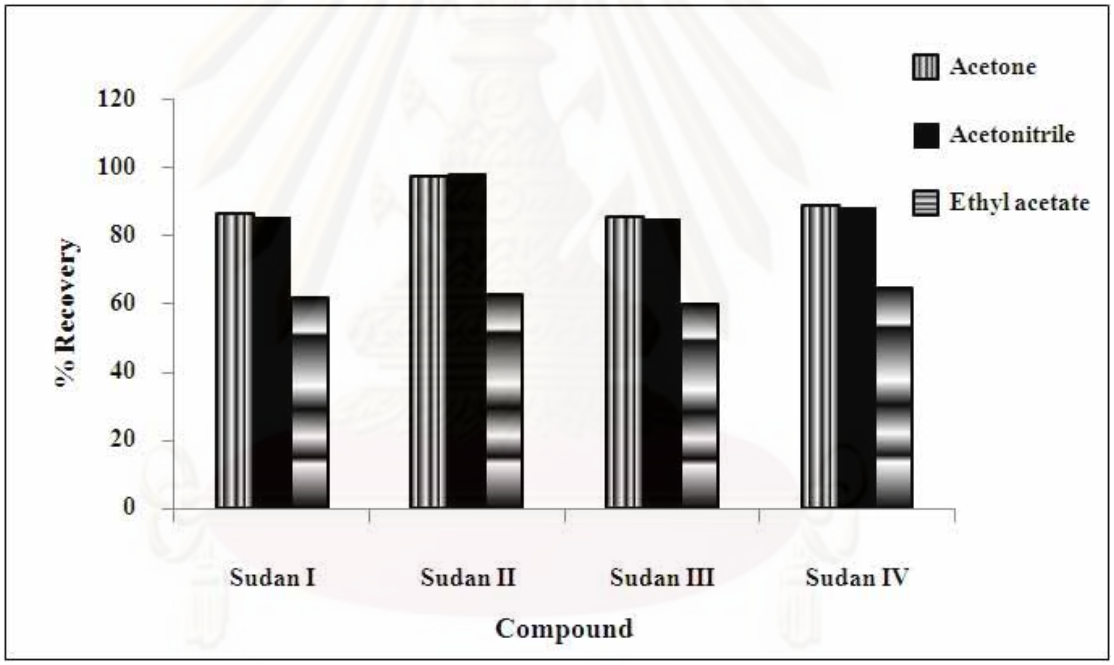


Figure 4.8 The recovery of the extraction of four Sudan dyes from chili powder sample with different organic solvents

4.5 Comparison of SPE Sorbent

Generally, SPE materials significantly affect the recovery efficiency. From the results shown in Table 4.9 and Figure 4.9, they were found that a C_{18} cartridge provided the highest recoveries of Sudan II, III and IV, but for Sudan I, a C_{18} cartridge

provided a lower recovery than a HCP-SC cartridge. This phenomenon can be explained by the polarity difference between Sudan I in comparison to the other dyes. The silica cartridge had the lowest recoveries because it was suitable for polar compounds and worked extremely well with non-polar solvents such as *n*-hexane or methylene chloride. On the other hand, Sudan dyes are non-polar compounds and acetone was used as an organic solvent, so the recoveries obtained from the silica cartridges were low. However, the C_{18} cartridge was chosen because it provided high recoveries for three dyes.

Table 4.9 Recoveries obtained for a 0.5 µg mL^{-1} standard mixture of four Sudan dyes using C_{18}, silica and HCP-SC cartridges

SPE sorbent	% Recovery			
	Sudan I	Sudan II	Sudan III	Sudan IV
C_{18}	81.52	92.05	85.10	89.33
Silica	68.09	65.12	62.21	60.11
HCP-SC	85.17	84.25	79.69	81.14

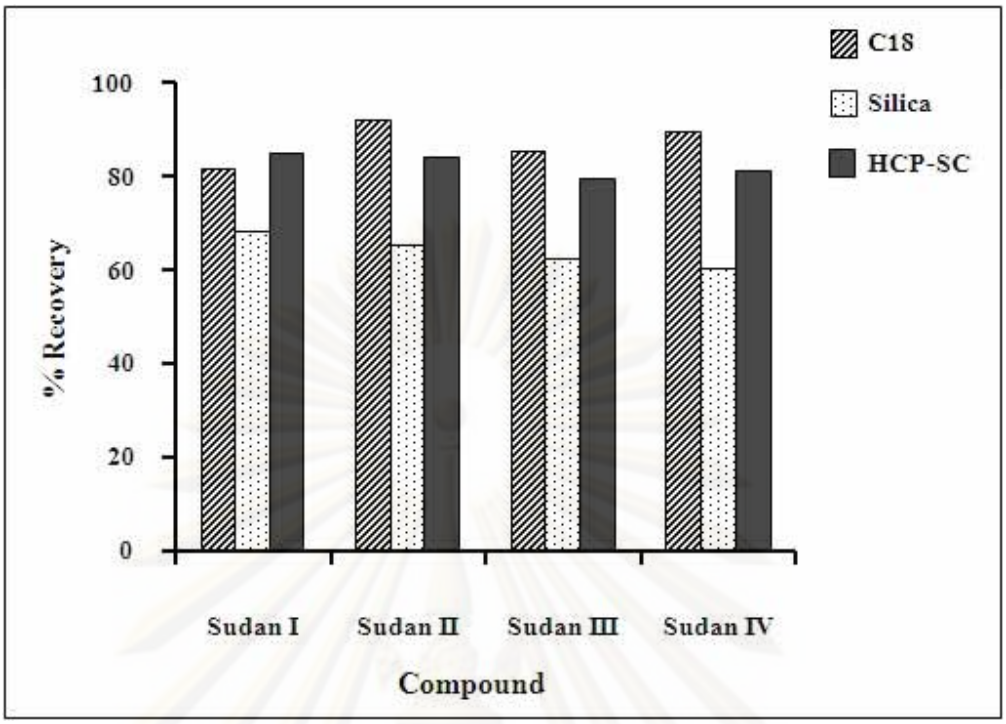


Figure 4.9 Recoveries obtained for a 0.5 µg mL^{-1} standard mixture of four Sudan dyes using C_{18}, silica and HCP-SC cartridges

4.6 Effect of the Ratio of Washing Solvent

Generally, the SPE washing step is important to remove interferences from the sorbent. If a suitable SPE washing solvent is used, a cleaned extract solution is achieved and the recoveries are improved. In this study, a cleaned extract was obtained by simply manipulating the ratio of methanol and water in the washing solvent. The results of the base-modified washing solvent on the percent recoveries of each compound are illustrated in Table 4.10 and Figure 4.10.

Table 4.10 The ratio of washing solvent (MeOH:H_2O) and recovery of four Sudan dyes

MeOH:H_2O	% Recovery			
	Sudan I	Sudan II	Sudan III	Sudan IV
90:10	95.96	91.07	81.17	87.22
80:20	96.02	90.20	83.03	87.00
70:30	95.11	90.01	84.10	88.19
60:40	94.55	89.50	85.02	90.57
50:50	94.09	89.00	86.14	92.03
40:60	93.21	87.15	86.56	92.50
30:70	92.13	85.87	87.03	94.15
20:80	91.45	85.23	87.50	95.30
10:90	90.00	83.21	88.01	95.55

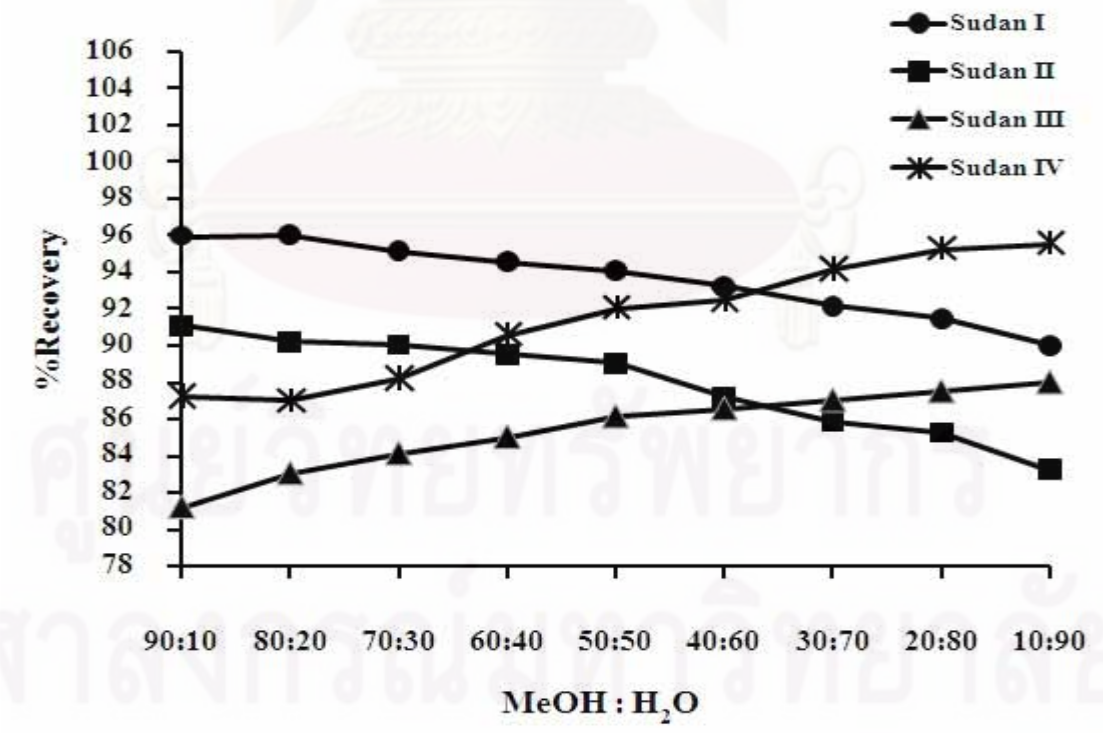


Figure 4.10 The ratio of washing solvent (MeOH:H_2O) and recovery of four Sudan dyes

4.7 On-line SPE-LC-MS

The most critical parameters influencing method performance were valve switching time and loading volume. These parameters were interdependent because their combination influenced recovery, retention time and chromatographic peak shape.

4.7.1 Loading Volume Dependence

The results of a loading volume of 0.05 μg mL^{-1} standard Sudan dyes on the on-line SPE are shown in Figure 4.11. At 5 and 10 μL, both were detected at 0.05 μg mL^{-1} but were not detected at lower concentrations. Higher loading volumes of 50 and 100 μL normally gave higher sensitivity but caused chromatographic peak broadening and the extraction cartridge duty cycle was diminished. Therefore, a loading volume of 20 μL was selected because this volume provided the best peak shape and gave a good signal-to-noise ratio.

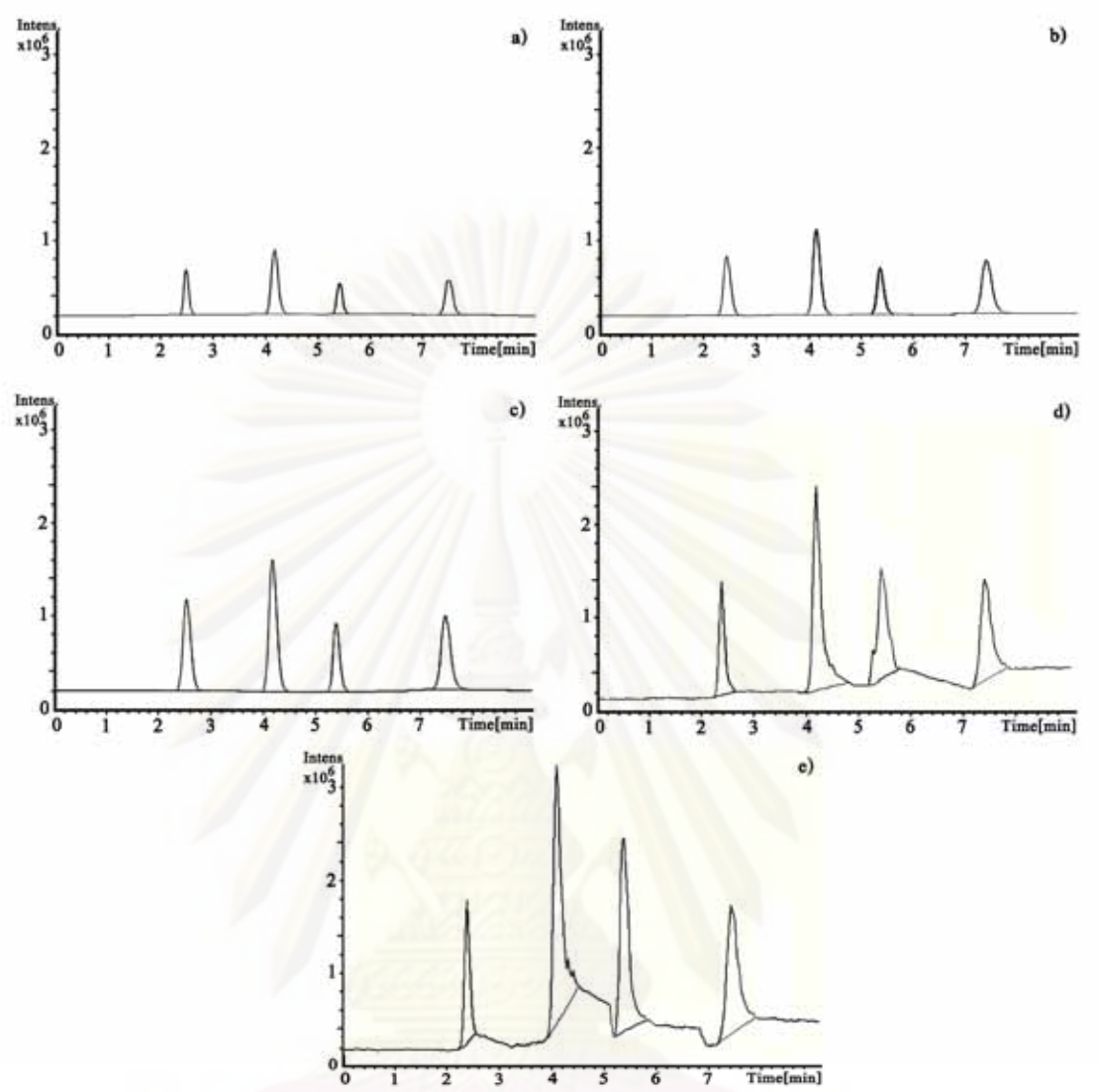


Figure 4.11 The on-line SPE-LC-MS chromatograms of all four Sudan dyes at loading volume (a) 5 µL, (b) 10 µL, (c) 20 µL, (d) 50 µL and (e) 100 µL

4.7.2 Switching Time Dependence

Figure 4.12 shows the chromatograms of all four Sudan dyes at 5, 15, 30 and 45 second and 1 minute switching times. At the fastest switching time, 5 seconds, no peaks were observed. The 45 second and 1 min switching times gave long retention times and peak broadening. Switching times of 15 and 30 seconds were compared by triplicate analyses of spiked sample at a concentration of 0.05 µg mL^{-1}. As can be seen from Table 4.11, a switching time of 30 seconds provided higher recoveries, so 30 seconds was selected for this method.

Table 4.11 Comparison of the recoveries obtained for a 0.05 μg mL^{-1} standard mixture solution of four Sudan dyes using switching time 15 and 30 second (n=3)

Compound	% Recovery	
	15 second	30 second
Sudan I	83.24	93.78
Sudan II	81.22	90.65
Sudan III	85.68	91.02
Sudan IV	80.11	90.34

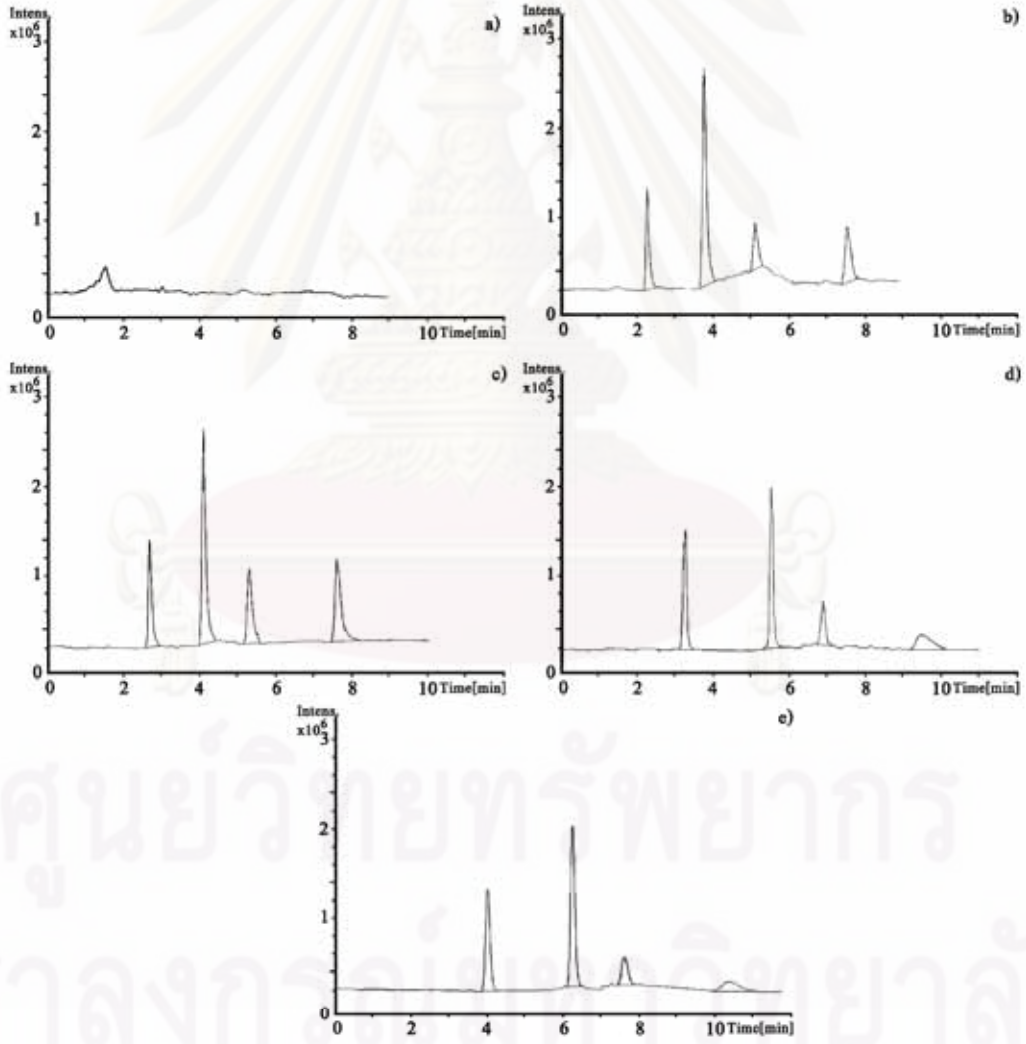


Figure 4.12 The on-line SPE-LC-MS chromatograms of all four Sudan dyes at switching time (a) 5 sec, (b) 15 sec, (c) 30 sec, (d) 45 sec and (e) 1 min

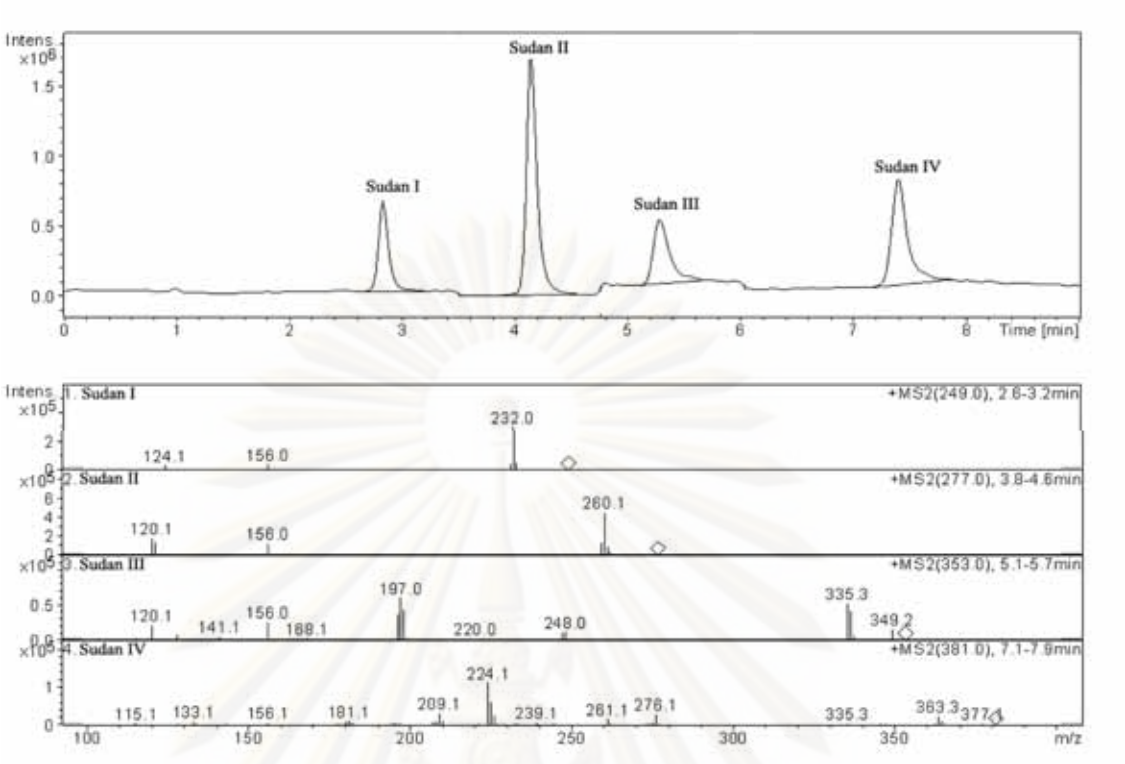


Figure 4.13 Typical on-line SPE-LC-MS chromatogram and mass spectrum of Sudan I-IV

From the study of the loading volume and switching time, the optimum on-line SPE-LC-MS conditions (loading volume at 20 µL and switching time at 30 second) were achieved. As the instrumental configuration in Figure 3.1, the solvent composition program of pump B and the switching valve program are shown in the Table 4.12.

Under the optimum on-line SPE-LC-MS conditions, Figure 4.13 showed the typical on-line-SPE-LC-MS chromatograms and mass spectrums for blank chili powder spiked with the standard mixture of Sudan I-IV (0.5 mg kg^{-1}). The four Sudan dyes were separated under 8 min, the total analysis time is 12 min (include re-equilibration) using the on-line SPE-LC-MS system.

Table 4.12 The on-line LC-MS optimization conditions (switching valve position)

Time (min)	Switching valve position	Pump B	
		%A	%B
0-0.5	Enrichment column	8	92
0.5-4	Analytical column	2	98
4-5	Analytical column	0	100
5-7	Analytical column	0	100
7-8	Analytical column	8	92
8-12	Enrichment column	8	92

4.8 Method Validation

Method validation is an important requirement in the practice of chemical analysis. The purpose is to study the method performance parameters and demonstrate a particular method for the quantitative measurement of analytes in a matrix (chili powder). The parameters for this validation included a standard calibration curve, linear range, matrix effect, LOD, LOQ, precision and accuracy.

4.8.1 Standard Calibration Curve and Linear Range

A mixture of four standard Sudan dyes in acetone that covered a concentration range of 0.005-20.0 μg mL^{-1} was prepared. Calibration curves of the four Sudan dyes were obtained from the relationship of the peak area versus concentration, and these calibration curves are shown in Figures 4.14, 4.15, 4.16 and 4.17. The coefficient of determination (R^2) and regression data in the order of each Sudan dye are summarized in Table 4.13.

Table 4.13 Calibration characteristics of Sudan I, Sudan II, Sudan III and Sudan IV by optimal conditions of on-line SPE-LC-MS

Compound	Slope	Intercept	Coefficient of determination (R^2)	Linear range ($\mu g\ mL^{-1}$)
Sudan I	10.4196	+0.0663	0.9999	0.005-1.0
Sudan II	21.2464	+0.0595	0.9997	0.005-1.0
Sudan III	3.7171	+0.2300	0.9997	0.01-4.0
Sudan IV	4.6051	+0.0762	0.9998	0.005-1.0

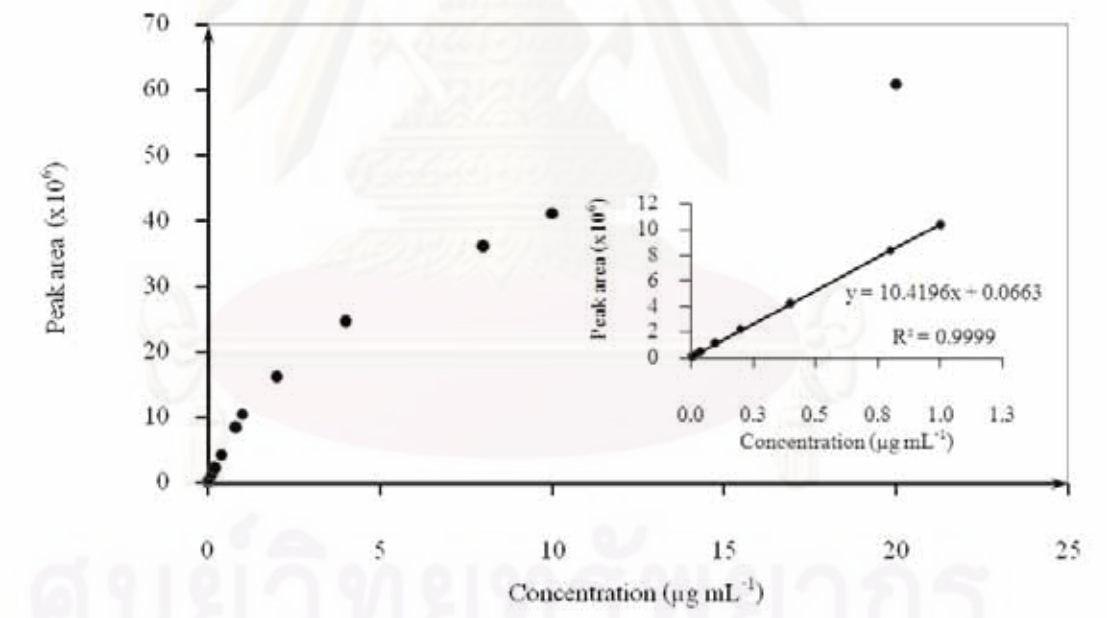


Figure 4.14 Calibration curve of standard Sudan I solution by on-line SPE-LC-MS

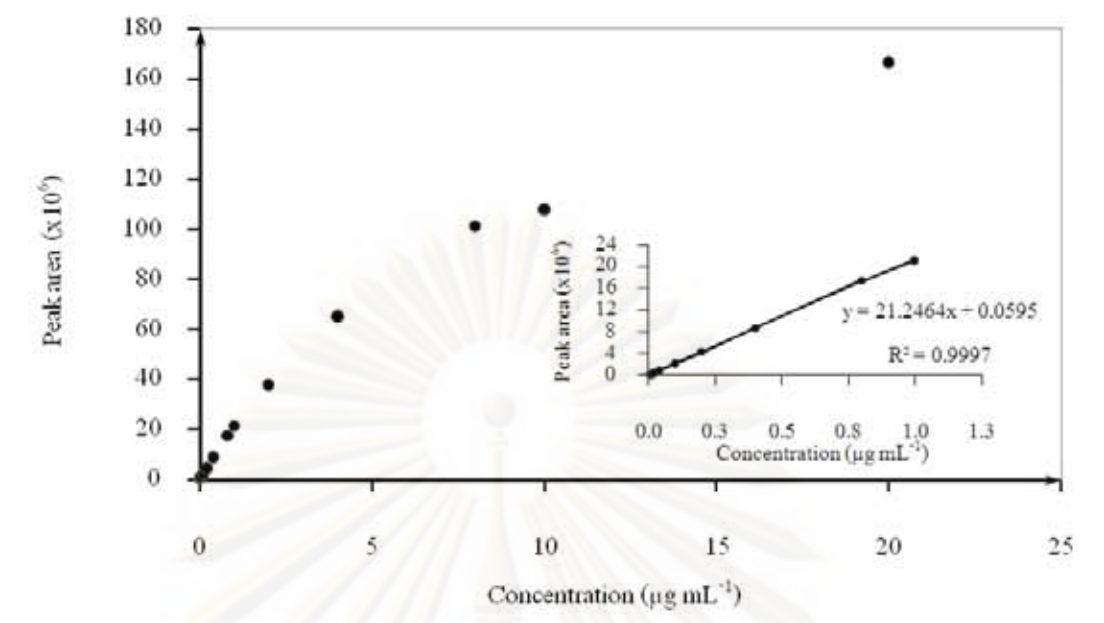


Figure 4.15 Calibration curve of standard Sudan II solution by on-line SPE-LC-MS

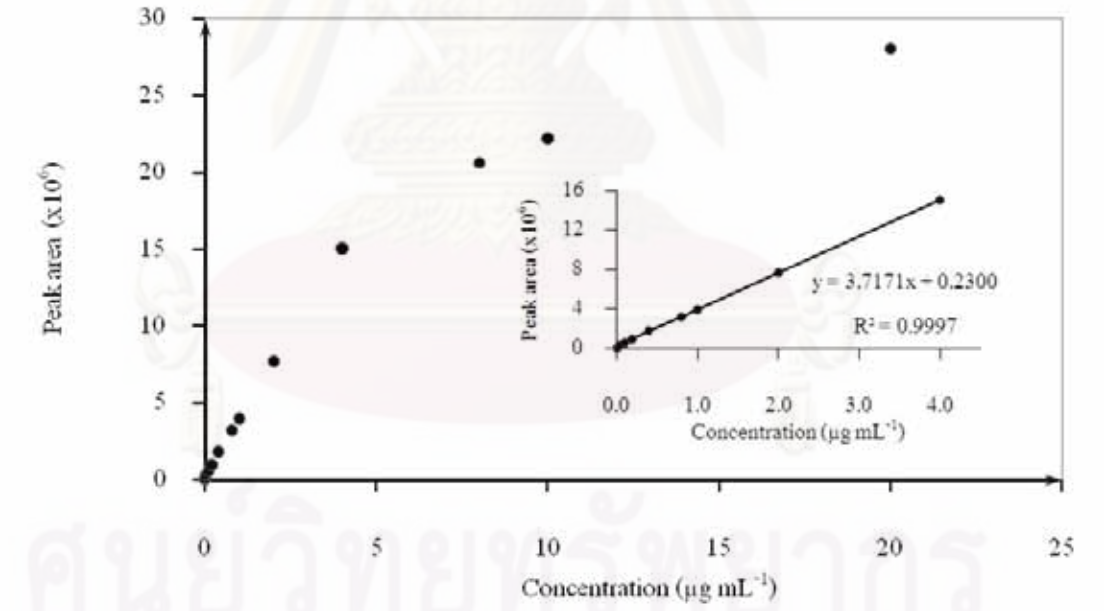


Figure 4.16 Calibration curve of standard Sudan III solution by on-line SPE-LC-MS

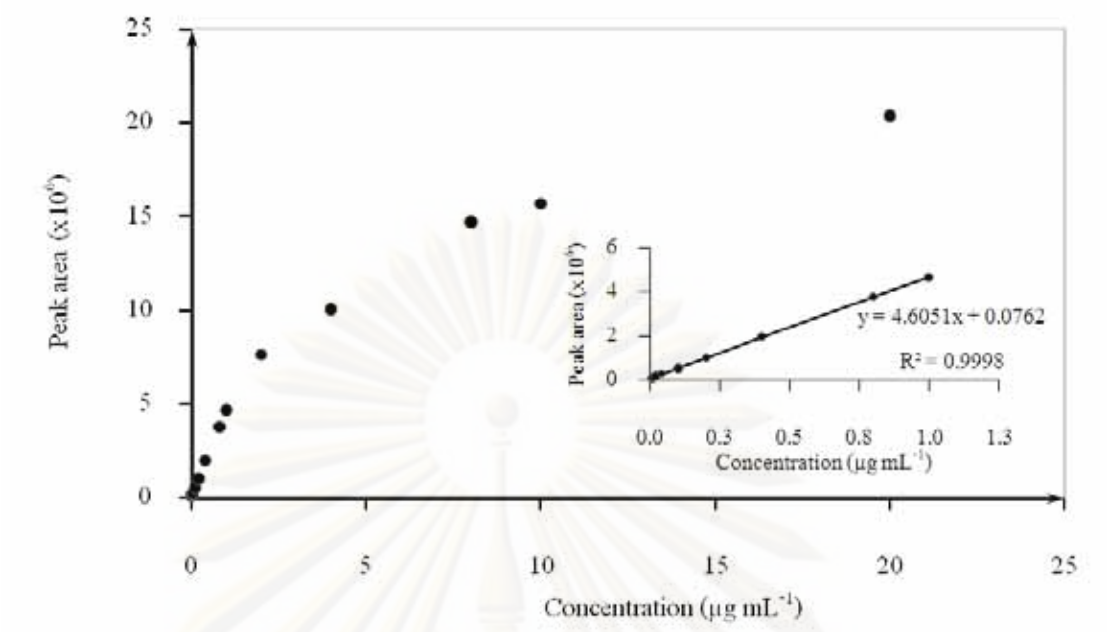


Figure 4.17 Calibration curve of standard Sudan IV solution by on-line SPE-LC-MS

The corresponding least-squares regression coefficient of determination (R^2) described in Table 4.13 were greater than 0.9997, which is acceptable for quantitative analysis. Furthermore, the sensitivity of each analyte that best showed the detector response is indicated by the slope value. A compound with a higher slope value gave a higher detector response and sensitivity.

4.8.2 Matrix Effect

The matrix is one of the most important factors affecting analytical measurements. When the analytical system is validated, the matrix must be considered. A test for the matrix effect can be made by adding analytes into a standard solution and comparing it with the matrix solution. Moreover, the two calibration curves should cover the same working range. The matrix effect here was studied by using a paired *t*-test with a mean 95% confidence limit. The *t*-values are given in Table 4.14. All results of the matrix calibration curve, coefficient of determination (R^2) and regression data in the order of each Sudan dye are shown in appendix B (Figure B1-B4).

Table 4.14 *t*-calculated values of two tailed paired *t*-test at 95% confidence level

Compound	Concentration ($\mu g\ mL^{-1}$)	Peak area		Pair *t*-test	
		Standard solution	Standard in chili powder	*t*-calculated	*t*-critical
Sudan I	0.005	68603	68598	1.95	2.31
	0.01	126486	126357		
	0.02	169589	169569		
	0.04	244639	244543		
	0.1	480510	480443		
	0.2	873534	873334		
	0.4	1714642	1716963		
	0.8	3345298	3344077		
	1.0	4171799	4171279		
Sudan II	0.005	110012	109997	2.22	2.31
	0.01	218203	218134		
	0.02	228768	228685		
	0.04	344860	344538		
	0.1	780502	780386		
	0.2	1491270	1490972		
	0.4	2890515	2892829		
	0.8	5808149	5804265		
	1.0	7255997	7253220		

Table 4.14 *(continued)*

Compound	Concentration ($\mu g\ mL^{-1}$)	Peak area		Pair *t*-test	
		Standard solution	Standard in chili powder	*t*-calculated	*t*-critical
Sudan III	0.01	65083	65033	1.36	2.31
	0.04	98526	98503		
	0.1	167980	167637		
	0.2	362888	362263		
	0.4	677691	677462		
	0.8	1301001	1300611		
	1.0	1657195	1666092		
	2.0	3216251	3215243		
	4.0	6284106	6283968		
Sudan IV	0.005	198867	198768	1.91	2.31
	0.01	248818	248648		
	0.02	285712	285642		
	0.04	355434	355417		
	0.1	550380	550336		
	0.2	885833	885578		
	0.4	1526813	1524666		
	0.8	2856644	2855893		
	1.0	3514343	3513985		

Tests were carried out at the same concentrations between the standard and matrix calibration curves. After the statistical treatments of peak area values were carried out by the paired *t*-test at a 95% confidence limit (Table 4.14), the calculated *t*-values of all of the compounds were lower than the critical *t*-values. These values were not significantly different between the standard calibration curve and the matrix calibration curve. Therefore, we concluded that the chili powder matrix did not significantly affect the signal of the analyte compounds.

4.8.3 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected at a signal-to-noise ratio of 3. The limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be quantitatively determined with an acceptable level of precision. It is also defined at various concentrations to be the analyte concentration corresponding to a signal-to-noise ratio of 10. The LOD and LOQ were obtained by determining the amount of Sudan I-IV in a chili powder matrix. The results are summarized in Table 4.15.

Table 4.15 LOD and LOQ of each Sudan I-IV in chili powder matrix

Compound	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Sudan I	0.03	0.05
Sudan II	0.03	0.05
Sudan III	0.04	0.1
Sudan IV	0.03	0.05

4.8.4 Method Precision

Method precision was evaluated at three different concentration levels in five replicates at the LOQ, medium and high concentration levels. Generally, precision is stated in terms of standard deviation or relative standard deviation (RSD). Relative standard deviation may be useful in this case; the acceptability of the precision values is based on the modified Horwitz equation.

Horwitz equation [36]:

$$RSD_r = 0.66 \times 2^{(1-0.5 \log C)}$$

where, RSD_r = the relative standard deviation calculated from results generated under repeatability conditions (with-in laboratory)

C = mass fraction: for 100% (pure materials), C = 1.00

for 1 µg/g (ppm), C = 0.000001

This is a generalized precision equation which that has been found to be independent of the analyte and matrix, and is solely dependent on the concentration for most routine methods of analysis.

From the Horwitz equation, the predicted percent RSD values of all analytes at the LOQ, medium and high concentration levels were determined and are tabulated in Table 4.16.

Table 4.16 Predicted %RSD of Sudan I, II, III and IV at LOQ, medium and high concentration levels

Compound	Predicted %RSD*		
	LOQ	Medium	High
Sudan I	16.572	10.56	7.47
Sudan II	16.572	10.56	7.47
Sudan III	14.930	8.57	6.06
Sudan IV	16.572	10.56	7.47

*Predicted %RSD is calculated from Horwitz equation

4.8.4.1 Results of Method Precision at LOQ level

The precision of this method is a measurement of the closeness expected between replicate tests under optimal conditions. Method precision at the LOQ level was studied by repeating the analysis on the same day and on two different days. The results of the method precision are summarized in Tables 4.17-4.19.

Table 4.17 % Recoveries and % RSD of spiked chili powder matrix at LOQ level on the first day (n=5)

Compound	% Recoveries					Mean	% RSD
	1	2	3	4	5		
Sudan I	94.66	94.71	94.54	94.82	94.42	94.63	0.164
Sudan II	99.96	99.82	99.96	99.90	100.05	99.94	0.087
Sudan III	99.80	99.85	99.73	99.84	99.77	99.80	0.048
Sudan IV	99.73	99.85	99.74	99.91	99.77	99.80	0.078

Table 4.18 % Recoveries and % RSD of spiked chili powder matrix at LOQ level on the second day (n=5)

Compound	% Recoveries					Mean	% RSD
	1	2	3	4	5		
Sudan I	94.69	94.65	94.54	94.69	94.48	94.61	0.101
Sudan II	99.71	99.98	99.60	99.76	99.44	99.70	0.202
Sudan III	99.64	99.74	99.64	99.67	99.57	99.65	0.065
Sudan IV	99.71	99.90	99.75	99.58	99.66	99.72	0.122

Table 4.19 Overall % recovery and % RSD of spiked chili powder matrix at LOQ level

Compound	% Recoveries		Mean	% RSD	*F*-test*	
	1	2			$F_{calculated}$	$F_{critical}$
Sudan I	94.63	94.61	94.62	0.015	2.656	9.605
Sudan II	99.94	99.70	99.82	0.170	5.399	
Sudan III	99.80	99.65	99.72	0.106	1.828	
Sudan IV	99.80	99.72	99.76	0.057	2.451	

**F*-values were calculated from variance values at 95% confidence limit

(1) Intra-day precision

In this study, the precision of the method was expressed as the percent relative standard deviation (%RSD). Based on the Horwitz equation, taking into account the concentration of the analytes at the LOQ level, the acceptable predicted RSD values were 16.572% for Sudan I, II, and IV and 14.930% for Sudan III. The experimentally determined % RSD values on the first day varied from 0.048-0.164 and from 0.065-0.202 on the second day. Both of these ranges were below and complied with the predicted values using the Horwitz equation. These results demonstrated that this

method was sufficiently precise at the concentration level of analytes being measured during the same day.

(2) Intermediate precision (Inter-day precision)

The precision of the method on different days (Table 4.19), $\%RSD_{overall}$, ranged from 0.015-0.170. From the *F*-test method at the 95% confidence limit, the critical *F*-value was 9.605. The calculated F-values of all of the analytes were less than the critical *F*-value. These results indicated that a good intermediate precision of this method was observed at the level of analytes being measured.

4.8.4.2 Results of Method Precision at a Medium Concentration Level

The method precision at a medium concentration level was studied by repeating the analysis during one day and on different days. The results of the method precision are summarized in Table 4.20-4.22.

Table 4.20 % Recoveries and % RSD of spiked chili powder matrix at medium concentration level on the first day (n=5)

Compound	% Recoveries					Mean	% RSD
	1	2	3	4	5		
Sudan I	99.41	99.37	99.34	99.36	99.43	99.38	0.037
Sudan II	90.12	90.22	90.10	90.13	90.13	90.14	0.048
Sudan III	101.53	101.77	101.73	101.75	101.45	101.65	0.143
Sudan IV	100.43	100.73	100.46	100.60	100.59	100.56	0.120

Table 4.21 % Recoveries and % RSD of spiked chili powder matrix at medium concentration level on the second day (n=5)

Compound	% Recoveries					Mean	% RSD
	1	2	3	4	5		
Sudan I	99.37	99.31	99.23	99.21	99.24	99.27	0.064
Sudan II	90.35	90.30	90.04	90.21	90.20	90.22	0.132
Sudan III	102.14	102.08	102.09	101.93	101.88	102.02	0.109
Sudan IV	101.54	101.30	101.24	101.40	101.24	101.35	0.127

Table 4.22 Overall % recovery and % RSD of spiked chili powder matrix at medium concentration level

Compound	% Recoveries		Mean	% RSD	*F*-test*	
	1	2			$F_{calculated}$	$F_{critical}$
Sudan I	99.38	99.27	99.33	0.078	3.029	9.605
Sudan II	90.14	90.22	90.18	0.063	7.488	
Sudan III	101.65	102.02	101.84	0.257	1.702	
Sudan IV	100.56	101.35	100.96	0.553	1.139	

**F*-values were calculated from variance values at 95% confidence limit

(1) Intra-day precision

The predicted RSD values for intra-assay precision were 10.56% for Sudan I, II and IV and 8.57% for Sudan III at the medium concentration level measured. The experimentally determined %RSD values on the first day varied from 0.037-0.143 and from 0.064-0.132 on the second day. Both of these ranges were below the %RSD values predicted by the Horwitz equation. These results indicated that the method was sufficiently precise at the concentration level of analytes being measured within the same day.

(2) Intermediate precision (Inter-day precision)

The precision of the method on different days, $\%RSD_{overall}$, is shown in Table 4.22 and varied from 0.063-0.553. Using the *F*-test method at the 95% confidence limit, the critical *F*-value was 9.605. The calculated *F*-values of all of the analytes was lower than the critical *F*-value. Therefore, the results indicated that a good intermediate precision of this method was observed at the level of analytes being measured.

4.8.4.3 Results of Method Precision at a High Concentration Level

The method precision at a high concentration level was studied by repeating the analysis during one day and on different days. The results of the method precision are summarized in Table 4.23-4.25.

Table 4.23 % Recoveries and % RSD of spiked chili powder matrix at high concentration level on the first day (n=5)

Compound	% Recoveries					Mean	% RSD
	1	2	3	4	5		
Sudan I	99.44	99.46	99.42	99.44	99.44	99.44	0.014
Sudan II	99.40	99.48	99.37	99.41	99.30	99.39	0.063
Sudan III	99.84	99.86	99.91	99.91	99.79	99.86	0.049
Sudan IV	99.47	99.44	99.48	99.50	99.51	99.48	0.027

Table 4.24 % Recoveries and % RSD of spiked chili powder matrix at high concentration level on the second day (n=5)

Compound	% Recoveries					Mean	% RSD
	1	2	3	4	5		
Sudan I	99.42	99.42	99.41	99.40	99.43	99.42	0.011
Sudan II	99.26	99.21	99.08	99.16	99.13	99.17	0.078
Sudan III	99.62	99.60	99.44	99.53	99.51	99.54	0.072
Sudan IV	99.49	99.40	99.52	99.39	99.43	99.45	0.057

Table 4.25 Overall % recovery and % RSD of spiked chili powder matrix at high concentration level

Compound	% Recoveries		Mean	% RSD	*F*-test*	
	1	2			$F_{calculated}$	$F_{critical}$
Sudan I	99.44	99.42	99.43	0.014	1.542	9.605
Sudan II	99.39	99.17	99.28	0.157	1.224	
Sudan III	99.86	99.54	99.70	0.227	2.163	
Sudan IV	99.48	99.45	99.47	0.021	4.492	

**F*-values were calculated from variance values at 95% confidence limit

(1) Intra-day precision

The predicted RSD values for the intra-assay precision were 7.47% for Sudan I, II and IV and 6.06% for Sudan III at the high concentration level studied. The experimentally determined %RSD values on the first day varied from 0.014-0.063 and from 0.011-0.078 on the second day. Both of these ranges were below the %RSD values predicted by the Horwitz equation. These results indicated that the method was sufficiently precise at the concentration level of analytes being measured within the same day.

(2) Intermediate precision (Inter-day precision)

The precision of the method on different days, $\%RSD_{overall}$, is shown in Table 4.25 and varied from 0.014-0.227. Using the *F*-test method at the 95% confidence limit, the critical *F*-value was 9.605. The calculated *F*-values of all of the analytes were lower than the critical *F*-value. Therefore, the results indicated that a good intermediate precision of this method was observed at the level of analytes being measured.

4.8.5 Method Accuracy

Accuracy is the measure of exactness of an analytical method or the closeness of agreement between the measured value and the true or accepted value. Accuracy is measured as the percent of analyte recovered by spiking samples with known amounts of standard compounds. In this study, the concentrations of analyte standard at the LOQ, medium and high levels were used to determine the method accuracy for the chili powder samples. The method accuracy of the percent recoveries of each compound are shown in Table 4.26.

Table 4.26 % Recoveries of method at LOQ, medium and high concentration levels for spiked chili powder sample

Compound	% Recovery		
	LOQ level	Medium level	High level
Sudan I	94.62	99.33	99.43
Sudan II	99.82	90.18	99.28
Sudan III	99.72	101.84	99.70
Sudan IV	99.76	100.96	99.47

The recovery of the spiked chili powder matrix at the LOQ level ranged from 94.62 to 99.82%, from 90.18 to 101.84% at the medium concentration level and from 99.28 to 99.70% at the high concentration level. These recovery values are acceptable according to the AOAC peer-verified methods, which recommend acceptable recovery values at the ppm concentration range of between 80-110%. The results obtained from the above studies indicated that the developed method provided good precision and accuracy for analysis of these analytes in chili powder.

4.9 Real Samples

Food samples including chili sauce, roasted chili paste and dried chili from local supermarkets were analyzed by on-line SPE-LC-MS, and none of the samples contained the dyes. To demonstrate method feasibility, recovery experiments were carried out using spiked samples with the Sudan dye standards at three levels. We found recoveries of greater than 90% for Sudan I-IV (Table 4.27).

Table 4.27 Recoveries for the spiked chili products (n=3)

Compound	Spiked level ($mg\ kg^{-1}$)	Recovery (%)		
		Chili sauce	Roasted chili paste	Dried chili
Sudan I	0.05	101.2	101.1	100.0
	1	95.6	95.5	95.5
	10	99.3	98.9	100.0
Sudan II	0.05	100.4	100.5	97.0
	1	97.2	94.6	101.4
	10	98.1	91.2	94.2
Sudan III	0.1	100.8	101.5	101.5
	4	99.7	96.8	97.9
	40	98.4	100.6	98.9
Sudan IV	0.05	100.7	101.4	100.2
	1	95.0	98.6	91.0
	10	98.8	97.8	99.6

CHAPTER V

CONCLUSIONS

5.1 Conclusions

In this study, a new method was developed for the simultaneous analysis of Sudan I-IV. The analysis was carried out using on-line SPE-LC-MS. This work also investigated optimal LC-MS parameters for analysis. The optimal ESI parameters achieved under positive ion detection mode were a nebulizer pressure of 45 psi, a drying gas flow rate of 10 L min^{-1} and a drying gas temperature of 325 ºC. The ICC target for the quadrupole ion trap mass analyzer was also studied, and the optimized level for this work was 100,000 ions.

Chromatographic separation was achieved by using a C_{18} analytical column with reversed-phase gradient elution with a ratio of 0.1% formic acid in water: 0.1% formic acid in ACN as a mobile phase at a flow rate of 0.4 mL min^{-1}. The elution order was Sudan I, Sudan II, Sudan III and Sudan IV. The analysis time for these dyes was 8 minutes.

The optimal extraction conditions utilized acetone as the extraction solvent. A C_{18} SPE sorbent and ratio of washing solvent of MeOH: H_2O of 50:50 were chosen as the optimal SPE conditions.

The optimal on-line SPE-LC-MS conditions were a loading volume of 20 µL and a switching time of 30 seconds. All four Sudan dyes were separated in under 8 minutes, and the total analysis time was 12 minutes (include re-equilibration) using the optimum on-line SPE-LC-MS system.

Method validation is establishment of the performance and limitations of a method. The on-line SPE-LC-MS method for the determination of Sudan I-IV was

validated based on the following parameters: LOD, LOQ, linearity range, matrix effect, precision and accuracy. The limits of detection (LOD) and limits of quantitation (LOQ) ranged from 0.03 to 0.04 ppm and 0.05 to 0.1 ppm, respectively. Studies of the matrix effect indicated that the matrix had no significant effect on the determination of Sudan dyes in chili powder. This method showed good analytical characteristics, had a good linear relationship with R^2 values of more than 0.9997 and the LODs were lower than those recommended by the EU and US, as shown in Table 5.1.

Table 5.1 Characteristics validation data consists of linearity range, coefficient of determination (R^2), limit of detection (LOD) and limit of quantiation (LOQ) of each compound in chili powder matrix

Compound	Linearity range ($\mu g\ mL^{-1}$)	R^2	LOD (ppm)	LOQ (ppm)
Sudan I	0.005-1.0	0.9999	0.03	0.05
Sudan II	0.005-1.0	0.9997	0.03	0.05
Sudan III	0.010-4.0	0.9997	0.04	0.1
Sudan IV	0.005-1.0	0.9998	0.03	0.05

The method precision and accuracy were evaluated at three concentration levels (LOQ, medium and high) for both intra-day precision and intermediate precision (inter-day precision) in the chili powder matrix. Satisfactory intra-day precision of this method on two different days was achieved with a relative standard deviation that was lower than the limited %RSD derived from the Horwitz equation. The statistical *F*-values of all of the analytes were less than the critical *F*-value at the 95% confidence level, which indicated a good intermediate precision of this method.

The overall recoveries of all analytes at the LOQ, medium and high levels were greater than 90%. This demonstrated the acceptability of the method accuracy according to the AOAC standard at the ppm concentrations studied (80-110%).

Furthermore, this new method could be applied to determine Sudan I-IV in other chili products such as chili sauce, roasted chili paste and dried chili. Its feasibility was demonstrated by using spiked samples at three different concentration levels. It was found that recoveries were greater than 90% for Sudan I-IV.

Moreover, the novelty of this method is the analysis time. Although the detection limit is not as good as other LC-MS (/MS) methods but the analysis time is better. As shown in Table 5.2, it was found that the proposed method has the shortest retention time than those of the LC methods using MS or MS/MS detection.

Table 5.2 Comparison of retention times of Sudan dyes with different methods

Method	Retention time (min)			
	Sudan I	Sudan II	Sudan III	Sudan IV
LC-ESI-MS/MS [6]	7.01	9.80	11.30	17.94
LC-ESI-Q-TOF-MS [7]	11.70	16.62	21.20	26.80
HPLC-DAD-ESI-MS [8]	17.79	20.52	23.33	29.75
GPC-LC-ESI-MS/MS [11]	16.5	22.5	25.3	30.8
DSSBME-and U-shaped HF-LPME-LC-ESI-Q-TOF-MS [12]	4.3-4.5	5.6-5.8	6.4-6.6	9.8-10.0
On-line SPE-LC-MS (this work)	2.7-2.9	4.0-4.2	5.2-5.4	7.3-7.5

Therefore, it can be concluded that the on-line SPE-LC-MS method for the determination of Sudan dyes in chili products with column switching provide reduced analysis time, minimized reagent and sample consumption and high recoveries. Finally, the proposed method can be used to analyze Sudan dyes in varieties of chili samples.

5.2 Suggestion for Further Work

In this study, only Sudan I-IV were studied. The proposed method could be extended to the determination of other oil-soluble synthetic dyes of red or orange color, such as Para red, Sudan red B, Sudan red G and Sudan orange G. These synthetic dyes should be of particular interest for the study using on-line SPE-LC-MS as well.

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APPENDICES

APPENDIX A

Mass Spectrum of Standard Sudan I-IV

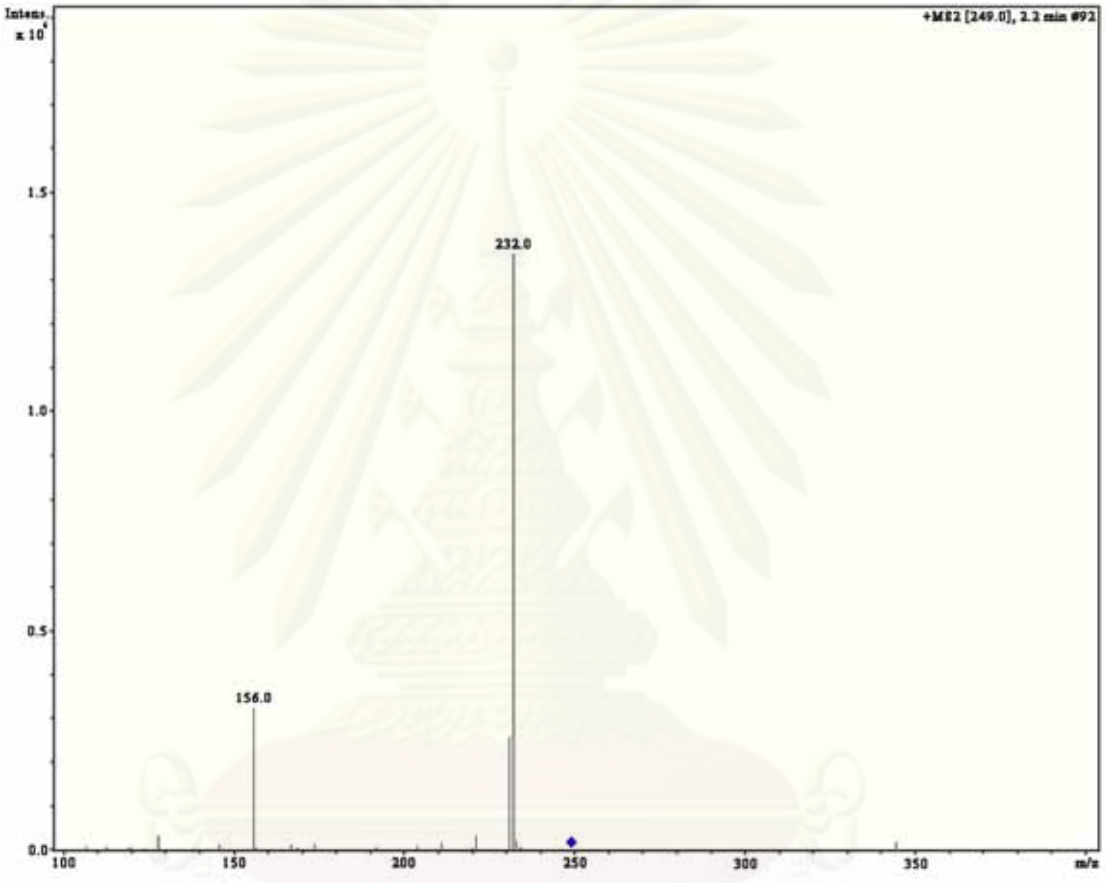

Figure A1 Mass spectrum of standard Sudan I 0.5 µg mL^{-1} analyzed by on-line SPE-LC-MS under optimal conditions

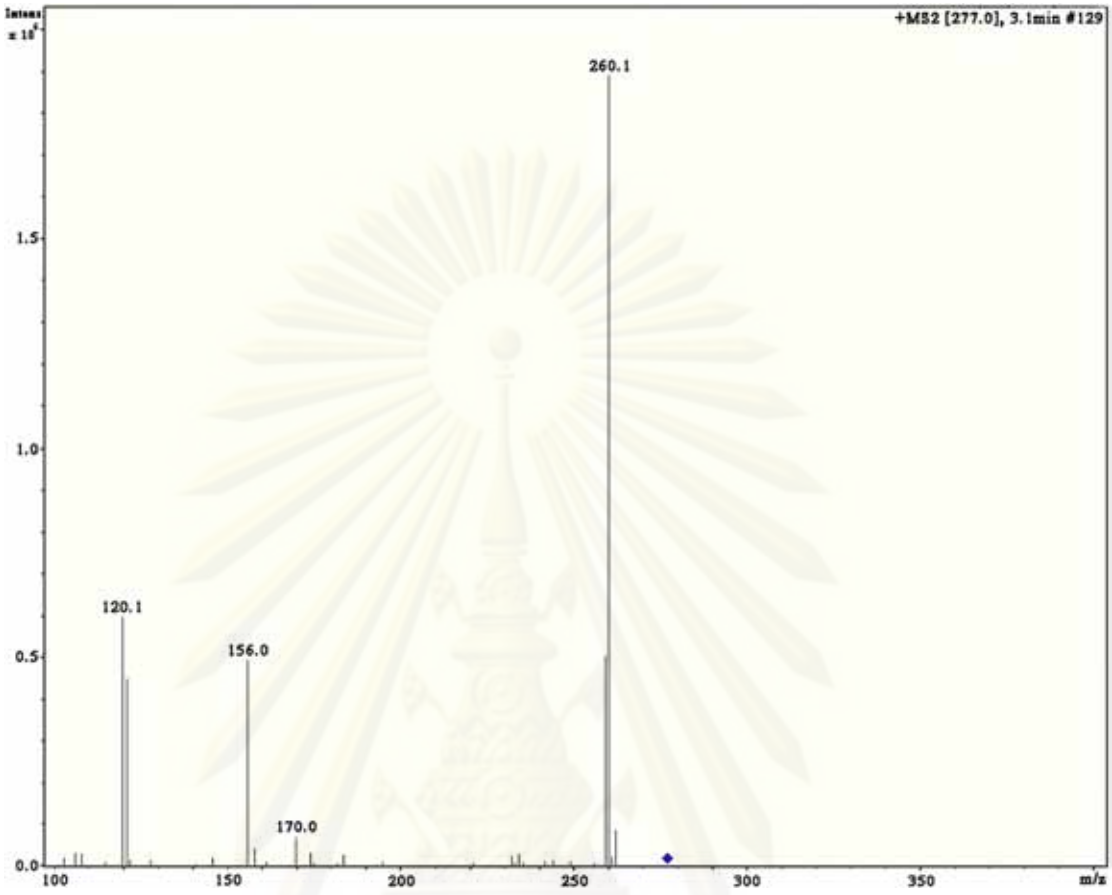

Figure A2 Mass spectrum of standard Sudan II 0.5 μg mL^{-1} analyzed by on-line SPE-LC-MS under optimal conditions

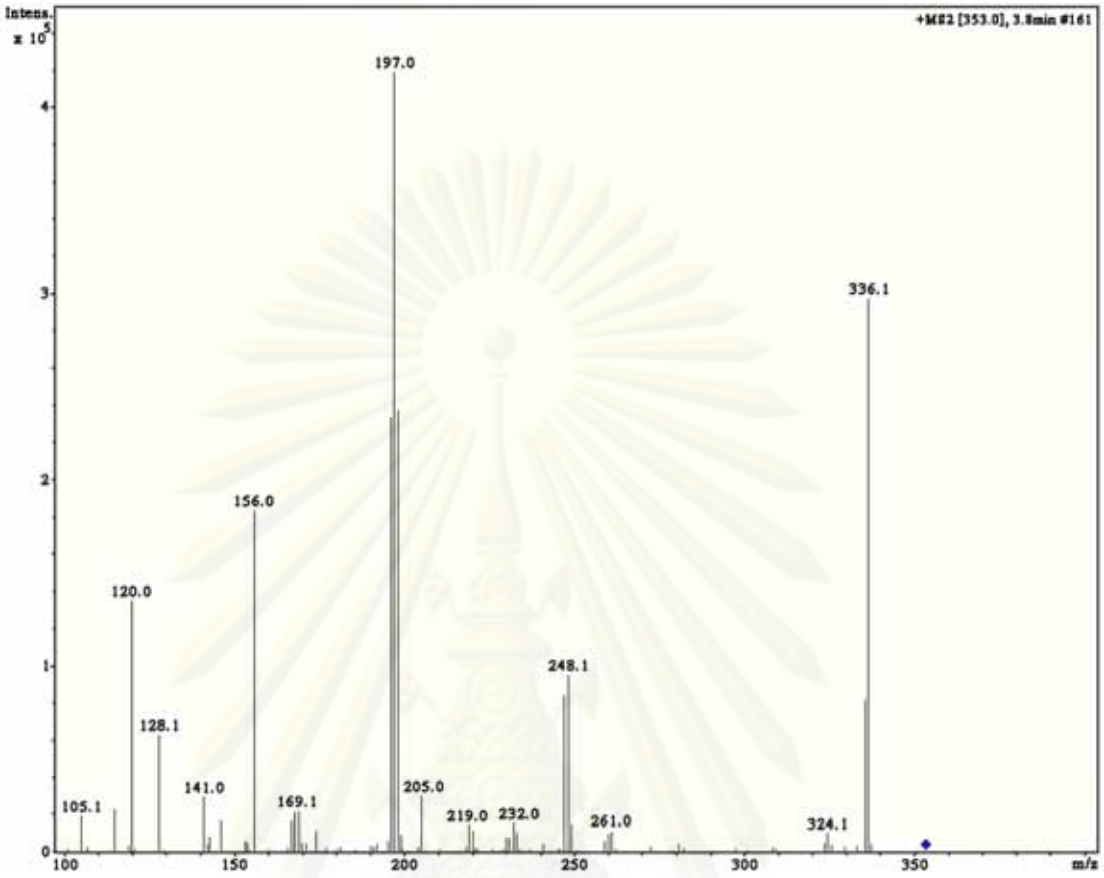

Figure A3 Mass spectrum of standard Sudan III 0.5 µg mL^{-1} analyzed by on-line SPE-LC-MS under optimal conditions

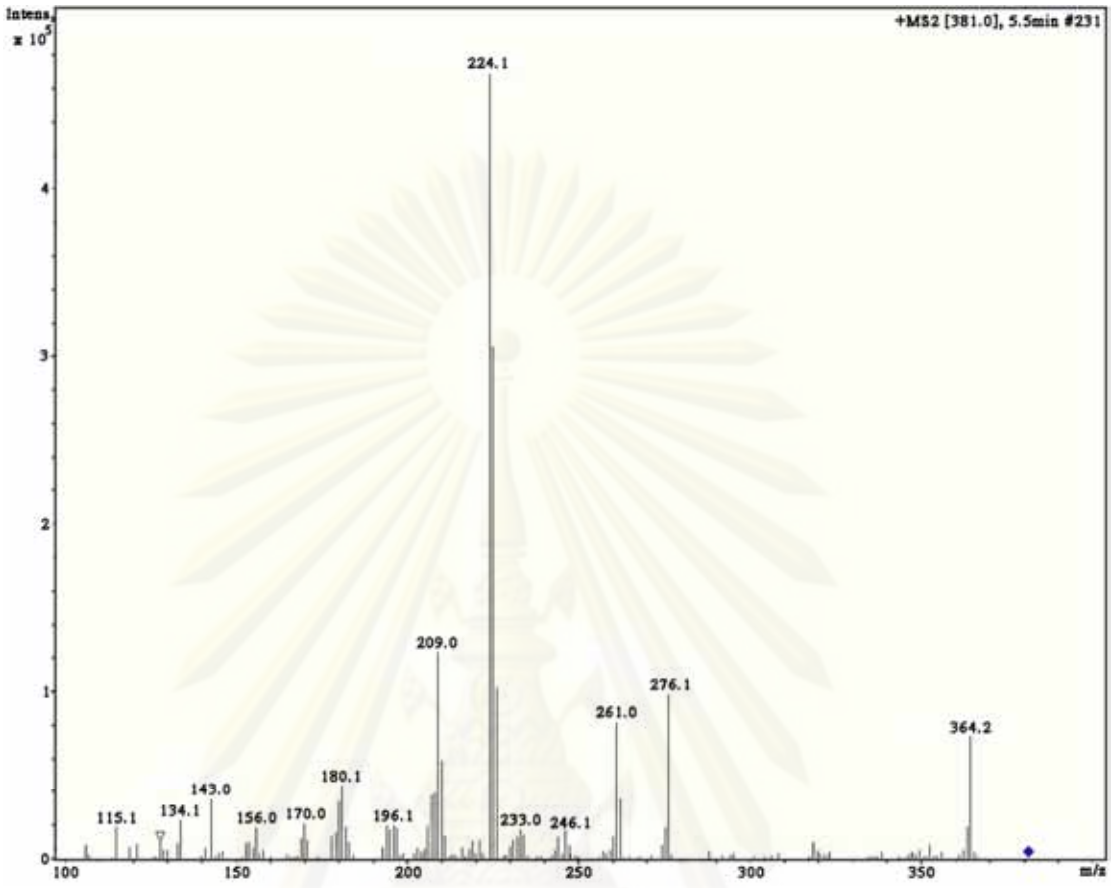

Figure A4 Mass spectrum of standard Sudan IV 0.5 µg mL^{-1} analyzed by on-line SPE-LC-MS under optimal conditions

APPENDIX B

Matrix Calibration Curve of Sudan I-IV

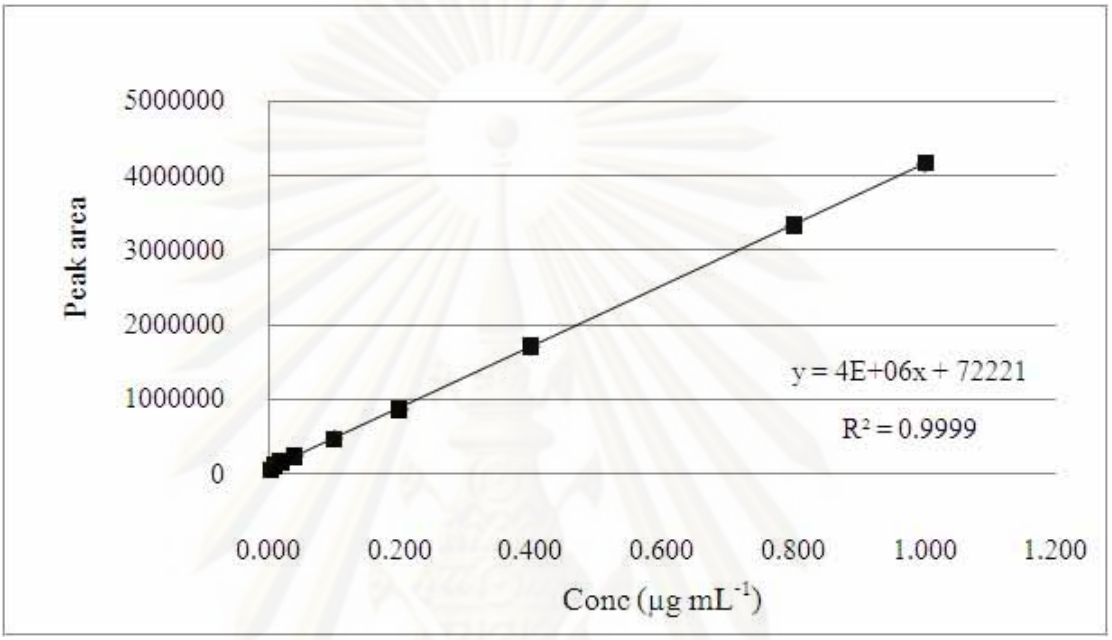

Figure B1 Matrix calibration curve of Sudan I

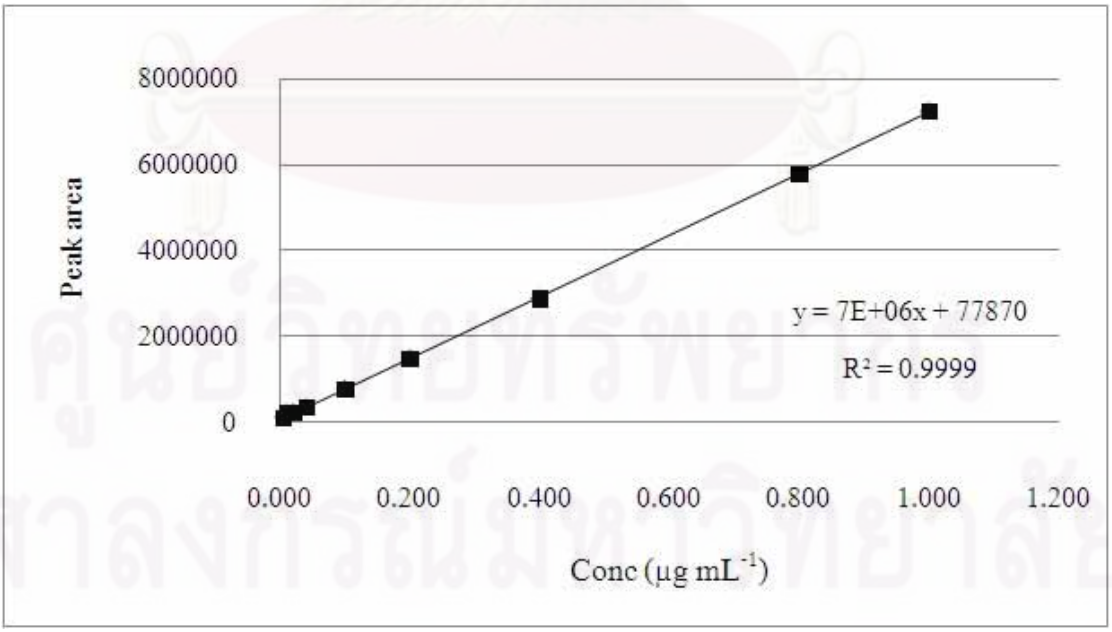

Figure B2 Matrix calibration curve of Sudan II

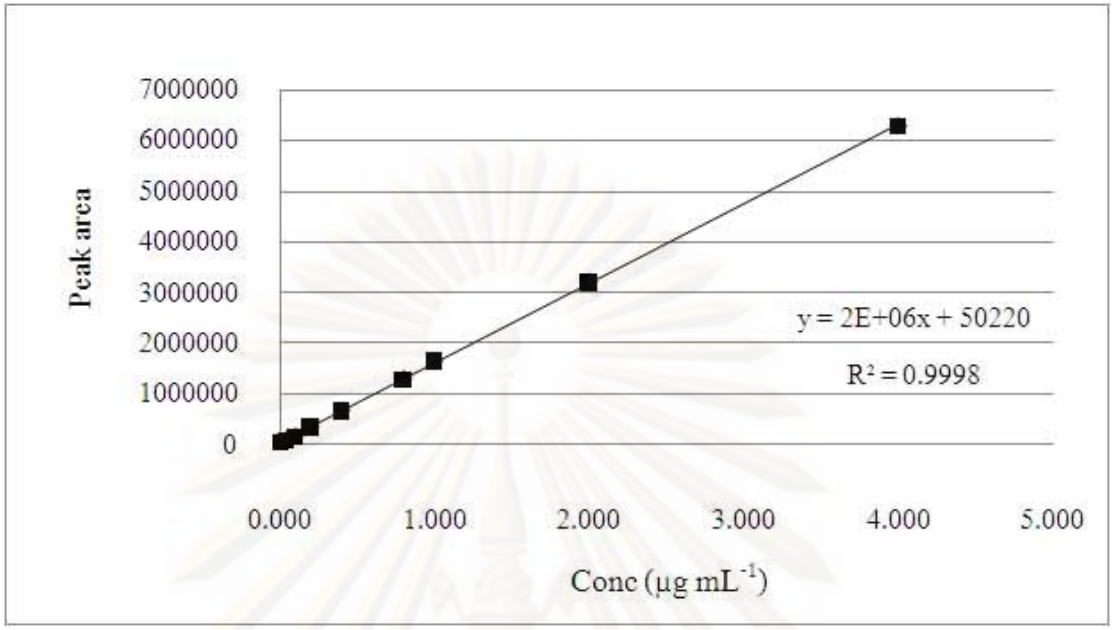

Figure B3 Matrix calibration curve of Sudan III

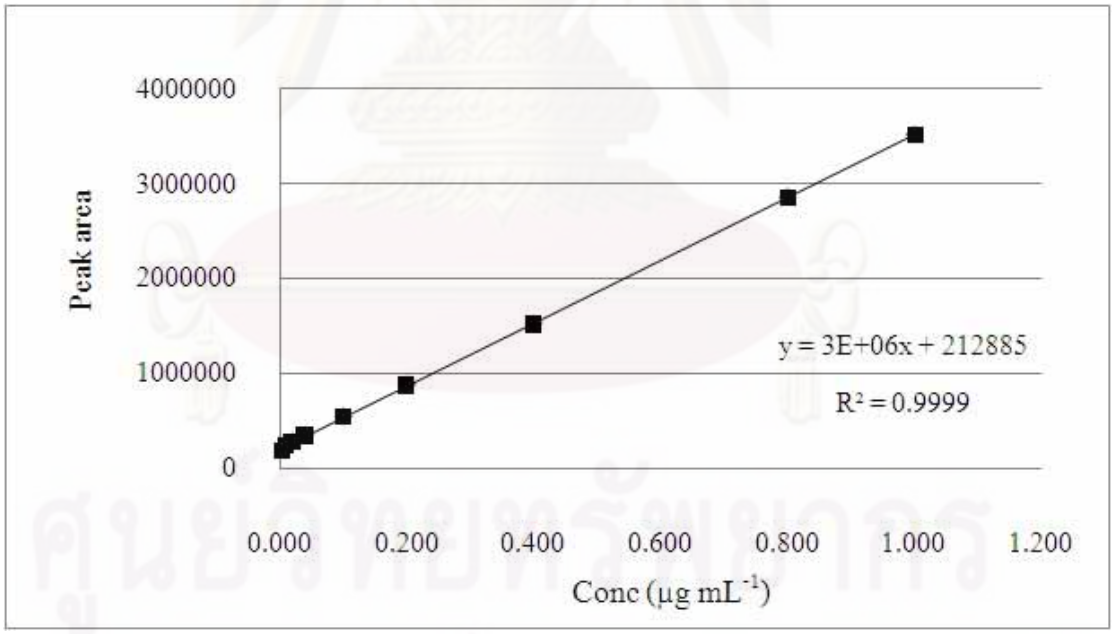

Figure B4 Matrix calibration curve of Sudan IV

APPENDIX C

Precision and Accuracy

Table C1 Expected %RSD calculated from Horwitz's equation

Analyte concentration	C	Expected %RSD
100% (100 g / 100 g)	1	$0.66 \times 2 \times (1^{-0.1505})$ = 1.3
10% (10 g / 100 g)	0.1	$0.66 \times 2 \times (0.1^{-0.1505})$ = 1.8
1% (1 g / 100 g)	0.01	$0.66 \times 2 \times (0.01^{-0.1505})$ = 2.6
0.1% (0.1 g / 100 g)	0.001	$0.66 \times 2 \times (0.001^{-0.1505})$ = 3.7
100 ppm (100 mg/kg)	$1 \times 10^{-4} = 0.0001$	$0.66 \times 2 \times (10^{-4})^{-0.1505}$ = 5.2
10 ppm (10 mg/kg)	$1 \times 10^{-5} = 0.00001$	$0.66 \times 2 \times (10^{-5})^{-0.1505}$ = 7.4
1 ppm (1 mg/kg)	$1 \times 10^{-6} = 0.000001$	$0.66 \times 2 \times (10^{-6})^{-0.1505}$ = 10.5
0.1 ppm (100 ppb)	$1 \times 10^{-7} = 0.0000001$	$0.66 \times 2 \times (10^{-7})^{-0.1505}$ = 14.9
0.01 ppm (10 ppb)	$1 \times 10^{-8} = 0.00000001$	$0.66 \times 2 \times (10^{-8})^{-0.1505}$ = 21.1
0.001 ppm (1 ppb)	$1 \times 10^{-9} = 0.000000001$	$0.66 \times 2 \times (10^{-9})^{-0.1505}$ = 29.8

Table C2 Acceptable recovery percentages as a function of the analyte concentration (The AOAC Manual for the Peer Verified Methods Program, 1993)

Analyte concentration	% Recovery
100%	98-102
>10%	98-102
>1%	97-103
>0.1%	95-105
100 ppm	90-107
10 ppm	80-110
1 ppm	80-110
100 ppb	80-110
10 ppb	60-115
1 ppb	40-120

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