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โดยไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี

นางสาวนันทนา กลิ่นสุนทร

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SAMPLE PREPARATION FOR DETERMINATION OF STEROIDS
ADULTERATED IN HERBAL MEDICINES BY
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Miss Nantana Klinsunthorn

A Thesis Submitted in Partial Fulfillment of the Requirements

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DETERMINATION OF STEROIDS ADULTERATED IN HERBAL
MEDICINES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)
อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.ธรรมนูญ หนูจักร, 59 หน้า.

ได้หาภาวะที่เหมาะสมและตรวจสอบความใช้ได้ของวิธีการเตรียมตัวอย่างด้วยเทคนิค
QuEChERS สำหรับการวิเคราะห์ด้วยไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟีของสเตอรอยด์ 9
ชนิดที่ปลอมปนในยาสมุนไพรชนิดน้ำ ได้แก่ triamcinolone, prednisolone, hydrocortisone,
methylprednisolone, betamethasone, dexamethasone, beclomethasone, fludrocortsone
acetate และ cortisone acetate ในขั้นตอนของ QuEChERS เริ่มจากการสกัดตัวอย่างที่เป็น
ของเหลว (2.0 มิลลิลิตร) ด้วยแอซีโตไนไตรล์ (2.0 มิลลิลิตร) ที่มีแอนไฮดรัสแมกนีเซียมซัลเฟต
(500 มิลลิกรัม) และโซเดียมคลอไรด์ (125 มิลลิกรัม) จากนั้นกำจัดสารรบกวนด้วย dispersive-
solid phase extraction โดยใช้ primary secondary amine (50 มิลลิกรัม) เป็นตัวดูดซับ
ร่วมกับแมกนีเซียมซัลเฟตเพื่อกำจัดน้ำส่วนที่เหลือ พบว่าร้อยละการคืนกลับในการสกัดสเตอรอยด์
ทั้ง 9 ชนิด ได้ผลเป็นที่น่าพอใจอยู่ในช่วง 91—113 % และได้เกณฑ์ที่ยอมรับของความเที่ยงในร้อย
ละการคืนกลับการสกัดภายในวันเดียวกันและต่างวันกัน โดยมีค่าเบี่ยงเบนมาตรฐานสัมพัทธ์น้อย
กว่าหรือเท่ากับ 3.4 % การเตรียมตัวอย่างด้วยเทคนิค QuEChERS ที่พัฒนาขึ้นนี้ให้การตรวจวัด
สเตอรอยด์ที่ปลอมปนอย่างน่าเชื่อถือด้วยขีดจำกัดของการตรวจวัดในช่วง 0.06—0.17 ppm จาก
การวิเคราะห์ตัวอย่างยาน้ำสมุนไพรในเชิงพาณิชย์จำนวน 6 ตัวอย่าง พบว่า 3 ตัวอย่างมีการ
ปลอมปนสเตอรอยด์ที่เป็น dexamethasone ปริมาณ 1.6 และ 8.8 ppm และ prednisolone
ปริมาณ 0.43 ppm

ภาควิชา ___เคมี___ ลายมือชื่อนิสิต _นันทนา กลิ่นสุนทร_
สาขาวิชา ___เคมี___ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก _____
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KEYWORDS : d-SPE / HERBAL MEDICINES / HPLC / QuEChERS / SAMPLE PREPARATION / STEROIDS

NANTANA KLINSUNTHORN: SAMPLE PREPARATION FOR DETERMINATION OF STEROIDS ADULTERATED IN HERBAL MEDICINES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. ADVISOR: ASSOC. PROF. THUMNOON NHUJAK, Ph.D., 59 pp.

QuEChERS sample preparation was optimized and validated for high-performance liquid chromatographic determination of nine steroids adulterated in liquid herbal medicines: triamcinolone, prednisolone, hydrocortisone, methylprednisolone, betamethasone, dexamethasone, beclomethasone, fludrocortsone acetate and cortisone acetate. In QuEChERS procedures, a liquid sample (2.0 ml) was first extracted with acetonitrile (2.0 ml) in the presence of anhydrous magnesium sulfate (500 mg) and sodium chloride (125 mg). The sample cleanup was then performed by dispersive-solid phase extraction using primary secondary amine sorbents (50 mg) as well as magnesium sulfate for the removal of residual water. Satisfactory extraction recoveries of 91–113 % for all nine steroids were obtained, along with an acceptable intraday and interday precision in extraction recoveries shown by relative standard deviation of $\leq$ 3.4 %. The QuEChERS sample preparation developed here allows the reliable detection of adulterated steroids with the limits of detection in the range of 0.06–0.17 ppm. Adulterated steroids in three out of six commercial herbal medicines were found, such as 1.6 and 8.8 ppm dexamethasone and 0.43 ppm prednisolone.

Department : _____Chemistry_____ Student's Signature _Nantana Klinsunthorn_

Field of Study : _____Chemistry_____ Advisor's Signature _Thumnoon Nhujak_

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LIST OF ABBREVIATIONS

ACN	acetonitrile
AOAC	Association of Official Analytical Chemists
BTS	betamethasone
BCS	beclomethasone
cm	centimeter
CSA	cortisone acetate
C 18	octadecylsilane
°C	degree celsius
DAD	Diode array detector
DXS	dexamethasone
EtOAc	ethyl acetate
FCA	fludrocortisone acetate
g	gram
g/cm^3	grams per cubic centimeter
GBC	graphitized carbon black
GC	gas chromatography
GC/MS	gas chromatography mass spectrometry
g/mL	gram per milliliter
HPLC	High performance liquid chromatography
HCS	hydrocortisone
HLB	hydrophilic-lipophilic balances copolymer
I.D.	internal diameter
LC	liquid chromatography
LC-MS	liquid chromatography tandem mass spectrometry
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
mm	millimeter
min	minute
mg	milligram

ml	milliter
M.W.	molecular weight
ml/min	milliliter per minute
ng/g	nanogram per gram
ng/ml	nanogram per milliliter
nm	nanometer
MPS	methylprednisolone
ppb	part per billion
ppm	part per million
PNL	prednisolone
PSA	primary secondary amine
QuEChERS	Quick Easy Cheap Effective Rugged Safe
rpm	round per minute
RSD	relative standard deviation
R^2	correlation coefficient
Rs	resolution
S.D.	standard deviation
S/N	signal to noise ratio
SPE	solid-phase extraction
TLC	thin-layer chromatography
TR	triamcinolone
UV-Vis	Ultraviolet -Visible
wt/wt	weight by weight
μg/l	microgram per liter
μg/ml	microgram per milliliter
μl	microliter
μm	micrometer
%	percentage

CHAPTER I

INTRODUCTION

1.1 Problem Definition

Herbal medicine is important in the treatment of diseases and becoming increasingly popular. This is because consumers perceive herbals as safe, harmless, and free from side effects [Lau *et al.*: 2003; Yee *et al*.: 2005; Bogusz *et al*.: 2006; Sazlina and Zation: 2009]. Currently, the production of herbal medicine is not well controlled or regulated. The herbal medicines may be adulterated with synthetic drugs such as steroids [Lau *et al*.: 2003; Bogusz *et al*.: 2006; Liang: 2006]. Steroids are a group of drugs intended to be mixed with herbal medicines to accelerate faster treatment. However, they cause various side effects such as osteoporosis, hyperglycemia, hypocalcemin, hypokalemia, high blood pressure and sugar, skin problems such as poor healing after injuries, and muscle wasting [Sweetman: 2007; Sazlina and Zation: 2009]. Steroids abuse is currently one of the most common public health problems in Thailand. Steroids are designated by Food and Drug Administration (FDA), Thailand, which is an Act for regulating these substances. The adulteration of steroids with herbal medicines is prohibited [Thai Drug Control Division: 2004]. Therefore, it is important to monitor the synthetic steroids in herbal medicines using an efficient analytical method. This will be the advantage for consurmers, risk assessment and health defect ending outcome of steroids. Herbs contains various chemical compositions that may interfere with determination of adulterated steroids, and therefore, sample preparation is an essential step prior to chromatographic analysis. High efficiency in the extract of steroids from the sample is required, and effective sample cleanup is needed to remove some matrices or substances that may interfere detection of the steroids of interest.

Nowadays, sample preparation with used is liquid-liquid extraction (LLE) and one of the common organic solvents used is chloroform. Disadvantages of the procedure include an emulsion formation which causes loss of compounds, resulting in lower recoveries, and the use of large volume of organic solvent, cumbersome glassware,

and high cost. In addition, chloroform, a best solvent used for extraction of traditional medicines is banned in some laboratories due to its hazard to health. In some cases of our samples, the emulsion occurred with chloroform and other solvents used for extraction. Therefore, it is necessary to develop a method for sample preparation in order to eliminate the interference of these substances prior to HPLC analysis, leading to the accurate results of the analysis with high precision.

1.2   Literature Review

In previous work, chromatographic method techniques for determination of steroids was reporting using thin layer chromatography (TLC*) [Huetos *et al.*: 1999; Gagliardi *et al.*: 2002], high-performance thin layer chromatography (HPTLC) [Vanoosthuyze *et al.*: 1993], and high-performance liquid chromatography (HPLC) [Santos-Montes *et al.*: 1993; Valvo *et al.*: 1994; Ku *et al.*: 1999, 2001; Huetos *et al.*: 1999; and Gagliardi *et al.*: 2002]. The detection method of steroids tested on TLC plates is based on spraying particular reagents on TLC plates and observing position and color of spots appeared [Huetos *et al.*: 1999; Gagliardi *et al.*: 2002]. In comparison with HPLC, TLC is simpler and cheaper but gives lower separation efficiency. A high-performance thin-layer chromatographic (HPTLC) method was used for multi-screening analysis of 26 corticosteroids standard. Development on preloaded Kiesel gel HPTLC plates with chloroform-methanol (92:8,v/v) yieled the best separation. After elution, plates were sprayed with different detection reagents, including resorcylaldehyde and sulfuric acid, heated for 10 min at 95^0C in an oven, and then examined under UV radiation at 366 nm. The resorcylaldehyde reagent is selective for the isomers, dexamethasone and betamethasone, which gives different color products [Vanoosthuyze *et al.*: 1993]. The identification and determination of steroids in Traditional Chinese Medicines [Ku *et al.*: 1999; Jin *et al.*: 2008], prednisolone adulterated in a Foreign Herbal Medicine [Ku *et al.*: 2001], pharmaceutical tablet formulations [Hashem and Jira: 2004], animal feed [Huetos *et al.*: 1999; Munuz-Valencia *et al.*: 2008], cosmetic products [Gagliardi *et al.*: 2001], and urine [Andersen *et al.*: 2008; Murry *et al.*: 2009; Saito *et al.*: 2010] were reported by using high-performance liquid chromatography (HPLC) with a UV detector [Santos-Montes *et al.*: 1993; Valvo *et al.*: 1994; Huetos *et al.*: 1999; Ku *et al.*: 1999, 2001; Gagliardi *et al.*: 2002; and Gonzalo-Lumbrears *et al.*: 2007] or a mass spectrometer [Andersen

et al.: 2008; Jin *et al.*: 2008; Murry *et al.*: 2009; Saito *et al.*: 2010]. However, prior to HPLC analysis, sample preparation was required to remove some matrices or substances that may interfere with detection of the steroids of interest to reduce separation efficiency and shorten the column life. The typical procedures of sample preparation in previous work were carried out by extracting the samples with water-insoluble organic solvent, and then followed by cleaning-up the extract with solid phase extraction (SPE) using a SPE cartridge [Huetos *et al.*: 1999; Ku *et al.*: 1999, 2001; Gagliardi *et al.*: 2002; Hashem and Jira: 2004; Gonzalo-Lumbrears *et al.*: 2007; Andersen *et al.*: 2008; Murry *et al.*: 2009; Saito *et al.*: 2010]. A Traditional Chinese medicine and and Foreign Herbal Medicine has reported [Ku *et al.*: 1999, 2001], the solvent extraction of herbal medicines with chloroform by sonication for 30 minutes and the cartridge SPE with a solid sorbent conditioning with different solvents such as methanol and chloroform, and then eluting with dichloromethane-isopropanal (6:4) and concentrated under reduced pressure to dryness and dissolved with ethanol before analysis by HPLC. However, the disadvantages of these procedures include large amounts of organic solvent used for extraction (25 to 50 mL), the expense of time for ultrasonicating or shaking (30 min), an expensive SPE cartridge, and several steps of SPE solvent elution [Ku *et al.*: 1999: 2001]. In addition, chloroform, a best solvent used for extraction of traditional medicines is banned in some laboratories due to its hazard to health. In some cases of our samples, the emulsion occurred with chloroform and other solvents used for extraction. Therefore, the friendly sample preparation should be established for analysis of herbal medicine samples.

Recently, the QuEChERS ("catchers") approach has been introduced for determination of pesticides in foods and agricultural samples, where QuEChERS stands for Quick, Easy, Cheap, Effective, Rugged, and Safe [Anastassiades *et al.*: 2003]. This approach contains two easy steps [Anastassiades *et al.*: 2003; Major: 2008]. First, the sample is extracted with organic solvent 1-10 ml in the presence of anhydrous salts by vortexing for 1-2 minutes and centrifuging for a few minutes (2-5 min). Second, the simple cleaning-up is carried out using dispersive SPE sorbents in a centrifuge tube. After sample clean up, an aliquot of the supernatant can be analysed directly or can be subjected to a concentration and solvent exchange step if nessary. In comparision with cartridge SPE the advantages of QuEChERS compared with the previous method, extraction process, is easier and faster. Insoluble solvent is used in

SPE cartridge. In dispersive-SPE [Thurman and Mills: 1998; Major: 2007], small amount of bulk SPE packing sorbents is added to the extract, for removing interference directly, not for conditioning solid sorbent.

The QuEChERS sample preparation technique was reported for determination pesticides residues in a variety of sample matrices such as vegetables and fruits [Lehotay *et al.*: 2005; Cunha *et al.*: 2007; Wang *et al.*: 2007; Banerjee *et al.*: 2007; Nguyen *et al.*: 2008; Cieslik *et al.*: 2011], food [Lehotay *et al.*: 2007; Hereegova *et al.*: 2006; Frenich *et al.*: 2008; Chung *et al.*: 2010; Wilkowska *et al.*: 2011], and olives and olive oil [Cunha *et al.*: 2007; Gilbert-Lopaz *et al.*: 2010], barley [Diez *et al.*: 2006].   In addition, extensive studies on this technique were extended to drugs in blood [Plossl *et al.*: 2006]. The QuEChERS technique has been accepted first as AOAC International Official Method 2007.01 [Lehotay: 2007].

QuEChERS sample preparation was used for determination of pesticide residues in fresh fruits and vegetables [Lehotay *et al.*: 2005; Banerjee *et al.*: 2007; Wang *et al.*: 2007; Nguyen *et al.*: 2008; Gilbert-Lopez *et al.*: 2010; Cieslik *et al.*: 2011], food [Lehotay *et al.*: 2007; Hereegova *et al.*: 2006; Frenich *et al*.: 2008; Chung *et al.*: 2010; Wilkowska *et al.*: 2011], and the amount of drug in the blood [Plossl *et al.*: 2006]. were reported using GC [Anastassiades *et al.*: 2003; Lehotay *et al.*: 2005; Diez *et al.*: 2006; Hereegova *et al.*: 2006; Plossl *et al.*: 2006; Cunha *et al.*: 2007; Paya, *et al.*: 2007; Nguyen *et al.*: 2008; Cieslik *et al*.: 2011; Wilkowska *et al.*: 2011], HPLC, [Lehotay *et al.*: 2005, 2007; Diez *et al*.: 2006; Banerjee *et al.*: 2007; Cunha *et al.*: 2007; Paya et al: 2007; Wang *et al.*: 2007; Frenich *et al*.: 2008; Chung *et al*.: 2010; Gilbert-Lopez *et al.*: 2010; Wilkowska *et al.*: 2011], with a couple mass spectrometer [Lehotay *et al.*: 2005; Hereegova *et al.*: 2006; Cunha *et al.*: 2007; Wang *et al.*: 2007; Banerjee *et al.*: 2007; Frenich *et al*.: 2008; Nguyen *et al.*: 2008; Chung *et al.*: 2010; Gilbert-Lopez *et al.*: 2010; Cieslik *et al.*: 2011; Wilkowska *et al.*: 2011]. The chief composition of samples is water, except for olives which contain fatty acid mainly and barley which is dry. Solvents used for QuEChERS extraction are for example, acetonitrile [Anastassiades *et al.*: 2003, Lehotay *et al.*: 2005, 2007; Cieslik *et al*.: 2006; Diez *et al.*: 2006; Hercegova *et al*.: 2006; Plossl *et al.*: 2006; Cunha *et al.*: 2007; Paya *et al.*: 2007; Frenich *et al.*: 2008; Gilbert-Lopez *et al*.: 2010; Wilkowska *et al.*: 2011], ethyl acetate [Anastassiades *et al.*: 2003; Diez *et al.*: 2006; Banerjee *et*

al.: 2007] and acetone [Anastassiades *et al.*: 2003; Diez *et al.*: 2006]. Advantages and disadvantage of these solvents are explained in Section 2.4.1.

Absorbents typically used for QuEChERS d-SPE include primary secondary amine (PSA) [Anastassiades *et al.*: 2003; Diez *et al.*: 2006; Cunha *et al.*: 2007; Lehotay *et al.*: 2007; Plossl *et al.*: 2006; Gilbert-Lopez *et al.*: 2010; Cieslik *et al.*: 2011; Wilkowska *et al.*: 2011], graphitized carbon black (GCB) [Anastassiades *et al.*: 2003; Cunha *et al.*: 2007; Lehotay *et al.*: 2007; Gilbert-Lopez *et al.*: 2010] and florisil [Cunha *et al.*: 2007]. More details of these absorbents are given in Section 2.4.3.

1.3   Aim and Scope

The aim of this research was to validate a QuEChERS sample preparation for HPLC determination of steroids adulterated in liquid herbal medicine. Initially work on HPLC separation of nine steroids, the gradient elution of ACN:water mobile phase was optimized on a Hypersil C18 column to achieve baseline resolution of nine steroids. Then the QuEChERS sample preparation was optimized by varying types of extracting solvent (ACN, EtOAc and acetone), the amount of salts added in the solvent extraction step (4:1 wt/wt $MgSO_4$:NaCl mg with 400:100 to 800:200 mg), types of sorbent (PSA, C18, alumina, GBC and HLB), and the amount of sorbents (25 to 100 mg). Next, the validation method for the QuEChERS method was evaluated by measuring limit of detection, limit of quantitation, standard calibration curves, linearity, accuracy, and precision. Finally, the QuEChERS method was applied for HPLC determination of steroid adulterated in real samples as liquid herbal medicines. It is expected that the QuEChERS method can be used for routine analysis at the Regional Medical Science Center 4th Samut Songkhram. Therefore, this work will be benefits for either academic or social contributions.

CHAPTER II

THEORY

2.1   Steroids

Corticosteroid is a class of steroid hormones, which is produced by the adrenal grands. It has important functions in controlling fluid balance in the body [Sweetman: 2007]. Two main types is cortisol and aldosterlone. Corticosteroids are often simply called steroid. Steroids used in the moden drug medicines are synthesized from cortisol. It is used in treating diseases such as rheumatoid arthritis, asthma, and allergies [Sweetman: 2007]. It also be used as replacement therapy some kinds of hormones deficiency. Morever, steroids are also used to treat some types of cancers. Although the use of medication or short-term or low dose does not cause serious side effects, but it is found that the use of a steroid in high dose or for a long term, it causes side-effects [Sweetman: 2007; Sazlina and Zation: 2009].

Currently, steroids are used in appropriately since they could effectively alleviate various symptoms. They can often be found in traditional medicine products which require certain activities such as reduce inflammation and relieve asthma symptoms. However, they could cause serious side effects, including the followings [Sweetman: 2007; Sazlina and Zation: 2009].

Cushing's syndrome: This is usually caused only for long-term, it can cause acne, puffiness of the face, facial hair in women and dark marks on the skin.

Muscle weakness: With very long-term use of steroids, wasting of leg muscles may occur. This can cause weakness. When stop using steroid some people have muscle cramps for a short time.

Ostoporosis (Bone thinning): When use of steroids for long-term, calcium may be lost from the bones and increased susceptibility to fractures and loss of height.

Eye changes: Occur when use of steroids for long-term, it can cause increasing the pressure in the eye, result glaucoma or cateracts, and can damage the eyes and nerves.

Irriation of the stomach lining: Steroids can irritate the lining of the stomach and may cause a stomach ulcer, or make one worse, can be severe to step through.

High blood sugar: That may need extra treatment for diabetes.patients. Steroids can occasionally raise blood sugar level and worsen diabetes symptoms.

High blood pressure: It taken in excess, steroids can raise blood pressure making body and mania retain salt and water rather than expel them.

Mood changes: Some people actually feel better in themselves when they take steroids. However, steroids may aggravate depression and other mental health problems.

Increased chance of infection: This happens mainly with high-dose or long-term treatment, such as from fungi, yeast and bacteria. Since steroids often affect immune system.

2.2.1 Physical and Chemical Properties of steroids

Steroids are a class of organic compounds with chemical structure derived from, cyclopentoperhydrophenanthrene as shown in Figure 2.1 [Sweetman: 2007]. The core structures of steroid includes seventeen carbon atoms, bonded together to have the form of four fused rings and three cyclohexane rings (designated as rings A, B, and C) and a cyclopentane ring (D)
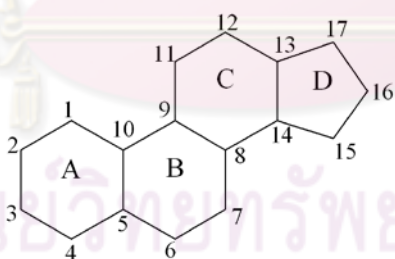
Figure 2.1 The basic steroid skeleton of cyclopentaperhydrophenanthrene.

Synthetic steroids used for treatment of illnesses are for example, triamcinolone (TR), prednisolone (PNL), hydrocortisone (HCS), methylprednisolone (MPS), betamethasone (BCS), dexamethasone (DXS), beclomethasone (BCS), fludrocortisone acetate (FCA) and cortisone acetate (CSA). The structure, physical and chemical properties of synthetic steroids [Sweetman: 2007] are shown in Figure 2.2 and Table 2.1.
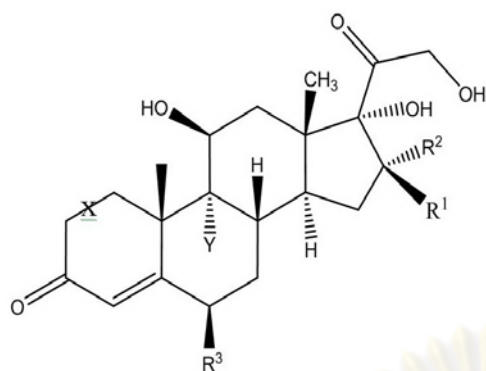
	X	R^1	R^2	R^3	Y
dexamethasone	CH=CH	H	CH_3	H	F
betamethasone	CH=CH	CH_3	H	H	F
prednisolone	CH=CH	H	H	H	H
methylprednisolone	CH=CH	H	H	CH_3	H
beclomethasone	CH=CH	CH_3	H	H	Cl
triamcinolone	CH=CH	H	OH	H	F
hydrocortisone	CH_2-CH_2	H	H	H	H

	X	R^1	R^2	Y
cortisone acetate	CH_2-CH_2	H	H	H

	X	R^1	R^2	Y
fludrocortisone acetate	CH_2-CH_2	H	H	F

Figure 2.2 The structure of synthetic steroids.

Table 2.1 Physical and chemical properties of steroids [Sweetman: 2007]

Compounds	MW	Colour	MP (^{o}C)	Solubility
triamcinolone	394.4	white	269-271	Soluble 1 in 500 of water and 1 in 240 of ethanol
prednisolone	360.4	white	240-241	Soluble 1 in 300 of water and 1 in 30 of ethanol
hydrocortisone	362.5	white	214	Soluble 1 in 100 of alcohol and slightly soluble in acetone and chloroform
methyprednisolone	374.5	white	228-237	Soluble 1 in 40 of ethanol and slightly soluble in chloroform
betamethasone	392.5	white to creamy-white	240	Soluble 1 in 75 of ethanol and very slightly soluble in ether
dexamethasone	392.5	white	268-271	Soluble 1 in 42 of ethanol and 1 in 65 of chloroform and soluble in acetone
beclomethasone	408.9	a white to creamy-white	212	Soluble 1 in 60 of ethanol and 1 in 8 of chloroform
fludrocortisone acetate	422.5	a white to pale yelow	233-234	Soluble 1 in 50 of ethanol, 1 in 8 of chloroform and 1 in 250 of ether
cortisone acetate	402.5	white	235-238	Soluble 1 in 5000 of water, 1 in 300 of ethanol and 1 in 4 of chloroform

Note: MW – Molecular weight   MP – Melting point

2.2 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a chromatographic technique widely used in analytical laboratories. Numerous analytical HPLC analyses have been developed for pharmaceutical, chemical, food, cosmetic and environmental applications. Normally HPLC is used in analytical chemistry to separate mixtures into their individual components, thus it can identify and quantify compounds. The separation principle is based on the interactions between analytes and the stationary phase as well as mobile phase. In general, a typical HPLC system consisting of mobile phase reservoirs, pump, injector, column, and detector. A schematic diagram of an HPLC system [Ardrey: 2003; Meyer: 2004], illustrating its major components, is shown in Figure 2.3. These components are discussed in details below.

Mobile phase reservoirs	Pump	Injector	Column	Detector

Figure 2.3 Schematic diagram of a typical HPLC instrument.

2.2.1 Mobile Phase reservoirs

Mobile phase is the part of the chromatographic system which carries the solutes through the stationary phase (column), and the distribution of the solutes occurs between these two phases The mobile phase may be polar (reversed phase) or a non-polar solvent (normal phase). In HPLC, the mobile phase must be chosen to complement the stationary phase so that the selected interactions are concentrated in the stationary phase. Morever, all liquid must be filtered by filter membrane prior to entering HPLC system in order to eliminate small paticles from the system. The solvent used as mobile phase is generally HPLC grade due to reaction between impurities in solvent and solute might be occured [Ardrey: 2003; Meyer: 2004].

2.2.2 Pump

The function of the pump is pushing mobile phase through the column with high pressure and controlling flow rate. The HPLC pump should produce, typical flow rates of 0.5-5.0 mL/min that operated at 3000-6000 psi. To operate at these pressures and remain sensibly inert enough to the wide variety of solvent used, HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. Two major categories of pump applied are constant flow or constant pressure. Constant flow pump (or constant volume pump) generates steady flow of liquid but pressure vary depends on the flow resistance while constant pressure pump (or pneumatic pump) applies a constance pressure to the mobile phase but flow rate changes with the flow resistance. Most HPLC instruments use reciprocating pumps for both maintaining a constance flow rate up to several milliliters per minute and obtaining high output pressure to push the mobile phase through the chromatographic column. Reciprocating pump generates pulsed flow that induces noise to the chromatogram. To eliminate this problem, a pulse damper is placed on the outer side of the pump [Ardrey: 2003; Meyer: 2004].

2.2.3 Sample Introduction (Injector)

The purpose of the sample introduction system is to apply the analytes carried out into the column in a narrow band. The analyte is injected into the HPLC system at injection port which commonly consists of an injection valve and a sample loop. The analyte is typically dissolved in the mobile phase before injection into the sample loop, then the sample is drawn into a syringe and injected into the loop via the injection valve. Valve rotor will close and open the loop in order to inject the sample into the stream of the mobile phase.

Normally, loop volume ranges between 10 µL and over 500 µL. The two types of injector are manual injector and auto injector. The manual injector can be done with only single injection and auto injector can be programmed to do up to 99 injections in a sequence but in modern HPLC systems, the sample injection is typically automated. The function of the injector is to place the sample into the high-pressure flow in as small volume as possible so that the sample enters the column as a homogeneous,

low-volume plug. To minimize spreading of the injection volume during transport to the column, the possible shortest length of tubing from the injector to the column should be used [Ardrey: 2003; Meyer: 2004].

2.2.4 Column (Stationary Phase)

HPLC column is normally stainless steel tube packed with a stationary phase inside. It is used to transport analyte and mobile phase, and provide the the environment in which separation is achieved. Typically, the column has a number of alternative lengths and diameters such as 10-35 cm for lengths and internal diameter of 1-10 mm. Column can also be packed with 3-10 μm diameter particles. There are many types of the stationary phase such as reversed phase, normal phase, size exclusion, and ion exchang etc. The majority of HPLC using reversed phase system is the column containing chemically modified silica stationary phase (non polar). Where mobile phase is generally a mixture of polar solvents like acetonitrile and water, the stationary phase is often a silica-based packing covalently bonded with hydrophpobic alkly chains of C8 (octyl group) or C18 (octadecyl group). The reversed phase column separates analytes on the basic of their hydrophobicity, with the more hydrophobic compounds being retained longer in the column [Ardrey: 2003; Meyer: 2004].

2.2.5 Detector

The detector for HPLC is the component that monitors the eluting compound and subsequently signals a peak on the chromatogram. The most popular HPLC detector based on spectroscopic measurement are UV-visible detector. The resulting chromatogram of UV-visible detector is a plot of absorbance as a function of elution time.

Nowadays, diode array detector (DAD) is commonly used as a chromatographic detector and an instrument gives a three-dimensional chromatogram showing absorbance as a function of wavelength and elution time. A DAD is a linear array of discrete photodiode on an integrated circuit (IC) chip. For spectroscopy, it is placed at the image plain of a spectrometer to allow a range of wavelengeh to be detected simultaneously. Array detectors are especially useful for recording the full UV-vis

absorption spectra of samples are rapidly passing through a sample flow cell. A DAD offers many advantages in terms of specificity, sentivity, speed and ruggedness. The data produced, comprising both retention time and absorption of spectra of eluting chemical entities, result in identification power at low cost [Lambert: 1997; Ardrey: 2003; Meyer: 2004].

2.3   Sample Preparation Techniques

Sample preparation is an important step in analytical techniques and is developed to improve the analysis hence time consuming should be minimized. This is necessary when a given sample cannot be directly analysis or when direct analysis generates poor results, typical problems with analysis are interferences and low sensitivity. The purpose of sample preparation is usually needed to eliminate interferences and increase sensitivity [Somenath: 2003]. Normally, the samples often contain two different parts as the analytes and matrix. The analytes are the compounds of interest that must be analyzed, while the matrix is the unwanted substance of the sample, which does not require any analysis. Sample preparation may target the matrix of the sample, the analytes or both. If the sample is solid, one common operation in sample preparation is dissolution of the sample by using appropriate reagent. Then, the matrix is usually modified during a cleanup and increase the concentration steps. An analyte also can be modified by chemical reactions such as derivatization in order to obtain better result of the chromatographic analysis.

2.4   QuEChERS

As previously mentioned in Section 1.2, QuEChERS sample preparation can be performed by two steps: solvent extraction in the presence of salts and d-SPE. More details of solvent extraction, addition of salt and d-SPE are discused in this section.

2.4.1 Solvent Extraction

As previously mentioned in Section 1.2, the solvents most commonly used in QuEChERS are acetonitrile, acetone, and ethyl acetate [Major: 2008]. Each solvent has some advantages and disadvantages in terms of selectivity and practical matters.

Ethyl acetate is suitable for extracting non-polar and a medium polar [Diez *et al.*: 2006], acetonitrile is suitable for extraction of non-polar, medium-polar and polar compounds [Diez *et al.*: 2006], while acetone is effciency for extraction of high polar [Diez *et al.*: 2006]. Ethyl acetate has the advantage of partial miscibility with water but in the extracts used in conjuction with lipids and waxes, obtains lower recoveries for acid-base pesticides, and provides less clean up in d-SPE. Acetonitrile does not extract as much lipophilic materials, e.g., waxes, fat, and lipophilic pigments [Anastassiades *et al.*: 2003; Diez *et al.*: 2006]. In addition, the miscibility of acetonitrile and acetone with water leads to a single-phase solvent extraction of the sample matrix. Except for samples with high sugar content, acetonitrile and water form two phases. Acetone needs a nonpolar co-solvent to induce a well-defined phase separation with water, while acetonitrile alone is sufficient to perform excellent extraction efficiency without the need to add nonpolar cosolvent that dilute the extract and make the extracts too nonpolar. In comparison between acetone and acetonitrile in the presence of salts, acetonitrile is the more appropriate solvent for QuEChERS because acetonitrile is separated more easily from water [Anastassiades *et al.*: 2003; Diez *et al.*: 2006], and allow the better removal of residul water with magnesium sulphate. In addition, acetonitrile is very useful in reversed-phase HPLC, but it may result in a large solvent expansion volume during GC vaporization, interferes with nitrogen phosphorus (NPD) detectors, and is less volatile than the other common organic solvents because of its low viscosity, therefore the process of evaporative concentration steps more time consuming [Anastassiades *et al.*: 2003; Diez *et al.*: 2006; Major: 2008].

2.4.2 Addition of Salts

As previously mentioned in Section 1.2, the purpose of salt ($MgSO_4$ and NaCl) addition to induce phase separation of water and organic solvent by promoting partitioning of the sulphate exceeded saturation concentration [Anastassiades *et al.*: 2003; Major: 2008]. The salting-out effect resulting from addition of NaCl usually leads to an increase in extraction efficiency of polar compounds, but this also depends on the nature of the solvents involved in the partitioning step. The concentration of salt can influence the percentage of water in the organic phase and can adjust in the polarity of the phase.

MgSO$_4$ and NaCl in the ratio 4:1 is widely used for separation and adjustment of the polar substances [Anastassiades *et al.*: 2003; Banerjee *et al.*: 2007; Cunha *et al.*: 2007; Diez et al: 2006; Hereegova *et al.*: 2006; Gilbert-Lopez *et al.*: 2010; Lehotay *et al.*: 2007; Nguyen *et al* 2007; Paya *et al.*: 2007; Wang *et* al.: 2007; Wilkowska *et al.*: 2011]. However, addition of magnesium sulfate to cause large amounts of exothermic. If the sample can degraded by heat, it is needed to extract at low temperatures to increase the efficiency of extraction [Anastassiades *et al.*: 2003]. In addition, the use of more MgSO$_4$ makes vortexing difficult due to the formation of conglomerates.

2.4.3 Dispersive Solid-Phase Extraction (d-SPE)

In d-SPE, an upper layer of sample extract is added to a centrifuge tube containing a small amount of SPE sorbent (for example, 50 mg of primary secondary amine) and the mixture will be shaken and mixed on a vortex mixer to distribute SPE material and facililate cleanup process easier. The sorbent is then separated by centrifugation or filtering, and the supernatant can be analyzed directly or can be subjected to a concentration and/or solvent exchange step if necessary [Major: 2008]. This approach is most convenient when the SPE sorbents act as a "chemical filter" to remove matrix components or adsorbs matrix co-extractives onto its surface but analytes are unretained. Table 2.2 lists six types of sorbents widely used in the QuEChERS d-SPE. Each type has different characteristic and effectiveness in cleanup matrix components in samples.

Table 2.2 Examples of sorbent type used in d-SPE

Type of sorbent	Characteristic and effectiveness
primary secondary Amine (PSA)	Weak anion exchange and modified silica functionalized with ethylenediamine groups. PSA is very similar to NH_2 material, but has a higher ionic capacity than NH_2 that removes fatty acids, other organic acids, sugars, pigments and compounds containing hydrogen bond [Anastassiades *et al.*: 2003; Lehotay *et al.*: 2007; Diez *et al.*: 2006; Plossl *et al.*: 2006].
alumina	Weak anion exchange with polar capability which can remove fatty acids, other organic acids, sugars and pigments [Anastassiades *et al.*: 2003; Lehotay *et al.*: 2007].
aminopropyl (-NH_2)	It is modified silica with an aminopropyl group which can retain sugar and other polar substances through hydrogen bonding and acidic compounds like free fatty acids through anion exchange [Anastassiades *et al.*: 2003; Lehotay *et al.*: 2007; Diez *et al.*: 2006; Plossl *et al.*: 2006].
florisil	Remove of interference in the sample containing high fat [Frenich *et al.*; 2008].
graphitized carbon black (GCB)	A strong affinity toward planar molecules and thus effectively removes pigments such as chlorophyll, carotinoids, sterols and structrally planar matrix components [Anastassiades *et al.*: 2003; Lehotay *et al.*: 2007; Cunha *et al.*: 2007].
Octadecylsilane (C 18)	Remove polar and non polar matrix compounds such as lipids and waxes [Anastassiades *et al.*: 2003; Lehotay *et al.*: 2007].

CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1 High performance liquid chromatograph, (a module 1200 Series, Agilent Technologies, USA) consists of a vacuum degasser, a quaternary pump, an autosampler, a column thermostat, and diode array detector

3.1.2 An HPLC column: Hypersil BDS, C_{18} column 4.6 mm $\times$ 300 mm I.D., 5 µm, Thermo Scientific, U.S.A.

3.1.3 A Milli-Q Ultrapure water system, Elga Ltd., England

3.1.4 An Analytical balance (5 digits), Model BP 210 D, Max 210 g, Sartorius AG Goettingen, Germany

3.1.5 An analytical balance (4 digits), Model LA 230 S, Max 230 g, Sartorius AG Goettingen, Germany

3.1.6 A vortex mixer, Model KMS1, IKA-works Industries, Willmington, U.S.A.

3.1.7 A centrifuge, Low speed, Hettich

3.1.8 Syringe filters, Nylon 13 mm, 0.45 µm, Vertical Chromatography Co., Ltd.

3.1.9 A 3 mL disposable Syringe , Nipro (Thailand) Co., Ltd

3.1.10 A 2 mL HPLC amber vials with PTFE caps, Agilent technologies, Palo Alto, U.S.A.

3.1.11 A 15 mL centifuge tubes with screw cap, Hycon, Plastics Inc

3.1.12 Micro-pipettes with 10-100, 50, 100, and 100-1000 µL, Eppendorf, Hamburg, Germany

3.2   Chemicals

3.2.1   Steroids Standards

All nine steroids used were purchased from Sigma-Aldrich (MO, USA): triamcinolone (TAL), prednisolone (PNL), hydrocortisone (HCS), methyprednisolone (MPS), betamethasone (BTS), dexamethasone (DXS), beclomethasone (BCS), fludrocortisone acetate (FCA) and cortisone acetate (CSA) with their purity of 98.5, 99.7, 99.6, 99.4, 99.9, 99.5, 99.0, 98.0 and 99.0%, respectively.

3.2.2   Organic Solvents

All organic solvents used were obtained from Merck (Darmstadt, Germany): acetonitrile (ACN) with an HPLC grade, ethyl acetate (EtOAc), methanol (MeOH) and acetone all with an analytical grade.

3.2.3   Salts and Sorbents used for QuEChERS

NaCl and anhydrous $MgSO_4$ salts were obtained from Merck (Darmstadt, Germany). The following d-SPE sorbents were obtained from Varian (Harbor city, USA): primary and secondary amine (PSA), octadecylsilane (C_{18}), alumina, graphitized carbon black (GCB), and hydrophilic-lipophilic balances copolymer (HLB).

3.2.4   Blank and Real Samples

The blank sample of brown liquid herbal medicines was obtained from Green Chart Natural Herbes (Thailand) Co, Ltd., and adulterated steroids were non detected using our HPLC analysis, while the real samples of liquid herbal medicines were obtained from the Regional Medical Sciences Center 4th Samut Songkhram and their brand names cannot be disclosed. It should be noted that the real and blank samples may contain different matrice. Active ingredients in the blank samples includes: leonurus sibiricus linn. 300 g., seed myristica fragans houtt. 150 g., cudrania javanensis inecul. 300 g., casesalpinia sappan linn. 300 g., and pericarb myristica fragans houtt.150 g. Active ingredients in the real samples are not known.

3.3 Preparation of Steroids Standard Solutions

3.3.1 Stock Standard Solutions

Stock standard solutions of 1,000 ppm each standard steroid were separately prepared by accurately weighting of approximately 0.0250 g of each standard, dissolving these in 5 ml of methanol and then making up with ACN to 25.00 ml in volumetric flasks. These stock standard solutions were kept in amber glass bottles with screw cap, and stored in a refrigerator at 4 ℃ prior to use. Each working standard solution containing nine steroids was prepared by pipetting the appropriate amounts of each stock solution.

3.4 The Optimization of HPLC Conditions for Steroids Analysis

HPLC analysis was performed on an Agilent 1200 series system (Agilent Technologies, USA), equipped with a G1315D PDA UV-vis detector scanning from 200 to 400 nm and monitoring at 240 nm, a G1311A quaternary pump, a G1322A vacuum degasser and a G1329A autosampler. The chromatographic separation of steroids was carried out using a Hypersil BDS C_{18} column (300 mm × 4.6 mm I.D., 5 μm), thermostated at 35 ℃, with a 1.0-ml/min flow rate of a (v/v) gradient elution of ACN:water mobile phase starting from 33:67 for 10 min and then using gradient to 50:50 within 10 min. All sample solutions were injected using a 10-µl sample loop.

3.5 QuEChERS Optimization

3.5.1 Typical QuEChERS procedure

Typically, anhydrous $MgSO_4$ and NaCl salts (4:1 wt/wt), with the desired amount, were filled in a 15-ml PTFE centrifuge tube. Then 2 ml of organic solvent and 2 ml of a liquid herbal medicine blank samples (as previously mentioned in Section 3.2.4) spiked with 10 ppm steroids, were added into the tube. The mixture was shaken vigorously for 10 sec, vortexed for 1 min, and then centrifuged for 5 min at 5000 rpm. A 1 ml aliquot from the upper part of the extract was transferred into a

microcentrifuge tube containing the desired amount of d-SPE sorbent and 50 mg
($\pm$10%) $MgSO_4$. The mixture was then shaken, vortexed and centrifuged for 5 min at
5000 rpm. The harvested solvent extract was then filtered using 0.45 μm nylon filters
prior to HPLC analysis. For each parameter of the QuEChERS opimization, the
experiment was carried out in tripcate.

3.5.2 Choices of Solvents

The QuEChERS extraction was performed as described in Section 3.5.1, except that
the three types of organic solvents (ACN, EtOAc, and acetone) were varied
for QuEChERS extraction with addition of 125 mg of NaCl and 500 mg anhydrous
$MgSO_4$ salts added, and then 50 mg of PSA sorbent was used for d-SPE. Results are
shown in Section 4.2.1.

3.5.3 The amount of Salts

The QuEChERS extraction was performed as described in Section 3.5.1, except that
the amounts of salts (4:1 wt/wt $MgSO_4$:NaCl) were varied at 800:200, 600:150,
500:0.125 and 400:100 mg, and ACN was used in the QuEChERS solvent extraction.
In addition, 50 mg of PSA sorbent was used for d-SPE. Results are shown in Section
4.2.2.

3.5.4. Choices of Sorbents

The QuEChERS extraction was performed as described in Section 3.5.1, using ACN
for the QuEChERS extraction with addition of 125 mg of NaCl and 500 mg
anhydrous $MgSO_4$ salts. Five types of d-SPE sorbent (50mg) were varied using PSA,
C18, Alumina, GCB, and HLB. Results are shown in Section 4.2.3.

3.5.5 Sorbent Mass

The QuEChERS extraction was performed as described in Section 3.5.1, using ACN
for the QuEChERS extraction with addition of 125 mg of NaCl and 500 mg

anhydrous $MgSO_4$ salts. The amounts of PSA sorbent for d-SPE were varied at 25, 50 and 100 mg. Results are shown in Section 4.2.4.

3.6 Method Validation

3.6.1 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Ten batches of blank sample were spiked with steroids at the concentrations near LOD and LOQ (C_D and C_Q) giving the average peak height at approximately 3 and 10 times over the baseline noise (S_D and S_Q), repectively. Therefore, the calculated LOD and LOQ are obtained from $3C_D/S_D$ and $10C_Q/S_Q$, repectively [Fajgelj and Ambrus: 2000; Gustavo and Angeles: 2007]. Results are given in Section 4.3.1.

3.6.2 Standard Calibration Curves and Linearity

Working standard solutions of nine steroids were prepared at six concentration levels starting from LOQ to 15 ppm each as present in Table 3.1. At each concentration, peak areas of all nine steroids were obtained from the duplicate HPLC runs. The calibration curves were established by plotting the peak area against the analyte concentration range. Results of slopes, intercepts, and coefficients of determination (R^2) of calibration linearity are shown in Section 4.3.2.

Table 3.1 Concentration levels of mixed standards (ppm) for calibration curves and linearity

Analytes	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
TAL	0.20	1.0	2.0	4.0	10.0	15.0
PNL	0.30	1.0	2.0	4.0	10.0	15.0
HCS	0.30	1.0	2.0	4.0	10.0	15.0
MPS	0.45	1.0	2.0	4.0	10.0	15.0
BTS	0.45	1.0	2.0	4.0	10.0	15.0
DXS	0.40	1.0	2.0	4.0	10.0	15.0
BCS	0.55	1.0	2.0	4.0	10.0	15.0
FCA	0.30	1.0	2.0	4.0	10.0	15.0
CSA	0.30	1.0	2.0	4.0	10.0	15.0

The QuEChERS method linearity was also determined by spiking standard solutions in the blank samples to contain nine steroids at the similar concentration levels used for calibration curves. Using triplicate for each concentration, the linear relationship between the average peak area and the concentration of standard spiked was determined, and the linearity was evaluated using an R^2 value.

3.6.3 Accuracy and Precision

The accuracy and precision in the QuEChERS extraction were investigated by spiking standard solutions in the blank samples at the four levels of 10 ppm and 2, 4 and 10 times the LOQ value as present in Table 3.2. The experiment for each concentration level was performed in ten replicates. The QuEChERS extraction recovery of the analyte spiked and its relative standard deviation (%RSD) were evaluated for the accuracy and precision, respectively. Intraday and interday precisions in the recovery were also determined using the blank sample spiked with the nine steroids at a concentration level of ten times their respective LOQ value. For the intraday precision, the recovery RSD value was obtained from 10 batches of the QuEChERS sample preparation, while that for interday precision was evaluated from five days. Results of the accuracy and precision are given in Section 4.3.3.

Table 3.2 Concentration of mixed standard solution (ppm) for the study of accuracy and precision

Analytes	Level 1	Level 2	Level 3	Level 4
TAL	0.4	0.8	2.0	10
PNL	0.6	1.2	3.0	10
HCS	0.6	1.2	3.0	10
MPS	0.9	1.8	4.5	10
BTS	0.9	1.8	4.5	10
DXS	0.8	1.6	4.0	10
BCS	1.1	2.2	5.5	10
FCA	0.6	1.2	3.0	10
CSA	0.6	1.2	3.0	10

3.6.4 Application to Real Samples

The QuEChERS sample preparation for real samples were performed in triplicate as detailed in Section 3.5.1, using ACN for the solvent extraction with addition of 500 mg $MgSO_4$ and 125 mg NaCl and following by d-SPE with 50 mg PSA sorbent and 50 mg $MgSO_4$. The HPLC analysis was carried out using HPLC conditions as previously mentioned in Section 3.4. Results are given in Section 4.4.

ศูนย์วิทยทรัพยากร

จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER IV

RESULTS AND DISCUSSIONS
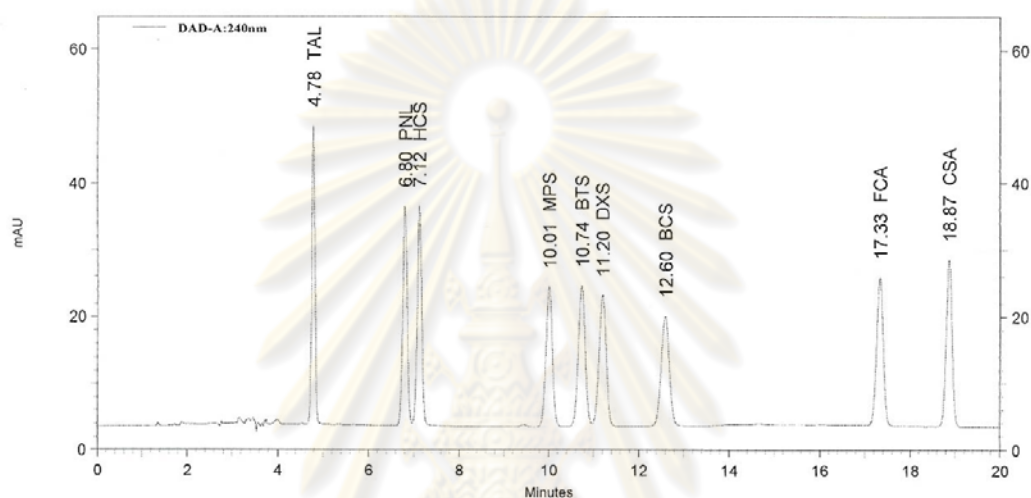
4.1 HPLC Conditions

Name	RT	Area	ESTD conc	Theoretical plates (USP)	Resolution (USP)
TAL	4.78	220752	10.05	21840	0.00
PNL	6.80	227042	10.10	21983	12.90
HCS	7.12	240904	10.13	21742	1.71
MPS	10.01	210365	10.01	22211	12.49
BTS	10.74	229084	10.16	21964	2.61
DXS	11.20	221913	10.09	22056	1.56
BCS	12.60	210968	10.21	21945	4.35
FCA	17.33	229735	10.01	62925	15.36
CSA	18.87	227001	10.01	95616	5.91

Figure 4.1 Representative HPLC separation of nine steroids at 10 ppm each. HPLC conditions: a Hypersil BDS C_{18} column (300 mm × 4.6 mm I.D., 5 µm) thermostated at 35°C, a 1.0-ml/min flow rate of a (v/v) gradient elution of ACN:water mobile phase starting from 33:67 for 10 min and then using gradient to 50:50 within 10 min, a 10-µl sample loop injection, and PDA UV detection scanning from 200 to 400 nm and monitoring at 240 nm.

In previous works, the reversed phase-HPLC separation of steroids were performed using a C18 column and ACN:water mobile phase [Ku *et.al.*: 1999; Gagliardi *et.al.*: 2002]. In this work, the gradient elution of ACN:water was optimized in order to achieve baseline resolution (R_s >1.5) of all nine steroids within appropriate time of analysis (20 min). Figure 4.1 shows a representative example of the HPLC separation of the nine steroids at 10 ppm. The following retention times (min) of nine steroids were obtained: 4.78 (TAL), 6.80 (PNL), 7.12 (HCS), 10.01 (MPS), 10.74 (BTS), 11.20 (DXS), 12.60 (BCS), 17.33 (FCA) and 18.87 (CSA), respectively. The spectra of the chromatographic peak at retention time of the steroids are also shown in Figure B-1 in Appendix B.

4.2 QuEChERS Optimization

4.2.1 Choices of solvents

ACN, EtOAc and acetone, commonly used in the QuEChERS technique as previously mentioned in Section 3.2.2, were compared for solvent extraction of a blank sample (three batches) of a commercial liquid herbal medicine spiked with the nine standard steroids at 10 ppm each. QuEChERS procedures were performed as previously mentioned in Section 3.5.2. The results are shown in Table A-1 (Appendix A) and Figure 4.2. In comparison with that obtained using EtOAc and acetone, ACN provided a better extraction efficiency for all nine steroids with recoveries in the range of 91−99 %, whilst acetone was the worst. Therefore, ACN was chosen as the extraction solvent for all further work. In addition, a final extraction solution in ACN is compatible with the water:ACN mobile phase used for the HPLC separation of steroids in this work.
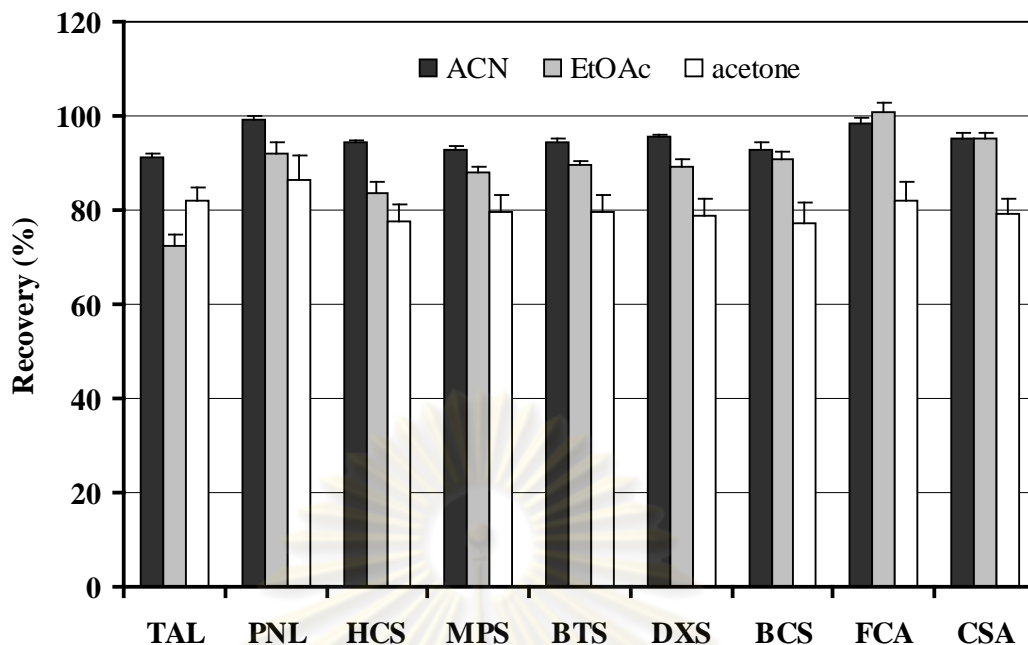
Figure 4.2 Average recovery (*n* = 3 batches) of steroids obtained from the QuEChERS, sample preparation using ACN, EtOAc and acetone for the solvent extraction with addition of 500 mg $MgSO_4$ and 125 mg NaCl, and following by d-SPE with 50 mg PSA sorbent and 50 mg $MgSO_4$. A blank sample was spiked with nine steroids at 10 ppm each.

4.2.2 The amount of Salts

As previously mentioned in Section 3.2.3, $MgSO_4$ and NaCl salts, particularly with a weight ratio of 4:1 is widely used to induce phase separation in the QuEChERS solvent extraction step [Anastassiades *et al.*: 2003; Banerjee *et al.*: 2007; Cunha *et al.*: 2007; Diez et al: 2006; Hereegova *et al.*: 2006; Gilbert-Lopez *et al.*: 2010; Lehotay *et al.*: 2007; Nguyen *et al.*:2007; Paya *et al.*: 2007; Wang *et al.*: 2007; Wilkowska *et al.*: 2011]. In this work, the amounts of salts (4:1 wt/wt $MgSO_4$:NaCl) were varied at 800:200, 600:150, 500:125 and 400:100 mg. ACN was used in the QuEChERS solvent extraction, and other QuEChERS conditions and procedures are previously mentioned in Section 3.5.3. As can be seen in Figure 4.3 (also Table A-2 in Appendix A), $MgSO_4$:NaCl salts with a weight ratio of 800:200, 600:150 and 500:125 mg give similar extraction recovery of each steroid (90-98, 90-97 and 91-99% , respectively), but lower recovery (83-91%) is seen from 400:100 mg of $MgSO_4$:NaCl salts. Therefore, 500 mg $MgSO_4$:125 mg NaCl was chosen in this experiment.

Figure 4.3 Average recovery (n = 3 batches) of steroids obtained from the QuEChERS sample preparation using the various amounts of $MgSO_4$:NaCl, 800:200, 600:150, 500:125 and 400:100 mg, for the ACN extraction, and following by d-SPE with 50 mg PSA sorbent and 50 mg $MgSO_4$. A blank sample was spiked with nine steroids at 10 ppm each.

4.2.3 Choices of Sorbents

In order to remove matrix components, d-SPE was performed using sorbent as PSA, C_{18}, alumina, GCB and HLB, as previously mentioned in Section 3.5.4. As results shown in Figure B-2 (Appendix B), when GCB or PSA was used as the d-SPE clean-up matrix, they were found to effectively remove the brown color of the ACN extract, whilst a slightly brown extract was still observed using C_{18}, alumina and HLB. This is consistent with the HPLC chromatograms from three latter sorbents that were found to contain more interference peaks. In addition, a poor recovery was found using HLB and GCB sorbents (67−82% and 12−68%, respectively) as shown in Figure 4.4 (also Table A-3 in appendix A), implying that the steroids were retained on these sorbents. Indeed, GCB has previously been reported to not only effectively remove pigments but sterols as well in food samples [Lehotay *et al.*: 2007; Hereegova *et al.*: 2006; Frenich *et al.*: 2008; Chung *et al.*: 2010; Wilkowska *et al.*: 2011]. Since steroids and sterols have somewhat similar structures, steroids, especially those with a high hydrophobicity (long retention time in Figure 4.1), may preferentially be retained on GCB. Overall, across the nine evaluated steroids, the PSA, C18 and alumina sorbents provided a comparably high recovery for each steroid (90−100%), except for the low

hydrophobic TAL that had a lower recovery (76%) using alumina. Taking account of the high recovery levels and effective removal of pigments, PSA was chosen as the d-SPE sorbent.
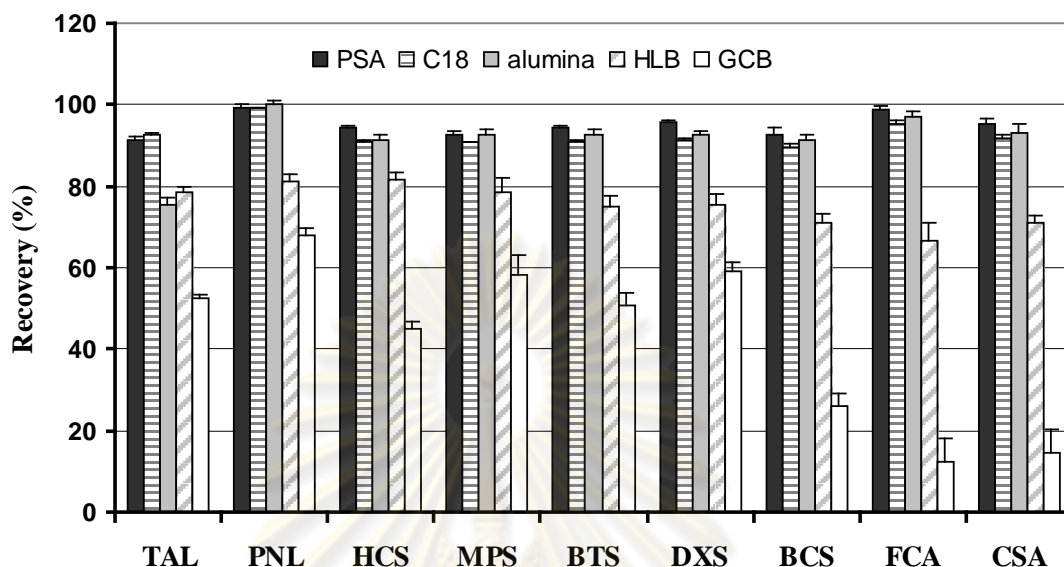
Figure 4.4 Average recovery (n = 3 batches) of steroids obtained from the QuEChERS sample preparation using 50 mg five different sorbents along with 50 mg $MgSO_4$ for d-SPE after the ACN solvent extraction with addition of 500:125 mg $MgSO_4$:NaCl. A blank sample was spiked with steroids at 10 ppm each.

4.2.4 Sorbent Mass

The amount of PSA was then varied at 25, 50 and 100 mg for the d-SPE with other QuEChERS procedures and conditions given in Section 3.5.5. PSA at 25 mg gave a poorer extraction of the brown color and a high number of interference peaks on the HPLC chromatogram as shown in Figure B-3 (Appendix B), whilst a comparable recovery of steroids was obtained with the use of 50 or 100 mg PSA (Figure 4.5 and also Table A-4 in appendix A). Therefore, 50 mg PSA was chosen for use as the d-SPE sorbent.

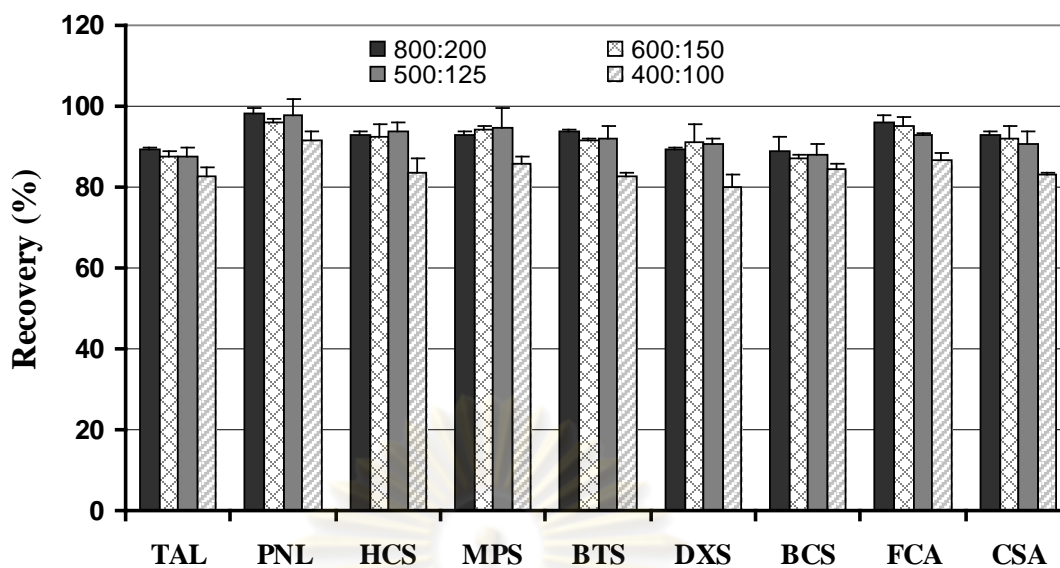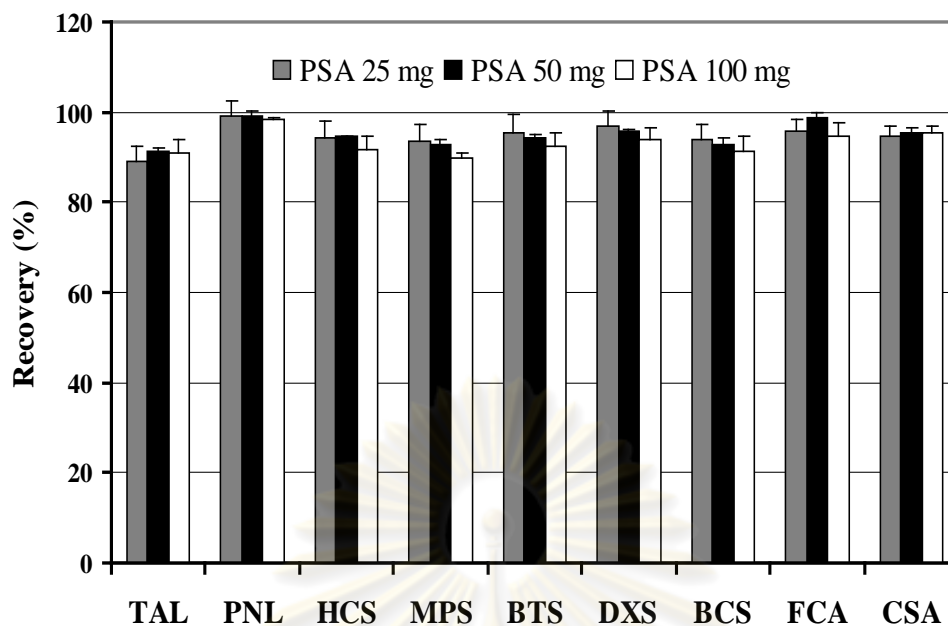**Figure 4.5** Average recovery (n = 3 batches) of steroids obtained from the QuEChERS sample preparation using various amounts of PSA along with 50 mg $MgSO_4$ for d-SPE after the ACN solvent extraction with addition of 500:125 mg $MgSO_4$:NaCl. A blank sample was spiked with steroids at 10 ppm each.

4.3 Method Validation

Method validation is a process used to confirm that an analytical procedure employed or developed for a specific test is appropriate for its quantitative analysis use. Thus, in this experiment, the following parameters were validated for the QuEChERS method: LOD, LOQ, standard calibration curve, linearity, accuracy, and precision.

The blank samples of liquid herbal medicines were spiked with known concentration levels, and then 2 ml of these solutions was extracted using the QuEChERS conditions optimized in Section 4.2: ACN (2.0 ml) for the solvent extraction with addition of 500 mg $MgSO_4$ and 125 mg NaCl, and 50 mg PSA sorbent for d-SPE with addition of 50 mg $MgSO_4$. Replicate measurements are given in each of the following sections.

4.3.1   LOD and LOQ

LOD is defined as the lowest concentration of analyte that can be detected but not necessarily quantified as an exact value [JCGM 200: 2008], while LOQ is defined as the lowest concentration of analyte that can be quantified with acceptable precision and accuracy [JCGM 200: 2008]. In chromatography, LOD and LOQ are typically obtained from the concentration of the analyte giving a signal-to-noise ratio (S/N) of 3 and 10, respectively [Taverniers *et al.*: 2004; Gustavo *et al.*: 2007].

In this work, the values of LOD and LOQ of nine steroids, after QuEChERS sample preparation, were obtained from the concentration of the analyte, giving a signal-to-noise ratio of 3 and 10, respectively, as the experiment previously mentioned in Section 3.6.1. From the results of ten batches of a blank sample spiked with steroids at the concentrations near LOD and LOQ (Table 4.1), the QuEChERS technique provided LOD and LOQ for nine steroids in a range of 0.06−0.17 and 0.20−0.55 ppm, respectively, which is sufficient for the HPLC determination of steroids intentionally mixed with herbal medicines.

In terms of the repeatability of LOQ, a HorRat value (a Horwitz ratio) obtained in a range of 0.2–0.5 meets the acceptance criteria for AOAC, where the HorRat is the ratio of the observed RSD calculated from the actual performance data, RSD_r (%), to the corresponding predicted relative standard deviation calculated from 0.67 times theoretical values determined by the Horwitz function for intra-laboratory precision, $PRSD_r$ (%) $= 0.67 \times 2C^{-0.1505}$, and where C is the concentration added, expressed as a mass fraction [Peeler *et al.*: 1989; Taverniers *et al.*: 2004; Horwitz and Albert: 2006].

Table 4.1 The limit of detections and limit of quantifications of nine steroids (n=10)

No.	Analytes	LOD (ppm)	LOQ (ppm)	HorRat at LOQ
1.	TAL	0.06	0.20	0.3
2.	PNL	0.09	0.30	0.2
3.	HCS	0.10	0.30	0.2
4.	MPS	0.13	0.45	0.4
5.	BTS	0.13	0.45	0.3
6.	DXS	0.13	0.40	0.4
7.	BCS	0.17	0.55	0.5
8.	FCA	0.09	0.30	0.4
9.	CSA	0.09	0.30	0.4

4.3.2   Standard Calibration Curves and Linearity

Table 4.2 Slope, intercept, and correlation coefficients from standard calibration curves and method calibration curves of nine steroids

Analytes	Concentration range (ppm)	Calibration of a linear plot					
		working standards			QuEChERS method*		
		Slope	Intercept	R^2	Slope	Intercept	R^2
TAL	0.2-15	19286	527	0.9991	19916	307	0.9999
PNL	0.3-15	22098	10	0.9998	21432	185	0.9978
HCS	0.3-15	23398	226	0.9999	22454	311	0.9999
MPS	0.4-15	21152	-394	0.9998	21152	-394	0.9998
BTS	0.4-15	22624	-720	0.9988	20982	-351	0.9996
DXS	0.4-15	22948	-313	0.9997	15674	-17.3	0.9997
BCS	0.55-15	20728	-848	0.9985	20790	-988	0.9999
FCA	0.3-15	22855	-616	0.9989	22868	-298	0.9994
CSA	0.3-15	22587	-494	0.9992	23258	-776	0.9997

*The steroid standards were spiked into the blank samples and then extracted using the QuEChERS method

As previously mentioned in Section 3.6.2., the calibration curves of standard steroids were obtained using linear plots of the peak area as the function of the analyte concentration at six levels in a range of LOQ to 15 ppm. Calibration curves are shown in Figure B-4 (Appendix B). The values of slope, intercept, and correlation coefficients are sumerized in Table 4.2. High linearity of the calibration plots was obtained with the R^2 values grather than 0.998. In addition, the high linearity of the QuEChRES method was also obtained with $R^2 > 0.997$. (Table 4.2 and also Figures B-5 (Appendix B).

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4.3.3   Accuracy and Precision in the QuEChERS method

Accuracy is defined as the closeness of agreement between a measured quantity value and a true quantity value of measurand [JCGM 200: 2008], while precision is defined as the closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions [JCGM 200: 2008],. By spiking the steroid standards into the blank samples at the four levels of 10 ppm and 2, 4 and 10 times the LOQ value as previously mentioned in Section 3.6.3, Table 4.3 shows the accuracy and precision in the QuEChERS method evaluated by measuring the extraction recovery, and its relative standard deviation, respectively, from ten replicates.

From Table 4.3 satisfactory accuracy of recovery, that is the range of 91−113%, was obtained, with 97% of the recovery data being within the acceptable recovery of 80−110% for the analyte concentration in the range 0.1−10 ppm [Taverniers *et al.*: 2004; Gustavo *et al.*: 2007]. An accepted level of precision was also obtained with RSD of < 5% and the HorRat values of 0.1−0.6 for all steroids.

Table 4.3 Accuracy and precision in the QuEChERS extraction recovery of steroids spiked in blank samples at four levels ($n = 10$ batches)

Analytes	% Recoveries (% RSD) at				HorRat at			
	2×LOQ	4×LOQ	10×LOQ	10 ppm	2×LOQ	4×LOQ	10×LOQ	10 ppm
TAL	113.0 (1.8)	100.2 (2.1)	99.5 (1.3)	91.1 (2.5)	0.2	0.2	0.1	0.3
PNL	102.5 (3.0)	98.2 (2.2)	98.5 (1.6)	99.3 (1.2)	0.3	0.2	0.2	0.2
HCS	98.1 (2.6)	95.3 (2.2)	96.1 (2.5)	94.5 (1.5)	0.2	0.2	0.3	0.2
MPS	97.5 (3.4)	96.0 (2.2)	97.5 (1.6)	92.8 (4.6)	0.3	0.2	0.2	0.6
BTS	103.1 (4.7)	98.4 (1.7)	97.0 (1.4)	94.4 (3.2)	0.4	0.2	0.2	0.4
DXS	98.9 (3.6)	95.4 (2.1)	96.2 (1.3)	95.6 (2.3)	0.3	0.2	0.2	0.3
BCS	97.4 (3.3)	99.4 (3.3)	94.4 (1.6)	92.8 (1.9)	0.3	0.3	0.2	0.3
FCA	101.3 (6.8)	97.5 (2.6)	98.4 (1.6)	98.6 (1.9)	0.6	0.2	0.2	0.3
CSA	102.1 (2.5)	98.8 (3.2)	98.5 (1.9)	95.4 (2.2)	0.2	0.3	0.2	0.3

  % RSD in parentheses

As previously mentioned in Section 3.6.3, intraday and interday precisions in the recovery were also determined using the blank sample spiked with the nine steroids at a concentration level of ten times their respective LOQ value. From results in Table 4.4, an acceptable intraday and interday precision in extraction recoveries was obtained with RSD of ≤ 3.4 % (ten replicate each day for five days), and the HorRat values of 0.1–0.6 for all steroids.

It should be noted that, using statistical ANOVA:Single Factor analysis at 95% confidence level [William: 1987] as shown in Table A-5 (Apendix A), non significant difference between day RSD (P-value > 0.05) is obtained for methylprednisolone, dexamethasone, beclomethasone and fludrocortisone acetate, while significant difference between day RSD (P-value < 0.05) for triamcinolone, prednisolone, hydrocortisone, betamethasone and cortisone acetate. Therefore, % RSD for intraday precision for the former set of the analytes using a single data set ($n = 50$ for each analyte), while that for the latter set of the analytes is calculated using % RSD = $100 S_r / \overline{x}$, where $\overline{x}$ is the average recovery and S_r is the square root of the within group mean square value obtained form the ANOVA data in Table A-5. In addition, % RSD for interday precision for the former set of the analytes is equal to % RSD for intraday precision, while that for the latter set of the analytes is calculated using equations [Joachim *et al.*: 2005] as given below Table A-6 (Apendix A).

Table 4.4 Intraday and interday precisions in the QuEChERS extraction recovery of steroids spiked in the blank samples at 10×LOQ

Analytes	% RSD in recovery from QuEChERS extraction (10 batches each day)					P-value	Intraday precision (% RSD)	Interday precision (% RSD)
	Intraday							
	Day 1	Day 2	Day 3	Day 4	Day 5			
TAL	1.9	3.1	2.9	3.0	3.5	0.016	2.8	3.2
PNL	1.4	2.1	2.5	1.6	1.9	0.009	1.9	2.2
HCS	1.6	2.2	3.4	2.5	4.0	0.003	2.8	3.4
MPS	1.4	2.3	2.4	2.4	2.8	0.256	2.3	2.3
BTS	2.0	2.3	2.4	3.4	3.1	0.038	2.6	2.7
DXS	1.9	2.9	3.9	2.3	4.2	0.193	3.2	3.2
BCS	1.9	3.4	1.4	2.1	2.5	0.450	2.4	2.4
FCA	1.5	4.4	4.6	2.7	2.2	0.100	3.4	3.4
CSA	1.6	3.3	3.6	2.3	3.0	0.021	2.7	3.2

4.4   Application to Real Samples

The developed QuEChERS sample preparation was used for HPLC determination of steroids adulterated in six real samples of liquid herbal medicines as previously mentioned in Section 3.6.4. Figure 4.6 shows HPLC chromatograms of real samples of liquid herbal medicines after the QuEChERS extraction. Samples L1, L2 and L3 were found to be adulterated with 8.8±0.2, 1.6±0.1 ppm dexamethasone and 0.43±0.01 ppm prednisolone respectively, while adulterated steroids were not detected, subject to the LOD of this assay system, in the other three samples. In our TLC routine analysis of these six samples, dexamethasone adulterated in L1 and L2 and prednisolone in L3 were also detected, but their extract amounts were not determined by TLC. In addition, by spiking dexamethasone in L3 (Figure B-6 in Appendix B) and comparing the UV- spectra of dexamethasone with that of the peak at retention time of 11.60 min (Figure B-7 in Appendix B), it can be indicated that the peak in L3 at retention time of 11.60 min does not belong to dexamethasone.
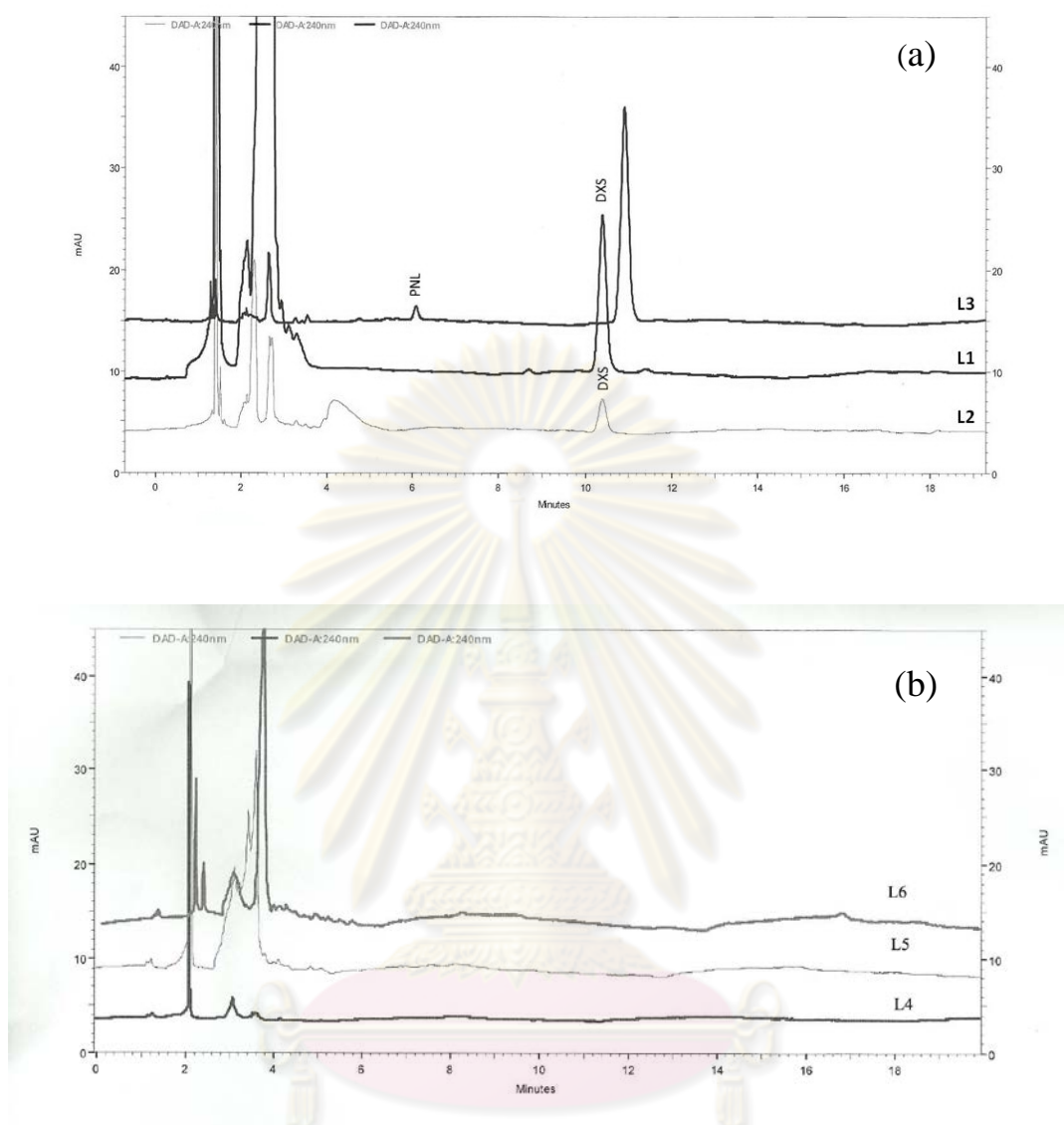
Figure 4.6 HPLC chromatograms of real samples of liquid herbal medicines after the QuEChERS extraction: (a) Samples with adulterated of steroids and (b) Samples with non-detected steroids.

CHAPTER V

CONCLUSION

Adulterations with synthetic steroids is one of common problems of herbal medicines and this can potentially cause serious adverse effects. It is important to determine the presence of synthetic steroid to ensure patient's safety. The effective analytical method is therefore critically important. In this work, QuEChERS sample preparation was validated for HPLC determination of nine steroids adulterated in liquid herbal medicine, including triamcinolone, prednisolone, hydrocortisone, methylprednisolone, betamethasone, dexamethasone, beclomethasone, fludrocortsone acetate and cortisone acetate.

In initial study, baseline resolution (R_S >1.5) of all nine steroids were achieved within 20 min using a Hypersil C18 column with 1.0-mL/min gradient elution of 35°C ACN:water mobile phase starting from 33:67 for 10 min and then using gradient to 50:50 within 10 min. In the QuEChERS sample preparation containing two steps: the solvent extraction and d-SPE cleanup, the following parameters were optimized: types of extracting solvent (ACN, EtOAc and acetone), the amount of salts added in the solvent extraction step (4:1 wt/wt $MgSO_4$:NaCl mg with 800:200, 600:150, 500:125, and 400:100), types of sorbent (PSA, C18, alumina, GCB and HLB), and the amount of PSA sorbents (25, 50, and 100 mg). Taking account of the effective removal of the interferences and the high recovery in the QuEChERS extraction of nine steroids spike in the blank samples with the known concentration levels, the appropriate QuEChERS parameters was obtained for sample preparation 2.0 ml of a sample solution using 2.0-ml ACN solvent extraction with addition of 500 mg anhydrous $MgSO_4$ and 125 mg NaCl, and then following by d-SPE cleanup with 50 mg PSA sorbent.

In order to evaluate the acceptability of the developed method, the following parameters were validated for the QuEChERS method: LOD, LOQ, standard calibration curve, linearity, accuracy, and precision. After the QuEChERS sample preparation, LOD and LOQ for nine steroids were found in a range of 0.06–0.16 and

0.20–0.55 ppm, respectively, which is sufficient for the HPLC determination of steroids intentionally mixed with herbal medicines. Using the concentration range starting from LOQ to 15 ppm, high linearity of calibration and the QuEChERS method, both with $R^2 > 0.998$, was obtained from the working standard directly dissolved in ACN, and the standard spiked in the blank samples, respectively. By spiking nine steroids in the blank samples at the four levels of 10 ppm, 2, 4, and 10 times the LOQ values, this QuEChERS method gave satisfactory accuracy with the extraction recovery in the range of 91-113%, with 97% of the recovery data being with in the acceptable recovery of 80-110%. In addition, an acceptable intraday and interday precision in extraction recoveries are shown by RSD of ≤ 3.4 %.

Using HPLC analysis with QuEChERS sample preparation, three out of six samples of liquid herbal medicine were found to be adulterated with 8.8±0.2, 1.6±0.1 ppm dexamethasone and 0.43±0.01 ppm prednisolone, respectively, while adulterated steroids were not detected, subject to the LOD of this assay system, in the other three samples.

In comparison with the previous method using solvent extraction with chloroform and cartridge SPE [Ku *et al*.:1999, 2001], QuEChERS sample preparation provides a fast, easier and cheaper method: high sample throughput of approximate 10–12 samples in 30 min (less time-consuming), two steps of simple extraction by hand-shaking and centrifuging, and low consumption of organic solvent and low cost of reagents used, (appoximately ten baht per sample), respectively. Disadvantages of the cartridge SPE [Ku *et al*.: 1999, 2001] include a large amount of organic solvent (25 mL) used, the expense of time for ultrasonicating or shaking (30 min), an expensive SPE cartridge, several steps of SPE solvent elution, and hazard chloroform used.

According to this preliminary study, the QuEChERS sample preparation method should be extended for HPLC determination of steroids aduterated in herbal medicines to cover another range of dosage from (solid) such as a powder, pills, capsule, and may be applied to pharmaceutical drugs namely betamethas dexamethasone, prednisolone, and prednisone drug tablet.

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APPENDICES

APPENDIX A

Table A-1 Average recovery of steroids obtained from the QuEChERS sample preparation using ACN, EtOAc and acetone for the solvent extraction of a blank sample spiked with nine steroids at 10 ppm each

Compounds	% Recovery ± SD (n=3)		
	ACN	EtOAc	Acetone
TAL	91.1 ± 1.0	72.5 ± 2.2	81.9 ± 3.1
PNL	99.3 ± 0.8	92.1 ± 2.4	86.4 ± 5.1
HCS	94.5 ± 0.2	83.5 ± 2.4	77.8 ± 3.5
MPS	92.8 ± 0.9	88.1 ± 1.1	79.7 ± 3.6
BTS	94.4 ± 0.7	89.6 ± 1.0	79.6 ± 3.6
DXS	95.6 ± 0.6	89.4 ± 1.3	78.9 ± 3.4
BCS	92.8 ± 1.5	90.8 ± 1.6	77.1 ± 4.5
FCA	98.6 ± 1.1	100.9 ± 1.9	81.9 ± 4.2
CSA	95.4 ± 1.1	95.2 ± 2.3	79.1 ± 3.2

Table A-2 Average recovery of steroids obtained from the QuEChERS sample preparation using the various amount of $MgSO_4$ and NaCl, 800:200, 600:150, 500:125 and 400:100 mg, for the solvent extraction of a blank sample spiked with nine steroids at 10 ppm each

Compounds	% Recovery ± SD (n=3)			
	800:200	600:150	500:125	400:100
TAL	90.4 ± 0.5	92.0 ± 1.3	91.1 ± 1.0	82.8 ± 2.1
PNL	98.4 ± 1.2	97.1 ± 0.9	99.3 ± 0.8	91.4 ± 2.4
HCS	93.1 ± 0.7	92.4 ± 2.0	94.5 ± 0.2	83.5 ± 3.6
MPS	92.7 ± 1.1	94.3 ± 0.9	92.8 ± 0.9	85.8 ± 1.6
BTS	93.6 ± 0.8	91.4 ± 0.5	94.4 ± 0.7	82.6 ± 1.2
DXS	94.5 ± 2.0	93.8 ± 1.5	95.6 ± 0.6	79.8 ± 3.4
BCS	91.6 ± 1.5	90.3 ± 0.7	92.8 ± 1.5	85.6 ± 1.2
FCA	96.2 ± 1.0	97.5 ± 1.8	98.6 ± 1.1	86.5 ± 2.2
CSA	95.4 ± 1.1	95.2 ± 1.4	95.4 ± 1.1	83.3 ± 0.4

Table A-3 Average recovery of steroids obtained from the QuEChERS sample preparation using five different sorbents for d-SPE of a blank sample spiked with steroids at 10 ppm each

Compounds	% Recovery ± S.D. (n=3)				
	PSA	C18	Alumina	HLB	GBC
TAL	91.1 ± 1.0	92.4 ± 0.7	75.5 ± 1.6	78.4 ± 1.4	52.3 ± 1.2
PNL	99.3 ± 0.8	99.0 ± 0.3	99.9 ± 1.1	81.0 ± 1.8	67.8 ± 2.0
HCS	94.5 ± 0.2	90.8 ± 0.4	91.5 ± 0.9	81.5 ± 1.8	44.9 ± 2.0
MPS	92.8 ± 0.9	90.7 ± 0.2	92.7 ± 1.3	78.5 ± 3.5	58.4 ± 4.8
BTS	94.4 ± 0.7	90.9 ± 0.4	92.5 ± 1.7	75.0 ± 2.6	50.9 ± 2.9
DXS	95.6 ± 0.6	91.3 ± 0.4	92.4 ± 1.2	75.3 ± 2.7	59.2 ± 2.2
BCS	92.8 ± 1.5	89.7 ± 0.8	91.2 ± 1.6	71.1 ± 2.0	26.0 ± 2.9
FCA	98.6 ± 1.1	95.5 ± 0.5	96.9 ± 1.6	66.7 ± 4.3	12.3 ± 5.8
CSA	95.4 ± 1.1	91.6 ± 08	93.2 ± 2.1	70.9 ± 1.7	14.5 ± 5.7

Table A-4 Average recovery of steroids obtained from the QuEChERS sample preparation varied amount of PSA for d-SPE of a blank sample spiked with steroids at 10 ppm each

Compounds	% Recovery ± S.D. (n=3)		
	25 mg	50 mg	100 mg
TAL	89.25 ± 3.3	91.14 ± 1.1	90.87 ± 3.2
PNL	98.99 ± 3.6	99.30 ± 0.8	98.20 ± 0.5
HCS	94.40 ± 3.5	94.53 ± 0.2	91.79 ± 3.0
MPS	93.40 ± 4.0	92.81 ± 0.9	89.75 ± 1.2
BTS	95.42 ± 4.0	94.36 ± 0.7	92.49 ± 3.1
DXS	96.90 ± 3.5	95.63 ± 0.6	94.03 ± 2.6
BCS	93.81 ± 3.5	92.79 ± 1.5	91.35 ± 3.5
FCA	95.74 ± 2.6	98.63 ± 1.1	94.60 ± 2.9
CSA	94.77 ± 2.1	95.40 ± 1.1	95.50 ± 1.3

Table A-5 ANOVA satistical analysis of nine steroids
A-5.1
triamcinolone
SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	996.31	99.631	3.762688
Day 2	10	985.25	98.525	9.263472
Day 3	10	978.2	97.82	7.825111
Day 4	10	958.3	95.83	7.976222
Day 5	10	959.05	95.905	11.54358

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	110.182068	4	27.54552	3.411541	0.016097	2.578739
Within Groups	363.33969	45	8.074215			

A-5.2
prednisolone
SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	985.17	98.51667	2.017593
Day 2	10	969.27	96.92667	4.202667
Day 3	10	974.90	97.490	5.919519
Day 4	10	968.47	96.84667	2.368444
Day 5	10	996.13	99.61333	3.404

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	55.38502	4	13.84626	3.86503	0.008785	2.578739
Within Groups	161.21	45	3.582444			

A-5.3
hydrocortisone
SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	977.10	97.71	2.47137
Day 2	10	966.8333	96.68333	4.727222
Day 3	10	967.9667	96.79667	11.04554
Day 4	10	957.4667	95.74667	5.616099
Day 5	10	1008.067	100.8067	16.03526

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	152.021	4	38.00526	4.763101	0.002731	2.578739
Within Groups	359.0594	45	7.979099			

A-5.4

methylprednisolone

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	924.0444	92.40444	1.580444
Day 2	10	927.1111	92.71111	4.542332
Day 3	10	918.7778	91.87778	4.932483
Day 4	10	915.4889	91.54889	4.692461
Day 5	10	935.8222	93.58222	6.804148

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	24.88468	4	6.22117	1.379303	0.256234	2.578739
Within Groups	202.9668	45	4.51037			

A-5.5

betamethasone

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	1042.689	104.2689	4.1809
Day 2	10	1057.867	105.7867	5.982025
Day 3	10	1046.156	104.6156	6.441926
Day 4	10	1047.4	104.74	6.193805
Day 5	10	1076.356	107.6356	10.8956

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	74.7714	4	18.69287	2.773896	0.038267	2.578739
Within Groups	303.24834	45	6.738852			

A-5.6

dexamethasone

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	1003.625	100.3625	3.562674
Day 2	10	995.15	99.515	8.177528
Day 3	10	999	99.90	15.02333
Day 4	10	1009.525	100.9525	5.447285
Day 5	10	1027.5	102.75	18.19778

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	64.24307	4	16.06077	1.593058	0.19259	2.578739
Within Groups	453.6774	45	10.08172			

A-5.7

beclomethasone

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	963.3091	96.33091	3.466064
Day 2	10	961.5455	96.15455	10.81802
Day 3	10	956.6727	95.66727	1.811427
Day 4	10	945.8727	94.58727	3.950564
Day 5	10	960.4	96.04	5.806487

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	19.43810909	4	4.859527	0.939854	0.449664	2.578739
Within Groups	232.6730248	45	5.170512			

A-5.8

fludrocortisone   acetate

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	958.6	95.86	1.961679
Day 2	10	976.7333	97.67333	17.18143
Day 3	10	966.3	96.63	19.31641
Day 4	10	938.5667	93.85667	6.347173
Day 5	10	953.5	95.35	4.323765

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	81.53542222	4	20.38386	2.074462	0.099957	2.578739
Within Groups	442.1741111	45	9.826091			

A-5.9

cortisone acetate

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	958.3	95.83	2.49542
Day 2	10	941.6333	94.16333	9.567519
Day 3	10	948.9	94.89	11.7489
Day 4	10	916.9667	91.69667	4.526037
Day 5	10	942.9667	94.29667	8.083815

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	94.07324444	4	23.51831	3.228613	0.020605	2.578739
Within Groups	327.7952222	45	7.284338			

Table A-6 Summarized ANOVA data for calculation of intraday RSD

Analytes	Within group MS (SD^{2}within)	Between group MS	SD^{2}between	SD$_{interday}$	Average Recovery ($\bar{x}$)	Interday RSD (%)
TAL	8.1	27.5	1.95	3.2	97.5	3.2
PNL	3.6	13.8	1.03	2.1	97.9	2.2
HCS	8.0	38.0	3.00	3.3	97.5	3.4
BTS	6.7	18.7	1.19	2.8	105.4	2.7
CSA	7.3	23.5	1.62	3.0	94.2	3.2

$$SD_{interday} = \sqrt{SD^2_{within} + SD^2_{between}}$$

$$SD^2_{between} = \frac{\text{between group MS} - \text{within group MS}}{n}$$

$$SD^2_{within} = \text{within group MS}$$

where the data of the within group MS and between group MS are obtained from the ANOVA data in Table A-5, and *n* is the number of replicate measurements (10).

APPENDIX B

λ max 241 nm   TAL (4.78 min)

λ max 245 nm   PNL (6.81 min)

λ max 246 nm   HCS (10.01 min)

MPS (10.73 min)

λ max 240 nm   DXS (12.59 min)

λ max 240 nm   BTS (11.20 min)

λ max 240 nm   BCS (17.32 min)

λ max 241 nm   FCA (18.86 min)
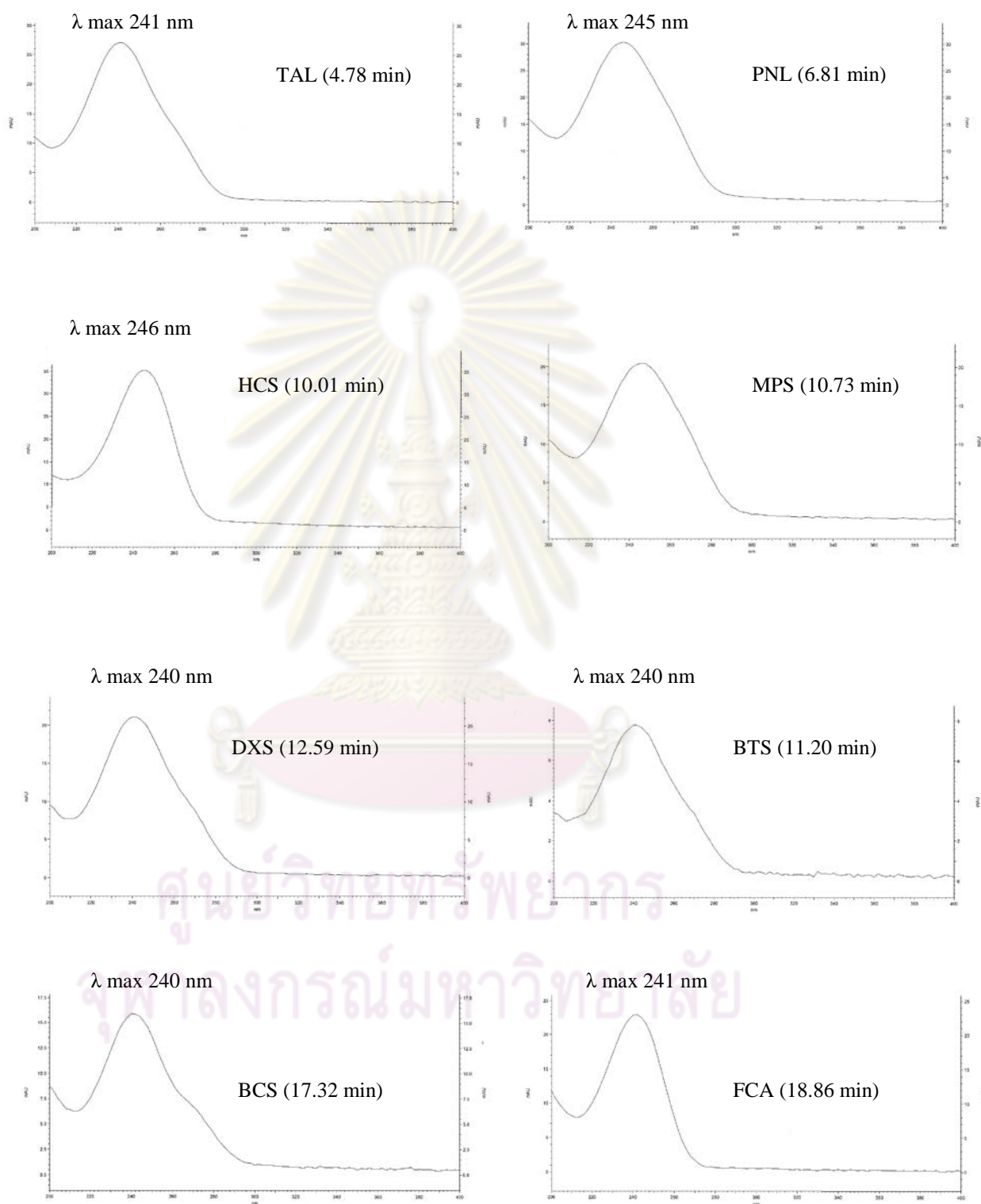
Figure B-1 UV spectra of nine steroids at their retention times.

Figure B-1 Continued.

λ max 241 nm

CSA (18.86 min)
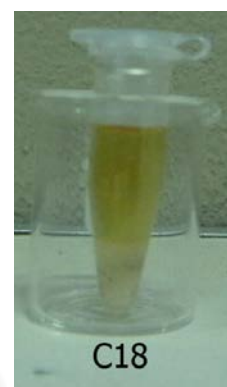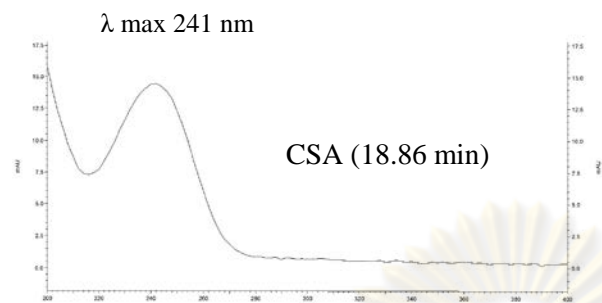
Figure B-2 The color of the ACN extract obtained from each sorbent for the QuEChERS sample preparation using 50 mg each sorbent + 50 mg MgSO$_4$ and after the ACN solvent extraction with addition of 500:125 mg MgSO$_4$:NaCl. A blank sample was spiked with steroids at 10 ppm each.
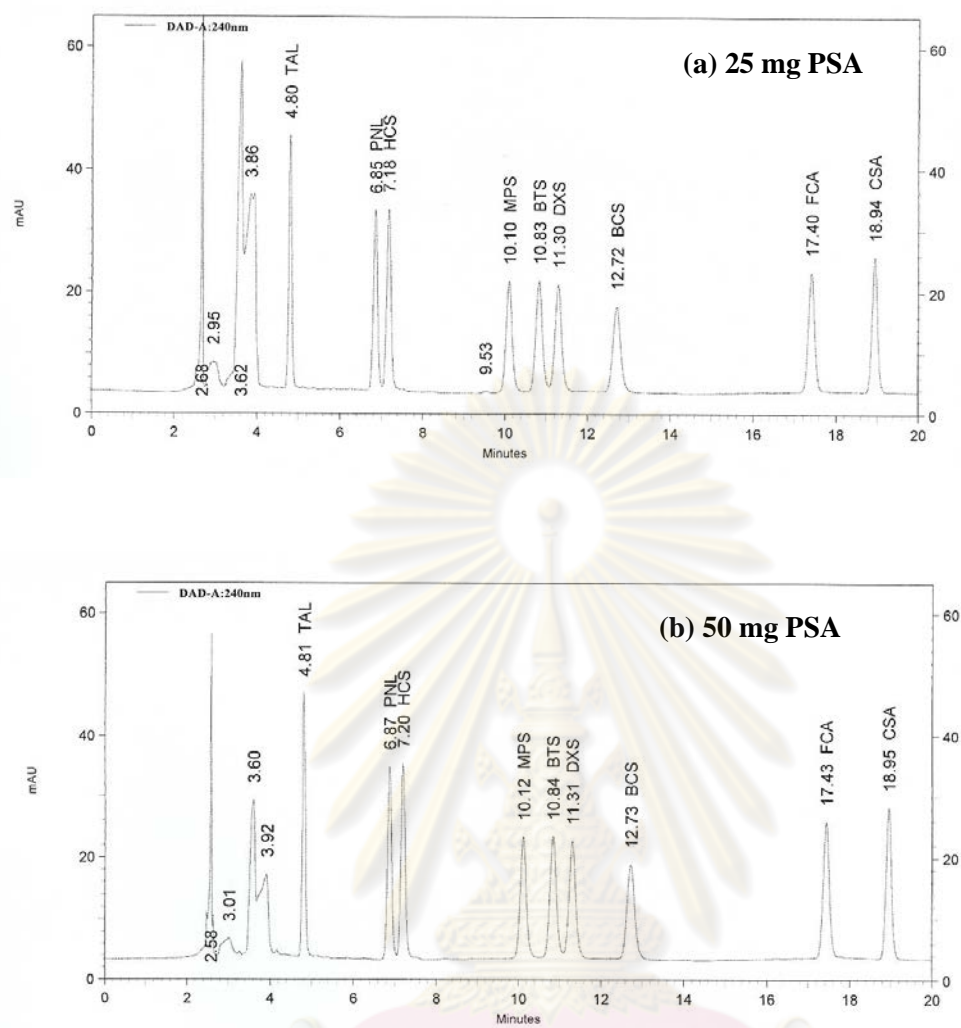
Figure B-3 The chromatogram of nine steroids obtained from the QuEChERS sample preparation using (a) 25 mg PSA + 50 mg MgSO$_4$ and (b) 50 mg PSA + 50 mg MgSO$_4$ and after the ACN solvent extraction with addition of 500:125 mg MgSO$_4$:NaCl. A blank sample was spiked with steroids at 10 ppm each.
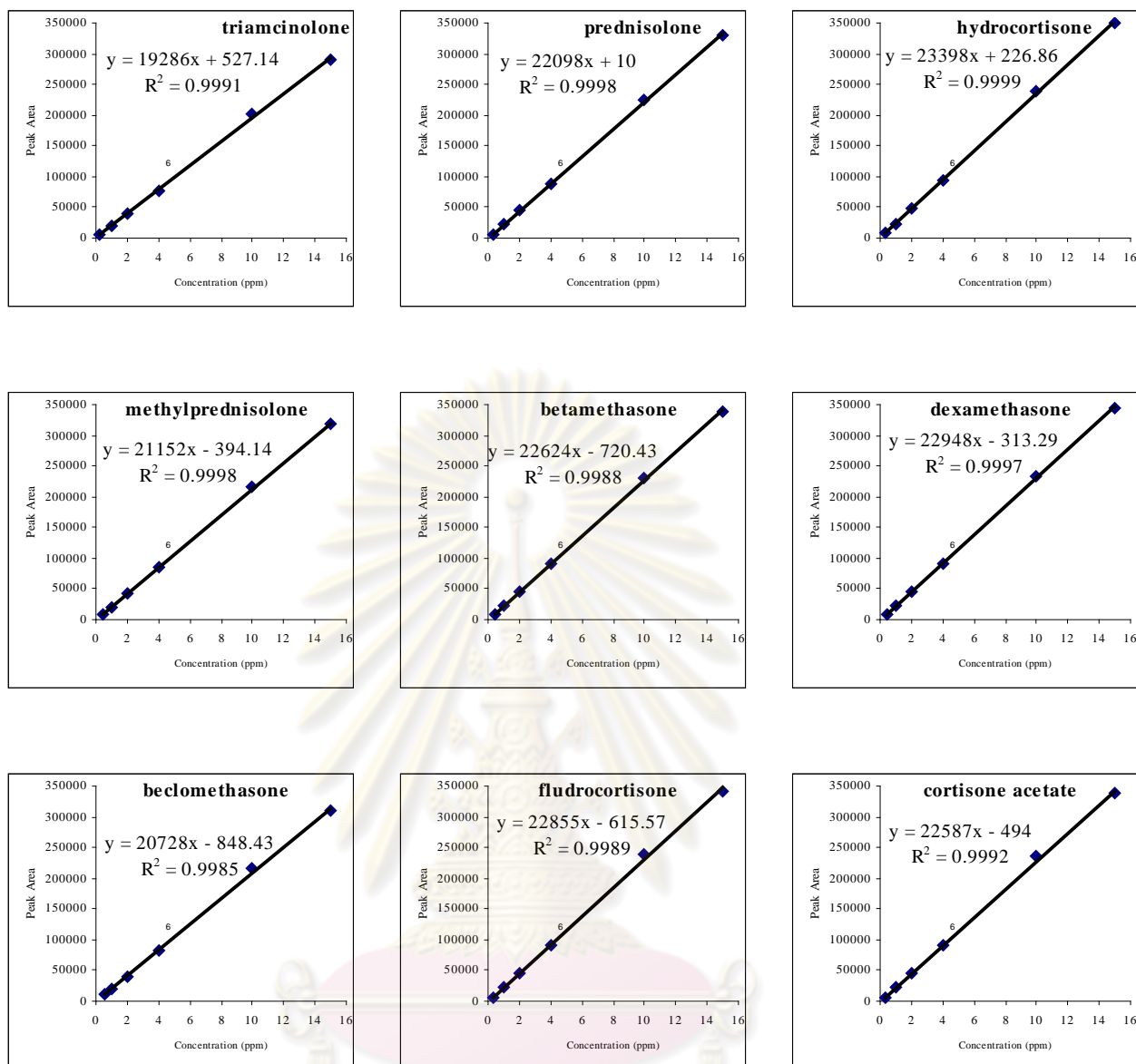
Figure B-4 Standard calibration curves of nine steroids.
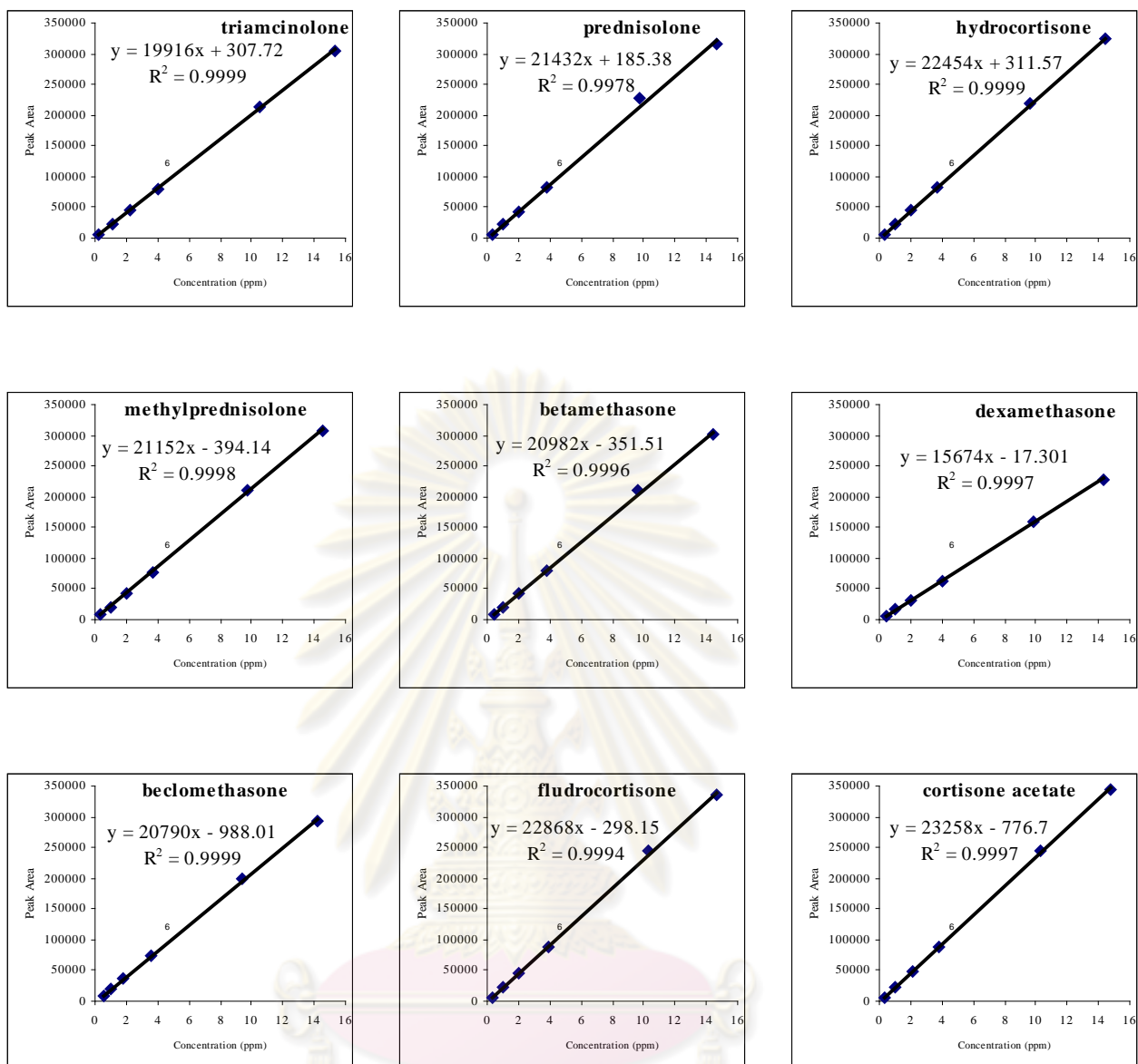
Figure B-5 Method calibration curves of nine steroids**.**
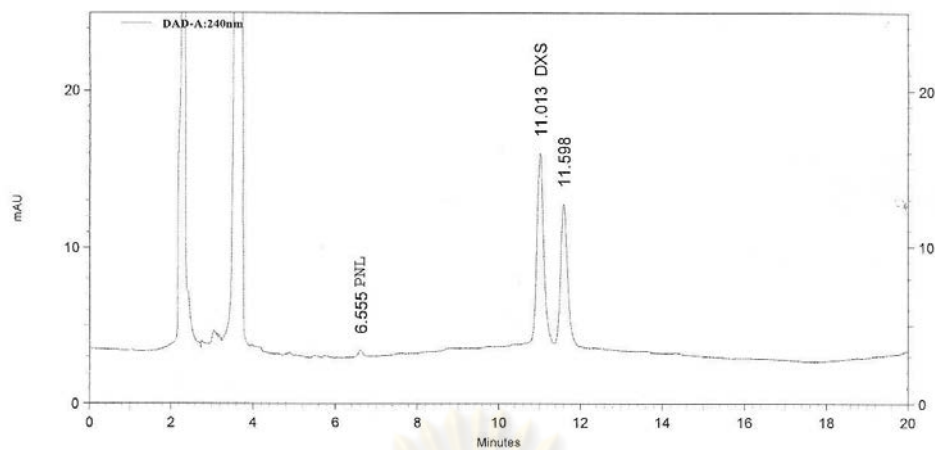
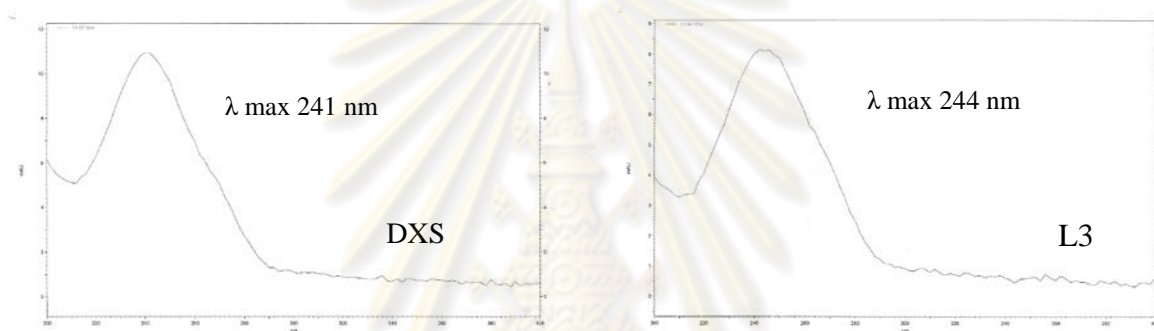**Figure B-6** HPLC chromatogram of 2-fold diluted sample L3 spiked with dexamethasone.

Figure B-7 UV spectra of dexamethasone (the retention time of 11.01 min) and unknown (the peak at retention time of 11.59) in sample L3.
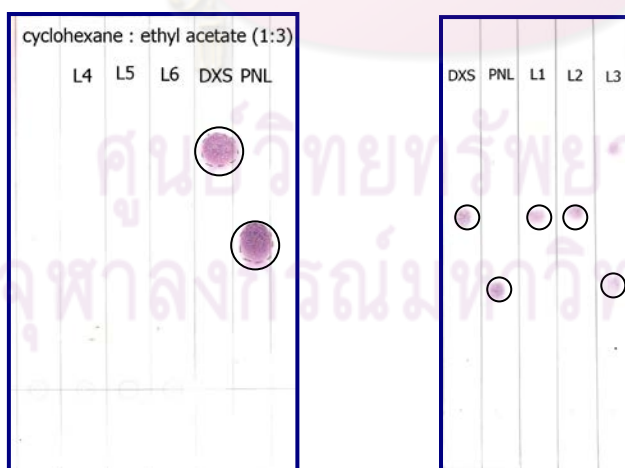

Figure B-8 Routine TLC analysis of six samples.

VITA

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