Determination of Rat Urinary Caffeine Metabolic Ratio and an Application in Studying of Activity of Betong Watercress on Caffeine Metabolism

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การหาค่าอัตราส่วนของเมแทบอไลท์ของคาเฟอีนในปัสสาวะของหนูขาวและการประยุกต์ใช้ในการศึกษาผลของผักน้ำเบตงต่อเมแทบอลิซึมของคาเฟอีน

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บทคัดย่อ

คาเฟอีนเป็นตัวชี้วัดที่ยอมรับในการประเมินการทำงานของเอนไซม์ CYP1A2 ในปัสสาวะ ค่าอัตราส่วนของเมแทบอไลท์ของคาเฟอีนที่ได้มาจากความเข้มข้นของคาเฟอีนและสารเมแทบอไลท์ในพลาสมาและปัสสาวะเป็นพารามิเตอร์ที่นิยมโดยเฉพาะในมนุษย์ กระบวนการเมแทบอลิซึมของคาเฟอีนในหนูมีความคล้ายคลึงกันกับในมนุษย์ จึงสามารถตรวจวัดกระบวนการเมแทบอลิซึมของคาเฟอีน จากค่าอัตราส่วนของเมแทบอไลท์ของคาเฟอีนในตัวอย่างปัสสาวะของหนูได้ เนื่องจากวิธีการสกัดคาเฟอีนและสารเมแทบอไลท์จากตัวอย่างปัสสาวะของหนูยังมีข้อจำกัด ดังนั้นการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อพัฒนาวิธีการสกัดโดยใช้เทคนิคการสกัดสารด้วยตัวดูดซับของแข็ง (SPE) เพื่อวิเคราะห์หาปริมาณคาเฟอีนและสารเมแทบอไลท์ของคาเฟอีน ได้แก่ 137- trimethyluric acid (สารเมแทบอไลท์หลัก), theobromine, paraxanthine และ theophylline ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง (HPLC) ซึ่งใช้วิธีตรวจวัดสัญญาณแบบอัลตราไวโอเลต การศึกษาครั้งนี้ยังมีวัตถุประสงค์เพื่อหาค่าอัตราส่วนของเมแทบอไลท์ของคาเฟอีนในตัวอย่างปัสสาวะของหนูและเป็นการศึกษาเบื้องต้นในการศึกษาอิทธิพลของการได้รับผักน้ำเบตงสดต่อค่าอัตราส่วนของเมแทบอไลท์ของคาเฟอีน

การพัฒนาเทคนิคการสกัดสารด้วยวิธี SPE ใช้คอลัมน์ Oasis® HLB ในการสกัดสารจากปัสสาวะของหนูขาว ทำการตรวจสอบความใช้ได้ของวิธีวิเคราะห์และนำมาใช้วัดความเข้มข้น ปริมาณ และอัตราส่วนของเมแทบอไลท์ของคาเฟอีน จากตัวอย่างปัสสาวะของหนูขาวที่ช่วงเวลาตั้งแต่ 0-9 ชั่วโมง เมื่อได้รับสารมาตรฐานคาเฟอีน (10 มิลลิกรัมต่อกิโลกรัม) ทางปากครั้งเดียว วัดค่าอัตราส่วนของคาเฟอีนและสารเมแทบอไลท์ของคาเฟอีนหลังจากได้รับ PEITC (2 มิลลิกรัมต่อกิโลกรัม) และผักน้ำเบตงสด (714 มิลลิกรัมต่อกิโลกรัม น้ำหนักตัว) ทางปากครั้งเดียว

จากการพัฒนาวิธีการสกัดสารด้วยเทคนิค SPE พบว่าให้ผลการสกัดที่ดี ตัวอย่างปัสสาวะจำเป็นต้องเจือจางก่อนการสกัดและล้างคอลัมน์ด้วย น้ำปราศจากไอออน, 1% ammonium hydroxide:3% methanol (อัตราส่วน 0.5:99.5) และ 1% ammonium hydroxide:3% methanol (อัตราส่วน 0.5:99.5) ชะสารด้วย methanol:acetronitrile (อัตราส่วน 80:20) ผลการตรวจสอบความใช้ได้ของวิธีวิเคราะห์พบว่าได้ค่าความเป็นเส้นตรงที่ดี ค่าร้อยละการคืนกลับสูง และมีค่าแม่นยำและความถูกต้องที่สูง ค่าความเข้มข้นต่ำสุดที่สามารถคำนวณปริมาณได้อย่างถูกต้องและแม่นยำของสารทุกตัวมีค่าอยู่ในช่วง 0.1-0.6 ไมโครกรัมต่อมิลิลิตร ค่าอัตราส่วนของคาเฟอีนและสารเมแทบอไลท์ของคาเฟอีนในหนูขาวหลังจากได้รับสารมาตรฐานคาเฟอีนทางปากครั้งเดียวพบว่าค่อย ๆเพิ่มขึ้นเมื่อเวลาผ่านไป และพบสูงสุดที่ช่วงเวลา 6-9 ชั่วโมง ค่าอัตราส่วนของคาเฟอีนและสารเมแทบอไลท์ของคาเฟอีนในหนูขาวหลังจากได้รับ PEITC และผักน้ำเบตงสดพบว่ามีแนวโน้มเพิ่มขึ้น ผลดังกล่าวอาจจะเสนอได้ว่ากระบวนการเมแทบอลิซึมของคาเฟอีนถูกชักนำโดย PEITC และผักน้ำเบตงสด

สรุปได้ว่าเทคนิค SPE-HPLC ที่ได้พัฒนามานี้สามารถที่จะนำไปใช้ได้ ในการหาค่าอัตราส่วนของสารเมแทบอไลท์ของคาเฟอีนในปัสสาวะ ซึ่งเป็นพารามิเตอร์สำหรับประเมินกระบวนการเมแทบอลิซึมของคาเฟอีนในหนู PEITC (2 มิลลิกรัมต่อกิโลกรัม) อาจจะกระตุ้นการเกิดเอ็น-ดีเมทิลเลชั่นและซี-8-ไฮดรอกซีเลชันของคาเฟอีนในกายภายหลังจากได้รับแบบครั้งเดียว ซึ่งแสดงผลโดยการเพิ่มขึ้นของอัตราส่วนของเมแทบอไลท์ของคาเฟอีนในปัสสาวะ ผักน้ำเบตงสด (714 มิลลิกรัมต่อกิโลกรัม น้ำหนักตัว) มีผลกระตุ้นการเกิดเอ็น-ดีเมทิลเลชั่นและซี-8-ไฮดรอกซีเลชันของคาเฟอีนเช่นกัน แต่น้อยกว่าเมื่อเปรียบเทียบกับ PEITC การศึกษาครั้งนี้ให้ผลเบื้องต้นว่า PEITC และผักน้ำเบตงสดอาจส่งผลกระทบต่อการทำงานของเอนไซม์ CYP1A2 ผลเหล่านี้อาจจะเป็นประโยชน์สำหรับข้อมูลการป้องกันการเกิดมะเร็งได้

Thesis Title Determination of Rat Urinary Caffeine Metabolic Ratio and an Application in Studying of Activity of Betong Watercress on Caffeine Metabolism

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ABSTRACT

Caffeine (CF) is accepted as a probe for determination CYP1A2 activity in urine. CF metabolic ratios calculated from plasma and urine concentrations of CF and its metabolites are widely used parameters especially in human. Metabolism of CF in rats is closely to that in humans, rat urinary CF metabolic ratio used to determine CF metabolism. Since a method for extraction of CF and its metabolites in rat urine is limited, the present study was aimed to develop the Solid-phase extraction (SPE) for the high performance liquid chromatography (HPLC) with ultra-violet (UV) detection to determine CF, 137-trimethyluric acid (137U, main metabolite), theobromine (TB), paraxanthine (PX), and theophylline (TP) in rat urine. The study was also aimed to determine urinary CF metabolic ratios in rats and preliminary study effect of Betong watercress on the metabolic ratios.

Oasis HLB cartridge was used. SPE procedures were tried. The analysis method was validated and used to determine concentrations, amounts, and metabolic ratios of CF and its metabolites in urine of rats given a single dose (10 mg/kg) of CF during 0-9 h post dose. The metabolic ratios of CF after pretreatment with a single oral dose of Phenethyl isothiocyanate (PEITC; 2 mg/kg) and Betong watercress homogenate (714 mg/kg BW) were also determined.

The results showed that the best SPE procedure was as follows. The urine sample was acidified and washed with water, 1% acetic acid:3% methanol-water (0.5:99.5 v/v) and 1% ammonium hydroxide:3% methanol-water (0.5:99.5 v/v). The analytes were eluted with methanol:acetronitrile (80:20 v/v). Method validation showed good linearity, high recovery of extraction, and high precision and accuracy.

The lower limits of quantification (LLOQs) were 0.1-0.6 µg/mL for all analytes. Urinary metabolic ratios after a single oral dose of CF were gradually increasing and were the highest at 6-9 h interval. PEITC and Betong watercress tended to increase urinary CF metabolic ratios. The results may suggest that CF metabolism was induced by PEITC and Betong watercress.

In conclusion, the SPE-HPLC method was valid. It was useful for investigating urinary CF metabolic ratio which was a parameter for assessing CF metabolism in rats. PEITC (2 mg/kg) may induced N-demethylation and C-8 hydroxylation of CF *in vivo* after single dose as shown by an increase of CF urinary metabolic ratios. Betong watercress homogenate (714 mg/kg BW) also induced N-demethylation and C-8 hydroxylation of CF but with a lesser extent compared with PEITC. This study provided prelimination results that they may affect on CYP1A2 activity. These findings may be benefit in chemopreventive data.

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List of Abbreviations

AAMU	Acetyl-6-amino-3-methyluracil
AFMU	Acetylamino-6-formylamino-3-methyluracil
AUC	Area under the curve
BaP	benzo[*a*]pyrene
BNF	5,6-benzoflavone
BW	Body weight
CF	Caffeine
CMR	CF metabolic ratio
CNS	Central nervous system
cyclic AMP	Cyclic 3′,5′-adenosine monophosphate
CYP	Cytochrome P450
DAsA	3-*O*-dodecylcarbomethyl ascorbic acid
DEV	Deviation
DMBA	7,12-dimethylbenz[*a*]anthracene
EAsA	3-*O*-ethylascorbic acid
g	Gram
GC	Gas chromatography
GC-MS	Gas Chromatography - Mass Spectrometry
GLS	Glucosinolates
h	Hour
HCA	Hydroxylated cinamic acid
HLB	Hydrophile-lipophile balance
HPLC	High performance liquid chromatography
HTHQ	1-*O*-hexyl-2,3,5-trimethylhydroquinone
i.e.	Id est
i.p.	Intraperitoneal
ITCs	Isothiocyanates
kg	Kilogram
L	Litre

List of Abbreviations (cont.)

LC	Liquid chromatography
LC-MS	Liquid chromatography–mass spectrometry
LD_{50}	Median lethal dose
LLE	Liquid-liquid extraction
LOD	Limit of detection
LLOQ	Lower limit of quantification
log P	Lipid partition coefficient
LOQ	Limit of quantitation
MDMA	3,4-methylenedioxymethamphetamine
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
MS	Mass spectrometry
mw.	Molecular weight
n	Number
NAT2	N-acetyltransferase 2
ng	Nanogram
NNN	*N′*-nitrosonornicotine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
OCS	Oral contraceptive steroids
p	*p*-value
PEITC	Phenethyl isothiocyanate
pK_a	Ionization constant
PX	Paraxanthine
QC	Quality controls
r	Correlation coefficients
rpm	Revolution per minute
RSD	Relative standard deviation

List of Abbreviations (cont.)

S.D.	Standard deviation
SEM	Standard error of the mean
SPE	Solid-phase extraction
TB	Theobromine
TP	Theophylline
UV	Ultraviolet
v/v	Volume by volume
µg	Microgram
µm	Micrometer
XO	Xanthine oxidase
1,3,7-DAU	6-amino-5-(N formylmethylamino)-1,3-dimethyluracil
1U	1-methyluric acid
13U	1,3-dimethyluric acid
1X	1-methylxanthines
17U	1,7-dimethyluric acid
3U	3-methyluric acid
3X	3-methylxanthines
37U	3,7-dimethyluric acid
7U	7-methyluric acid
7X	7-methylxanthines

CHAPTER 1

Introduction

1.1 Background and rationale

Cytochrome P450 1A2 is an important microsomal enzyme responsible for the metabolism of xenobiotic compounds including several drugs such as certain antidepressants and antipsychotics (Rendic, 2002) or chemical carcinogens including dietary heterocyclic amines and polycyclic aromatic hydrocarbons (Gooderham *et al.*, 1996; Hammons *et al.*, 1997). This enzyme involves in bioactivation of chemical procarcinogens to carcinogens. The activity of this enzyme can be altered by various xenobiotics either by induction or inhibition. As a result, it can regulate toxicity of drugs and carcinogens. As the major role of this enzyme is bioactivation, an assessment for variation of CYP1A2 activity is useful for prediction of chemical carcinogenesis *in vivo*.

Metabolic ratios of caffeine (CF) is most commonly used as an *in vivo* parameter for assessing the activity of CYP1A2 in humans because this enzyme involved in CF metabolism, more than 90% of the primary metabolism of it (Georga *et al.*, 2001; Labedzki *et al.*, 2002; Caubet *et al.*, 2004; Yubero-Lahoz *et al.*, 2012). CF is mainly metabolized via CYP1A2 by demethylation to theobromine (TB), paraxanthine (PX), and theophylline (TP) and hydroxylation to 1,3,7-trimethyluric acid (137U) (Faber *et al.*, 2005). PX is a major metabolite in humans (Caubet *et al.*, 2002) while 137U is the main metabolite in rats (Kot and Daniel, 2008). All metabolites and the parent compound are excreted in urine.

An appropriate analytical method is needed to measure CF and its metabolites in biological samples. A number of analytical methods have been reported for the analysis of CF and its metabolites in different biological samples including gas chromatography (GC) (Kumazawa *et al.*, 1999) and high performance liquid chromatography (HPLC) (Grant *et al.*, 1983; Caubet *et al.*, 2002; Weimann *et al.*, 2005). Among previous studies, HPLC with ultraviolet (UV) detection is the most

frequently used technique for determining CF and its metabolites in human biological fluids. The samples were prepared using one of the following methods; protein precipitation (Blanchard *et al.*, 1990; Noh *et al.*, 2011), liquid-liquid extraction (LLE) (Grant *et al.*, 1983; Begas *et al.*, 2007), and solid-phase extraction (SPE) (Georga *et al.*, 2001; Arinobu *et al.*, 2009). However, those methods still produced some interferences that could contaminate the analytical column and reduce the resolution of separation. In this case, the SPE method may be more attractive because it is faster, easier, and lower solvent consuming than LLE. In addition, it leads to reduction in pollution and offering a wide sorbent selection. Therefore, SPE is widely used to sample preparation especially for biological samples.

Urinary CF metabolic ratios is one of parameters used to assess CYP1A2 activity *in vivo*. Therefore, the method for the extraction and quantification of CF and its primary metabolites, i.e. TB, PX. TP, and 137U, with high accuracy and precision is necessary. A rat model can be employed in evaluating of CYP1A2 activity (Jorritsma *et al.*, 2000). In addition, the chromatographic method for analyzing 137U in urine sample is limited. Such method is required in case of determination of CYP1A2 activity in rat model. The present study is therefore aimed to develop and validate the SPE method for the HPLC-UV technique to determine CF and it metabolites; TB, PX, TP, and 137U, in rat urine. The SPE-HPLC method is used for determining urinary CF metabolic ratio in rats and also applied for preliminary studying the effects of Betong watercress on urinary CF metabolic ratios.

CHAPTER 2

Literature Review

2.1 Caffeine

CF is a methylxanthine alkaloid found in the leaves, seeds or fruits of over 63 plants species. The highest amounts are found in guarana (4-7%), tea leaves (3.5%), coffee beans (1.1-2.2%), cola nuts (1.5%) and cocoa beans (0.03%) respectively. It is also present in a variety of beverages (coffee, tea and soft drinks) and some foods (chocolate and desserts). CF is used both recreationally and medically for central nervous system stimulant effects and some broadly used drugs contain CF for combination with aspirin, ephedrine and acetaminophen.

2.1.1 Physicochemical properties

CF (1,3,7-trimethylxanthine; $C_8H_{10}N_4O_2$; MW. 195.15 g; Table 2.1) is an odorless, white solid, fleecy masses and glistening needles of powder with a bitter taste. It has a volatility of 0.5% and a vapour pressure of 101 kPa at 178 °C. CF is a weak base in the gastric fluid (pH = 2-3), the ionization constant (pK_a) is 14 and the lipid partition coefficient (log P) is 0.85 (Peri-Okonny *et al.*, 2005). It has a melting point with a range of 235-239 °C and a boiling point of 178 °C. The density of CF is higher than that of water (1.23 g/cm^3 at 18 °C) and 1 g dissolves in 46 mL water, 5.5 mL water at 80 °C, moderate soluble in alcohol, acetone and chloroform; slightly soluble in ether and benzene (Merckmillipore, 2014).

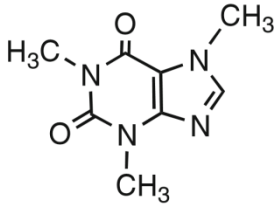
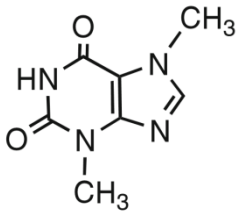
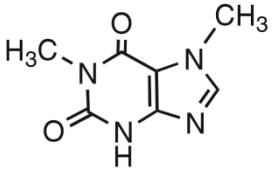
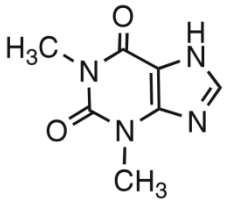
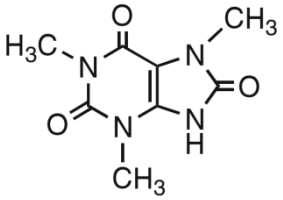
TB (3,7-dimethylxanthine; $C_7H_8N_4O_2$; MW. 180.16 g; Table 2.1) is a principle alkaloid in theobroma cacao (the cacao bean) and other plants. Pure TB is an odorless white crystalline; the color has been listed as either white or colourless. TB is a weak base and pK_a is 9.9 (Peri-Okonny *et al.*, 2005). It has a melting point with a range of 345-350 °C, a boiling point of 483.47 °C and a density of 1.419 g/cm^3 at 25 °C. TB is soluble in DMSO (<1 mg/mL at 25 °C), ethanol (<1 mg/mL at 25 °C), 0.5M sodium hydroxide (50 mg/mL), and water (<1 mg/mL at 25 °C). TB is practically insoluble in benzene, ether, chloroform, and carbon tetrachloride (Chemicalbook, 2008; SantaCruzBiotechnology, 2014).

PX (1,7-dimethylxanthine; $C_7H_8N_4O_2$; 180.16 g; Table 2.1) is off-white to pale yellow solid. PX is a weak base and pK_a is 8.50 (Peri-Okonny *et al.*, 2005). It has a melting point with a range of 294-296 °C and a density of 1.6 g/cm^3. PX is soluble in ethanol (0.6 mg/mL), 0.1N sodium hydroxide (2 mg/mL), water (1 mg/mL), and 1N ammonium hydroxide (49.00-51.00 mg/mL) (SantaCruzBiotechnology, 2014; Sigma-aldrich, 2014).

TP (1,3-dimethylxanthine; $C_7H_8N_4O_2$; MW. 180.16 g; Table 2.1) is an odorless white crystalline powder with a bitter taste. TP is a weak base and pK_a is 8.60 (Peri-Okonny *et al.*, 2005). It has a melting point with a range of 274-275 °C, a boiling point of 454 °C and a density of 1.35 g/cm^3 at 25 °C. TP is soluble in water (5.5 mg/mL at 19.9 °C) and clear colorless solution at 50 mg/1M ammonium hydroxide, moderately soluble in 0.1M sodium hydroxide and 0.1M hydrochloric acid (SantaCruzBiotechnology, 2014; Sigma-aldrich, 2014).

137U (1,3,7-trimethyluric acid; $C_8H_{10}N_4O_3$; MW. 210.19 g; Table 2.1) is a weak acid and pK_a is 5.89 (Peri-Okonny *et al.*, 2005). Pure 137U is an odorless white crystalline. It has a melting point of 300 °C and a density of 1.6 g/cm^3. 137U is soluble in water (5.5 mg/mL at 15 °C) (Chemspider, 2014; Sigma-aldrich, 2014).

Table 2.1 Molecular structures and some physicochemical characteristics of CF and its metabolites (Ashihara *et al.*, 2011)

No.	Analyte	Molecular mass	Molecular structure
1	CF	195.15	
2	TB	180.16	
3	PX	180.16	
4	TP	180.16	
5	137U	210.19	

2.1.2 Pharmacokinetics

1) Absorption

CF is absorbed mainly form the small intestine and partly (20%) from the stomach. The bioavailability in humans is 99% (Chvasta and Cooke, 1971; Fredholm, 2011). In both animals and humans, the peak plasma CF concentrations are reached between 15-120 min after oral ingestion (dose 5-8 mg/kg for humans) and the mean plasma values are 8-10 mg/L (Arnaud and Welsch, 1982; Bonati *et al.*, 1982; Fredholm, 2011). CF pharmacokinetics is independent on the route of administration. Its pharmacokinetics is comparable after oral or intravenous administration of CF in humans and animals, leading to superimposable plasma concentration curves and suggesting that there is no significant hepatic first-pass effect (Bonati *et al.*, 1982; Bonati *et al.*, 1984; Fredholm, 2011).

The dose-independent pharmacokinetics in animals for CF was described with one-compartment open model. Latini and coworkers (1978) studied CF pharmacokinetics in rats after oral administration of CF at three different doses 1, 10, 100 mg/kg. They reported that the ratio of brain:plasma concentrations changed with the dose. The AUC changed about 45-746 times in plasma and about 10-100 times in brain. These findings indicated dose-dependent absorption kinetics of CF.

2) Distribution

CF is poorly bound to plasma protein (10-30%) at a wide range of concentrations (Patwardhan *et al.*, 1980; Blanchard and Sawers, 1983a). Its volume of distribution is 0.8-0.9 L/kg in infants, 0.5-0.75 L/kg in adults and 0.9 L/kg in rats. CF distributes relatively uniformly throughout all body tissues, including breast milk, saliva, and serum (Bonati *et al.*, 1984; Fredholm, 2011). Nakazawa and coworkers (1985) reported that volume of distribution of CF in rats given CF decreased during pregnancy. CF is no physiological barriers limited through to the entire body. Therefore, it passes across the blood brain barrier, the placenta into amniotic fluid and the fetus, and breast milk. (Berger, 1988; Kot and Daniel, 2008). McCall and coworkers (1982) reported that CF may enter the brain by simple diffusion and carrier-mediated transport.

3) Metabolism and excretion

3.1) Metabolic pathways

CF metabolism in humans includes multiple and separate pathways with demethylation to dimethyl- and monomethylxanthines, and C-8 oxidation of these methylxanthines into methylurates and uracil formation. The rate of CF metabolism relates mainly to CYP1A2 and to a lesser extent xanthine oxidase (XO) and N-acetyltransferase 2 (NAT2) (Fenster *et al.*, 1998). As shown in Figure 2.1, CF is oxidized at a few position of its molecular structure; CYP1A2 catalyses the N-1, N-3, and N-7 demethylation of CF to form TB, PX, and TP respectively, accounting for about 11, 80, and 4% for the major part of CF metabolism (Lelo *et al.*, 1986). Minor pathways of CF metabolism in humans include formation of the C8-hydroxylated metabolite to form 137U. Each of these dimethylxantines undergoes an N-monodimethylation reaction to give the corresponding monomethylxanthines; 1-methylxanthines (1X), 3-methylxanthines (3X), and 7-methylxanthines (7X). After that, CF and xanthine are hydroxylated to their corresponding uric acid; 1,3-dimethyluric acid (13U), 1,7-dimethyluric acid (17U), 3,7-dimethyluric acid (37U), 1-methyluric acid (1U), 3-methyluric acid (3U), and 7-methyluric acid (7U).

Other non-microsomal enzymes participate in CF metabolism. PX is further metabolized via two parallel but independent reactions. The polymorphic NAT2, catalyses the C8-N9 bond scission and the acetylation of PX to produce 5-acetylamino-6-formylamino-3-methyluracil (AFMU). AFMU is converting to 5-acetyl-6-amino-3-methyluracil (AAMU) in urine (Fredholm, 2011).

CF metabolism in the rats is similar in term of pathway to that in humans (Figure 2.2). It is primarily metabolized via CYP1A2-mediated the N-1, N-3, and N-7 demethylation to TB, PX, TP and the C-8-hydroxylation to 137U (70%) which is a major metabolite in CF metabolic pathway in rats (Kot and Daniel, 2008). Each primary metabolite either by N-demethylation or C-8-hydroxylation products of CF are metabolized further to form methylxanthines, methylurates and uracil for excretion. Characteristics of CF biotransformation in rats is the formation of 6-amino-5-(N formylmethylamino)-1,3-dimethyluracil (1,3,7-DAU), a metabolite with an opened imidazole ring, in amounts of quantitative importance that was not found in

human (Schrader *et al.*, 1999). CF metabolism decreases during pregnancy, resulting in higher serum concentrations.

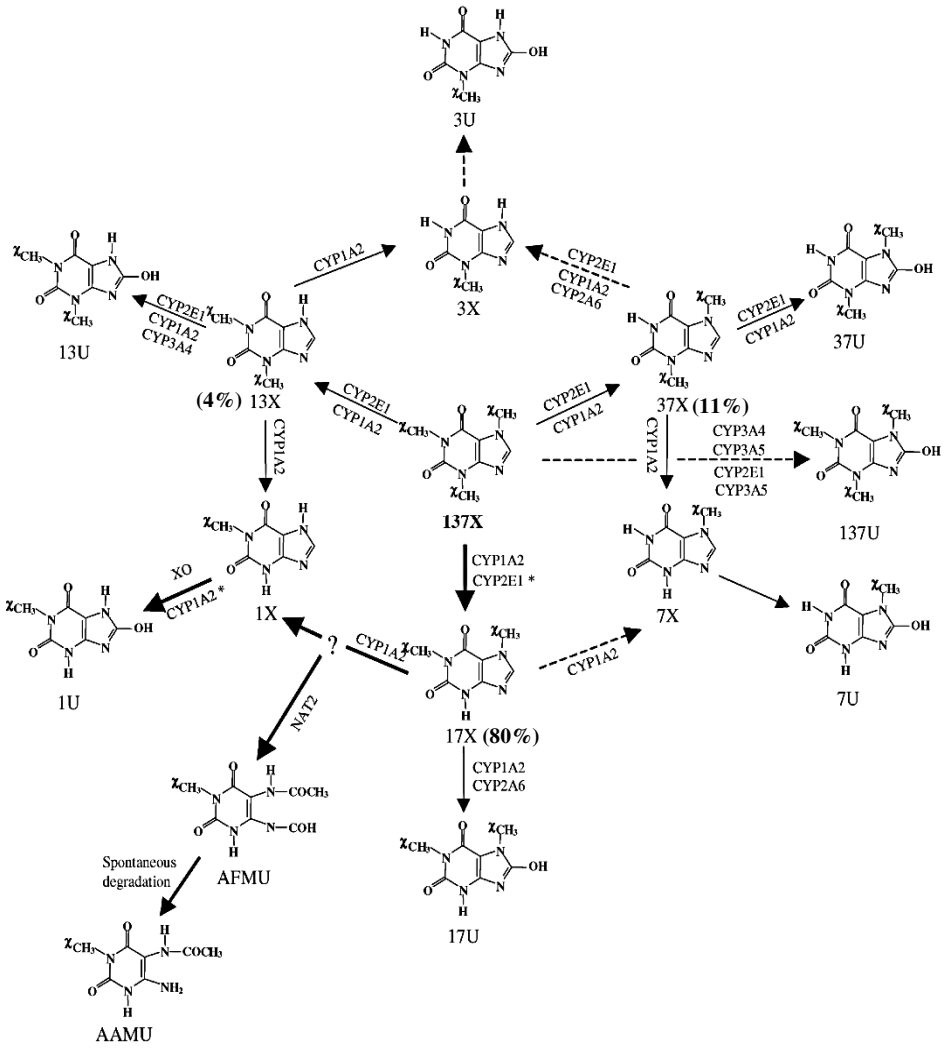


Figure 2.1 CF metabolic pathway in human liver (Caubet *et al.,* 2002)

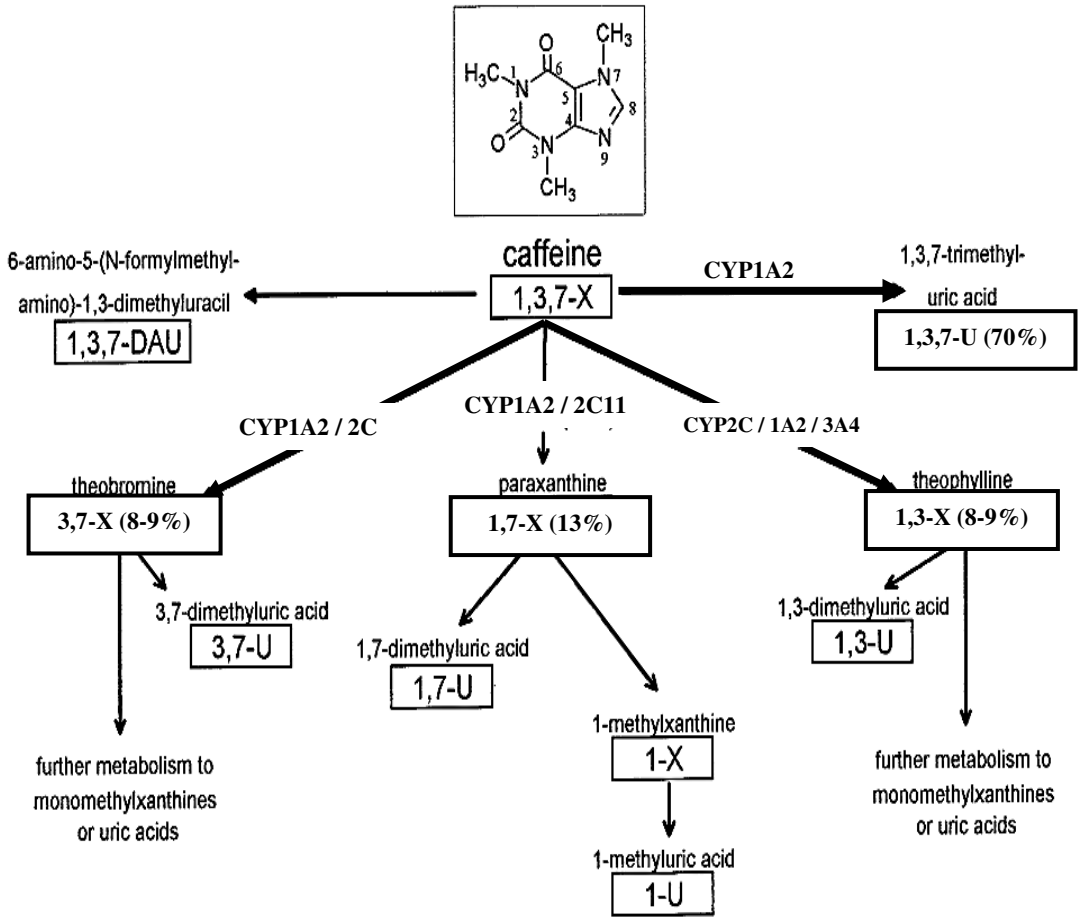


Figure 2.2 CF metabolic pathway in rat liver (Jorritsma *et al.*, 2000; Kot and Daniel, 2008)

3.2) Excretory route

CF and its metabolites are mainly excreted via urine approximately 90% and 95% in humans and rats, respectively. CF is reabsorbed in the renal tubule about 0.5-2% of CF at the dose of 7.5 mg/kg. (Fredholm, 2011). For higher CF intake (1 g, 10-12 cups of coffee), the recovery of CF in urine is from 0.74-0.91% of the dose and the urinary concentration is 14 mg/L. A good correlation is found between urinary and plasma CF concentrations (Birkett and Miners, 1991). CF elimination half-life ranges from 0.7-1.2 h in rats and mice, 3-5 h in monkeys at dose 10 mg/kg (Bonati *et al.*, 1984), 2.5-4.5 h in young and elderly humans at dose 5 mg/kg (Arnaud, 1987; Blanchard and Sawers, 1983b). The elimination half-life can be prolonged during the neonatal period for the full-term newborn infant approximately 80±23 h (Aranda *et al.*, 1977; Le Guennec and Billon, 1987) and can be over 100 h for the premature infants (Parsons and Neims, 1981). The elimination half-life is prolonged about 4 h during the first trimester to 18 h during the third trimester of pregnancy and 4.5 h in women taking oral contraceptives when compared to women taking no oral contraceptives (Patwardhan *et al.*, 1980). In adult males, the elimination half-life is reduced about 30-50% in smokers when compared with non-smokers (Hart *et al.*, 1976; Murphy *et al.*, 1988). CF is eliminated by first order kinetics in healthy humans. Its elimination is described by a one-compartment open model in the dose between 2-10 mg/kg (Newton *et al.*, 1981; Bonati *et al.*, 1982).

2.1.3 Assessment of CYP1A2 activity using CF a marker

1) Cytochrome P450 (CYP)

CYP is a superfamily of constitutive and inducible enzymes. These enzymes are responsible for the metabolism of several endogenous and exogenous compounds, including drugs and carcinogens (Coon *et al.*, 1992; Conley and Hinshelwood, 2001). Although more than 40 different CYP genes are present in the human genome, about 6 genes involve for more than 90% of the oxidation of drugs (CYP1A2, 2A6, 2C9, 2D6, and 3A4) or carcinogens (CYP1A1, 1A2, 1B1, 2A6, 2E1, and 3A4) (Rodriguez-Antona and Ingelman-Sundberg, 2006; Pelkonen *et al.*, 2008).

CYP1A2 is one of the major phase I enzymes in the liver. It accounts for about 15% of total liver P450 content (1994 Shimada). This enzyme plays an important role in the biotransformation of many clinically used drugs such as TP (Tanaka *et al.*, 2014), imipramine (Lemoine *et al.*, 1993), clozapine (Bertilsson *et al.*, 1994), propranolol (Masubuchi *et al.*, 1994), and olanzapine (Callaghan *et al.*, 1999). It is the most important catalyst in the metabolism of CF and has been implicated in the bioactivation of carcinogenic substance such as aromatic and heterocyclic amines and polycyclic aromatic hydrocarbons (Boobis *et al.*, 1994; Hammons *et al.*, 1997). The high degree of inter-individual variability of CYP1A2 expression and activity in human liver is probably due to both genetic and environmental factors. Thus, CYP1A2 activity may influence individual susceptibility to cancer risk.

2) CF metabolic ratio

Phenotyping by specific probe substrates of CYP isoform is widely used today to assess the *in vivo* activity of drug-metabolizing enzymes (Perera *et al.*, 2013). Causes for variation in enzyme activity are genetic polymorphisms, induction, or inhibition by medication or environmental factors, physiological status such as age and gender, and pathologic conditions such as liver disease (Fuhr *et al.*, 2007). Ideal a probe drug should have a wide safety margin for *in vivo* use and be exclusively metabolised by the enzyme of interest, preferably to a single primary metabolite (Pelkonen *et al.*, 1998; Frye, 2004). Phenotyping can be performed to determine the enzyme activity using probe drugs such as CF for CYP1A2, mephenytoin or omeprazole for CYP2C19, dextromethorphan or debrisoquine for CYP2D6, and chlorzoxazone for CYP2E1 (Streetman *et al.*, 2000). Phenotype classification *in vivo* is based on drug to metabolic concentration ratio in biological fluids (metabolic ratio or MR). Many probes have been tried to measure CYP1A2 metabolic activity, including phenacetin (Bartoli *et al.*, 1996), tacrine (Larsen *et al.*, 1999), melatonin (Hartter *et al.*, 2001), TP (Caubet *et al.*, 2004) and tizanidine (Backman *et al.*, 2008), and CF (Hakooz, 2009).

Currently, CF is a first-choice substance for assessment CYP1A2 activity *in vivo*. Although CYP2E1, CYP2A6, CYP2C8/9, and CYP3A4 are also involved in CF metabolism, 90% of its metabolism in both humans and animals is regulated by CYP1A2. CF is a useful enzymatic probe because of its rapid and complete gastrointestinal absorption, distribution throughout total body water, its low plasma protein binding, as well as for its short half-life, negligible first-pass metabolis, its complete biotransformation in the liver, and minimal renal excretion (Arnaud, 1987; Krul and Hageman, 1998). In addition, CF is a relatively safe, low toxic and known drug for human subjects. CF metabolic ratio is well determined marker of CYP1A2 metabolic activity. Moreover, metabolic ratios of CF and its metabolites are also used for determination of NAT2, XO and CYP2A6 activities (Nyeki *et al.*, 2001; Begas *et al.*, 2007; Hakooz, 2009). These approaches utilize CF concentration in plasma or saliva (CF clearance), as well as plasma, saliva, or urinary CF metabolite ratio. Therefore, metabolism of CF mediated by CYP1A2 can be

determined by measuring metabolic ratios in urine, saliva, plasma, and serum (Kalow and Tang, 1993; Fuhr and Rost, 1994).

The metabolic ratios from plasma and salivary samples have been proposed as valid measures of CYP1A2 activity. The ratio of PX to CF concentrations in either plasma or saliva at 3 to 10 h following administration of CF was the most widely study to measure CYP1A2 activity (Fuhr and Rost, 1994). Fuhr and Rost (1994) conducted a clinical trial comprising 78 subjects to validate the use of the PX/CF ratios in plasma and saliva for measuring activity of CYP1A2. The PX/CF ratios in plasma and saliva especially at 5-7 h after administration of CF were the most closely resemble to systemic CF clearance with correlation coefficients (r) typically higher than 0.85. They suggested the ratios of PX/CF in plasma and saliva appear a valid and inexpensive method of assessing CYP1A2 activity *in vivo*.

Carrillo and coworkers (2000) examined the validity and appropriateness of several CF-based methods for performed CYP1A2 activity in plasma, saliva, and urine. Enzyme activity was assessed by the ratio of PX/CF in plasma and saliva, and the ratio of (AFMU+1U+1X+17U+PX)/CF in urine. Participants were given 100 mg CF orally. The subjects consisted of 25 (12 men and 13 women) healthy volunteers and divided into two groups (smokers and nonsmokers). Among indices that appear to be relatively simple and inexpensive to measure, they recommended the plasma or saliva 17X/137X ratio determined at 4 hours after dose and the urinary ratio of (AFMU+1U+1X+17U+PX)/CF in a sampling interval of at least 8 hours, starting at time zero since CF intake. CYP1A2 activity was in smokers significantly higher metabolic ratio than nonsmokers ($p < 0.0001$) in plasma sample. They suggested that a higher activity of CYP1A2 depend on smoking. These indices are simple, reliable, and relatively inexpensive estimates of CYP1A2 activity to be used in the study of human populations.

Bozikas and colleagues (2004) determined of CF and its metabolites in urine and saliva. The study was to assess indirectly the impact of smoking on the metabolism of psychotropic drugs in a group of patients with schizophrenia, by measuring CYP1A2 activity. The authors assessed the metabolic ratio of CF and metabolites in urine such as (AFMU+1U+1X)/17U and saliva such as (PX/CF).

Participants 40 patients were divided into two groups such as smokers and nonsmokers. The metabolic ratio of CF metabolites in urine and saliva of smokers with schizophrenia were significantly higher than that of nonsmoking patients. These results suggest that CYP1A2 activity is higher in smokers.

Backman and coworkers (2006) studied the effect of rofecoxib on CYP1A2 activity in humans, using CF as probe substrates. Nine healthy male nonsmokers took 25 mg of rofecoxib and 100 mg of CF. Plasma was collected and CMR (PX/CF) was calculated for assessment of CYP1A2 activity. Rofecoxib significantly increased the CMR by 2.4-fold.

Yubero-Lahoz and colleagues (2012) studied the activity of CYP1A2 after CYP2D6 inhibition by 3,4-methylenedioxymethamphetamine (MDMA) in healthy subjects. CMR PX/CF was calculated to assess the CYP1A2 activity in male and female subjects before and after MDMA intake. The study indicated that CYP1A2 activity was higher in both genders, especially in females after CYP2D6 inhibition.

Urinary CF metabolic ratios are frequently used in human to assess CYP1A2 enzyme activity (Table 2.2). Two of these ratios have been frequently employed as a measure of CYP1A2 activity in humans; ratio 1: (PX+17U)/CF (Butler *et al.*, 1992; Kalow and Tang, 1993) and ratio 2: (AFMU+1X+1U)/17U (Campbell *et al.*, 1987). Butler and coworkers (1992) used the ratio of N-3 demethylation: (PX+17U)/CF with the measurement of PX and the 8-hydroxylated metabolite (17U) at 4-5 hours following the administration of a standard CF dose.

Table 2.2 Urinary CF metabolic ratio used to measure CYP1A2 activity *in vivo*

Ratio	Reference
(AFMU+1X+1U)/PX	Grant *et al.* (1983)
(AFMU+1X+1U)/17U	Campbell *et al.* (1987)
(PX+17U)/CF	Butler *et al.* (1992)
(AFMU+1U+1X+17U +PX)/CF	Carrillo and Benitez (1996b)
PX/CF	Furge and Fletke (2007)

Krul and Hageman (1998) reported an analysis of urinary CF metabolites. A standardized procedure was used for oral intake of CF and for urine collection. CYP1A2 activity was calculated from the CF metabolic ratio (CMR) (AFMU+1X+1U)/17U, N-acetyltransferase (NAT) from the ratio AFMU/1X, XO from the ratio (1U/(1X+1U) and CYP2A6 from the ratio 17U/(17U+17X+1U+1X+AFMU). CMR were determined for four groups of subjects; healthy, non-smoking females using oral contraceptives (n=55) and non-users (n=55), healthy nonsmoking males (n=59) and children (n=57). A significantly higher CYP1A2 ratio was found for males (4.87±60.47) compared to females (3.62±60.91; p = 0.005). For the other enzyme activities, no significant differences were found between the groups of subjects in this study.

Caubet and coworkers (2004) determined levels of CF and its fourteen metabolites in urine by using HPLC-MS. CMRs were determined after an oral bolus of labelled CF in 20 healthy subjects with different characteristic CYP1A2 activity. Eight men, 4 non-smokers (group A), 4 smokers (group B), and 12 women, 4 non-smokers (group C), 4 non-smokers taking oral contraceptive steroids (OCS) (group D) and 4 smokers taking OCS (group E) were included. The CMRs were calculated and used as an index of CYP1A2 activity (7X+37U+TB)/CF, (PX+17U)/CF, (3X+13U+TP)/CF, and (TP+PX+TB)/CF. Women taking OCS intake decreased significantly all ratios in non-smoking women (group D versus group C). On the contrary, smoking habit increased significantly all ratios in women taking OCS (group E versus group D). Smoking habit in women taking OCS resulted in an overall metabolic activity not significantly different from that measured in non-smoking women taking no OCS.

Begas and coworker (2007) used CMRs (AFMU+1U+1X)/17U, 17U/PX, and 1U/(1X+1U) for assessment *in vivo* activity of enzyme CYP1A2, CYP2A6, and XO, respectively, in urine of subjects. Subjects (21 men, 23 women) were divided into two groups according to their smoking status: smokers (>10 cigarettes/day) and non-smokers. Urine samples were analyzed 6 h after 200 mg pure CF consumption. The median values of CYP1A2 activities were significantly different between non-smokers (CMR = 3.57) and smokers (CMR = 5.83; $p < 0.001$).

CYP2A6, XO activities were not significantly different both non-smokers and smokers. These results showed that smoking affected only CYP1A2 but not CYP2A6.

Rat CMR was also reported. Jodynis-Liebert and Matuszewska (1999) measured of CF and its metabolites and assessed CYP1A2 activity in male Wistar rats pretreated with toluidine (1, 10, 60 mg/kg BW) and dinitrotoluene (DNT) (100 and 200 mg/kg BW). CMRs in plasma: TB/CF, PX/CF, TP/CF, (TB+PX+TP)/CF were calculated and compared to those of control rats. Toluidines caused a significant increase of all ratios. Toluidines seemed to be an inducer of CYP1A2. Similaly, Jorritsma and coworker (2000) examined the usefulness of urinary CF metabolites as a measure of CYP1A activity in rats. Male and female Wistar rats were treated with [1-Me-^{14}C]-CF (10 mg/kg i.p.), before and 48 h after administration of the potent CYP1A inducer 5,6-benzoflavone (BNF) (80 mg/kg, i.p.). It was found that the CMR (1U+1X)/PX increase after administration of BNF.

2.1.4 Pharmacodynamics

1) Mechanism of action

CF acts through several mechanisms, including blocking of adenosine receptors, inhibiting of phosphodiesterase and mobilizing of extracellular calcium. The most important mechanism of action of CF is the competitive antagonism of adenosine receptors: A_1 and A_2. Adenosine is an endogenous neuromodulator with mostly inhibitory effects. It inhibits the release of neurotransmitters from presynaptic sites. Therefore, CF is an antagonist of adenosine receptors leading to augmenting of neurotransmitters such as norepinephrine. CF increases intracellular cyclic 3′,5′-adenosine monophosphate (cyclic AMP) by inhibiting of phosphodiesterase, the enzyme that degrades cyclic AMP. CF also increases the release of various neurotransmitters such as dopamine and serotonin in the brain.

2) Pharmacological effects

- Central nervous system

CF is the central nervous system (CNS) stimulant. Main mechanisms of action of CF on the CNS have been described. The targets of CF are the cerebral cortex and the brain stem. Low to moderate doses (20–200 mg) of CF cause the most behavioral effects alertness and decrease fatigue (Juliano and Griffiths, 2004). Zwyghuizen-Doorenbos and coworkers (1990) investigated the effects of CF on alertness. Subjects were given CF (250 mg twice/day) performed significantly better in an auditory vigilance test than did the placebo group. Several studies suggest that CF improves performance on vigilance tasks and simple tasks which require sustained response vigilance at dose of CF 200 mg (Regina *et al.*, 1974) and 75-300 mg (Clubley *et al.*, 1979). On the other hand, Sicard and coworkers (1996) reported increased anxiety after administration of high dose (600 mg) CF.

CF also has effects on sleep. It reduced total sleep time, increased latency to sleep, and reduced percent stage 3-4 sleep in a dose-related manner (Okuma *et al.*, 1982; Hicks *et al.*, 1983; Zwyghuizen-Doorenbos *et al.*, 1990). However, REM sleep was not affected.

The spinal cord is stimulated at very higher doses (2-5 g) of CF. Very high doses, from accidental or suicidal overdose, cause medullary stimulation and convulsions and may lead to death (Mycek *et al.*, 1997).

- Cardiovascular system

At low concentrations, CF effects appear to result from inhibition of presynaptic adenosine receptors in sympathetic nerves increasing catecholamine release at nerve endings. The higher concentrations (> 10 μmol/L, 2 mg/L) associated with inhibition of phosphodiesterase and increases in cAMP. These actions produce slight tachycardia, an increase in cardiac output and peripheral resistance. CF elevates the stress hormones cortisol, epinephrine, and norepinephrine cause to increased blood pressure slightly.

Rachima-Maoz and colleagues (1998) reported that CF (250 mg) increases systolic and diastolic blood pressure in old (58 years) hypertensive patients.

The effect of CF on blood pressure in habitual CF consumers and abstainers has been investigated in more than 50 acute and 19 repeated dose clinical trials in healthy and hypertensive subjects. The results of the acute studies showed that CF increase in systolic about 5-15 mmHg and diastolic about 5-10 mmHg blood pressure, at doses more than 250 mg/person (Nawrot *et al.*, 2003).

Most *in vitro* studies on the direct vasomotor action of xanthine derivatives in isolated vessel preparations have revealed a vasodilator action (Brodmann *et al.*, 2003; Lo *et al.*, 2005).

- Gastrointestinal system

CF and all methylxanthines stimulate secretion of both gastric acid and digestive enzymes from the stomach and the small intestine causing stomach and intestinal injury. However, even decaffeinated coffee has a similar stimulant effect on secretion gastric acid proving that CF is not only compound in coffee that influences gastric juice flow (Cohen and Booth, 1975). From the data reported by Brown and coworkers (1990), coffee stimulates motility and promotes defecation in the distal colon.

- **Respiratory system**

CF stimulates respiratory centers promoting increased respiratory rate. It is a weak bronchodilator and it also reduces respiratory muscle fatigue. In clinical tests on adults with asthma, at fairly low doses (5mg/kg of body weight), CF provides a small improvement in lung function (Welsh *et al.*, 2010). TP, a one metabolite of CF, is one of the most widely prescribed medications for the treatment of asthma worldwide. CF is the primary treatment of the breathing disorders (Mathew, 2011) and may be effective in preventing bronchopulmonary dysplasia in premature infants (Kugelman and Durand, 2011). Mechanism of action of CF in reducing apnea may be through blockade of adenosine A_{2A} receptors on GABAergic neurons.

- Renal system

CF and TP are weak diuretics that enhanced renal excretion of water and electrolytes such as sodium, chloride, and potassium. This effect caused by both increased glomerular filtration rate and reduced tubular sodium reabsorption because CF blocked adenosine A_1 receptors in the proximal tubular reabsorption (Rieg *et al.*, 2005; Osswald and Schnermann, 2011). This effect can result in dehydration. The diuretic potency of CF appears to be also modulated by age and habituation, with old age and previous exposure to CF causing further decreases in the diuretic effectiveness of CF.

3) Withdrawal symptom

CF withdrawal symptoms may appear within 12-24 hours after abrupting of CF, peaking at approximately 48 hours, and usually lasting from 2-9 days (Juliano and Griffiths, 2004). CF withdrawal symptoms reported in human are headaches, feelings of weariness, weakness and drowsiness, impaired concentration, fatigue and work difficulty, depression, anxiety, irrritability, increased muscle tension, occasionally tremor, and nausea and vomiting, as well as withdrawal feelings (Nehlig, 1999). Headaches are most commonly symptoms seen as the most problematic side effect of withdrawal, and are caused by a temporary increase in cerebral blood pressure (Jones *et al.*, 2000). Administration CF > 250 mg/day can produce headaches, irritability, lethargy, and occasional nausea. Significant withdrawal symptoms have been observed at longterm intakes as low as 100 mg/day, although they are more common with higher intakes (Dews *et al.*, 1999).

4) Toxicity

Toxicity of CF occurs primarily through nonselective inhibition of adenosine receptors. At overdose, it acts through $beta_1$- and $beta_2$-adrenergic stimulation secondary to release of endogenous catecholamines.

- Acute effect

An acute overdose of CF usually in excess of about 300 mg, dependent on body weight and level of CF tolerance, can result in a state of CNS over-stimulation called CF intoxication.

The median lethal dose (LD_{50}) given orally in rats is 200-400 mg/kg and 185 mg/kg in mice. The LD_{50} given inhalation in rats is 4.94 mg/L/4h or less than 2000 mg/kg. The LD_{50} of CF in humans is dependent on weight and individual sensitivity and estimated to be about 150- 200 mg/kg of body mass or roughly 80-100 cups of coffee for an average adult taken within a limited time frame that is dependent on half-life. Ingestion of 15-30 mg/kg has resulted in significant toxicity. At doses 200-300 mg, CF enhances arousal and performance of both cognitive and psychomotor skills (Smith, 2002).

Symptoms of acute toxicity of CF are usually anorexia, tremor, and restlessness, followed by nausea, vomiting, tachycardia, and agitation. With serious intoxication, delirium, seizures, supraventricular and ventricular tachyarrhythmias, hypokalemia, and hyperglycemia may occur. CF poisoning occasionally causes rhabdomyolysis and acute renal failure (Higdon and Frei, 2006).

- Chronic effect

Chronic or long-term effects result from the ingestion of high doses of CF (500-600 mg/day) that leads to “caffeinism” which includes a wide variety of symptoms such as nervousness, irritability, anxiety, tremulousness, muscle twitching, insomnia, palpitations, and hyperreflexia (Carrillo and Benitez, 1996a).

2.1.5 Analytical methods for determination of CF and its metabolites in biological matrices

Methods of analysis of CF and its metabolites in a wide range of matrixes such as in foods, beverages, pharmaceutical products, and biological fluids have previously been reported. The extraction of CF and its metabolites is usually required to remove proteins and other impurities (as shown in Table 2.3). The analytical requirements for the development of CF and its metabolite is increasing and results in a continuous search for new, reliable, precise, simple, fast and low-cost methods of analysis.

1) Sample extraction

The extraction techniques for CF and its metabolites include protein precipitation, LLE, and SPE.

- Protein precipitation

Schrader and coworkers (1999) determined ^{14}[1-Me- C]CF and its eight major radiolabelled metabolites in rat urine using HPLC. The sample was prepared by protein precipitation with acetic acid (100%) and then centrifuged for 5 min at 15,000 g. This extraction leads to a shorter sample preparation time and allowed a quantification of the radiolabelled metabolites based only on the measured radioactivity in the corresponding peak without consideration of any extraction efficiency.

Kamimori and coworkers (2002) evaluate the rate of absorption and relative bioavailability of CF from a Stay Alert® chewing gum and capsule formulation in human plasma. Plasma samples were added 250 L of 0.8 M perchloric acid for precipitate protein. The resulting solution was vortexed for 10 s and centrifuged at 6,000 rpm for 5 min. The supernatant was injected into the HPLC system for determination CF.

Alvi and coworkers (2011) determined CF in human plasma by using HPLC with photodiode array detector. Plasma sample was precipitated with perchloric acid. The supernatant was centrifuged at 12000 rpm for 10 min.

The advantages of protein precipitation are simple and cost effective. In addition, instruments are easily available. The disadvantages are skill requirement for effective results.

- Liquid liquid chromatography (LLE)

LLE is a traditional and primarily used for extracting CF and its metabolites from many samples such as beverages (Shrivas and Wu, 2007) and biological fluids such as urine (Zambonin *et al.*, 2004), and plasma (Yubero-Lahoz *et al.*, 2012), saliva (Perera *et al.*, 2010), and sweat (Tsuda *et al.*, 2000). The basic technique is utilizing two immiscible liquids with often water and an organic solvent for the removal of an analyte, referred to as the solute. Extraction of CF and its metabolite by LLE has been reported by several approaches which wide variety of solvents have been used, including chloroform:isopropanol mixture (80:20) (Krul and Hageman, 1998), (85:15) (Bendriss *et al.*, 2000), or (90:10) (Ventura *et al.*, 2003), dichloromethane:methanol (90:10) (Delbeke and De Backer, 1996), dichloromethane:isopropanol (90:10) (Davis *et al.*, 1993), ethyl acetate:isopropanol (92:8) (Nordmark *et al.*, 1999), (93:7) (Rasmussen and Brosen, 1996), dichloromethane in acidic pH (Tanaka, 1992), chloroform (Pons *et al.*, 1988), and chloroform:ethanol (82.5:17.5) (Falk and Lau, 1991). Two-component extraction mixtures of organic solvents are often used for increase the distribution ratios. The extraction efficiency of the separation can be improved by solvent selection, solvent volume, pH, and by using the salting-out effect.

Several studies have reported the use of LLE techniques for extracting of CF and metabolites. First, Grant and coworkers (1983) was extracted CF and its metabolites from human urine sample using chloroform:isopropanol (85:15 v/v). The percentage recoveries were 90-100%. Second, Bendriss and coworkers (2000) extracted CF and its metabolites in human urine also using chloroform:isopropanol (85:15 v/v). The recovery of extraction was up to 77% (AFMU 1X, 1U, PX and 17U), except for 1U which was about 50%. Finally, Begas and coworkers (2007) extracted CF and its metabolites in human urine. The mean recoveries of AFMU, 1U, 1X, 17U, and 17X at the different concentrations tested were 73.6, 65.2, 94.3, 91.7, and 97.5%, respectively. A chloroform:isopropanol (9:1) mixture was used to extraction CF and

PX from human urine prior to their determination by HPLC with UV detector. The average recoveries of both compounds were rage from 96-108% (Ou-Yang *et al.*, 1998). Ventura and coworkers (2003) also used chloroform:isopropanol (9:1) for extracted CF from human urine. An extraction recovery was 81.4%.

Perera and coworkers (2010) used only ethyl acetate to extract CF and PX in human plasma and saliva samples. Samples were vortex mixed for 5 min followed by centrifugation at 4000g for 10 min. The organic layer was transferred to a tube and then it was dried under a stream of nitrogen at 45°C. Extraction recoveries from both matrixes were approximately 70%.

- **Solid phase extraction (SPE)**

SPE is an effective sample preparation method and a widely used for sample isolation, concentration and clean-up of selected analytes from several matrices. It was a replacement for LLE. SPE extraction is low intrinsic cost, shorter processing time and low solvent consumption (Poole, 2003). SPE involves partitioning between a liquid or mobile phase (sample matrix or solvent with analytes) and a solid or stationary phase (sorbent). The method is based on the distribution of the desired component to be isolated between the mobile and stationary phases as a result of absorption processes taking place in a SPE column (Zwir-Ferenc and Biziuk, 2006).

The general procedure involves four steps (Figure 2.3). The first step, the solid sorbent is equilibrated or preconditioned using an appropriate solvent to wet the sorbent. The second step, the loading solution containing the analyte is percolated through the sorbent. The third step, the sorbent is then washed to remove impurities with an appropriate solvent. The final step, the analyte is collected into collection tube during this elution step by an appropriate solvent. The separation ability of SPE is based on the preferential affinity of desired analytes to a stationary phase or sorbent. The stationary phase is selected so that the impurities in the sample are un-retained on the sorbent while the analytes of interest is retained on it. Analytes that are retained on the stationary phase can then be eluted from the solid phase extraction cartridge with the appropriate solvent (Camel, 2003).

The most frequently used design in SPE is cartridge in from of syringe or disk. The cartridges are available with a variety of stationary phases, each of which can separate analytes according to different chemical properties. Most of the sorbent materials in stationary phase are based on silica and other materials are based on polymer and carbon (Chen *et al.*, 2009). Sorbent can be divided into three types depending on their primary retention mechanism: reversed phase, normal phase, ion exchange. Reversed phase SPE separates a polar or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase (Bulletin, 1998). The stationary phase of a reversed phase SPE cartridge is hydrocarbon chains, which retain compounds of mid to low polarity due to the hydrophobic effect. These nonpolar-nonpolar attractive forces are called van der Waals forces, or dispersion forces. The analyte can be eluted by washing the cartridge with a non-polar solvent, which disrupts the interaction of the analyte and the stationary phase (Zwir-Ferenc and Biziuk, 2006). The materials are used as reversed phase: carbon-based media, polymer-based media, and bonded silica media. A stationary phase of bonded silica sorbent is most widely used, such as octyl (C_8), octadecyl (C_{18}), ethyl (C_2), butyl (C_4), phenyl, cyclohexyl (CH) and cyanopropyl (CN) (Masqué *et al.*, 1998).

Normal phase SPE separates a polar analyte, a mid- to nonpolar matrix (e.g. acetone, chlorinated solvents, and hexane), and a polar stationary phase, such as LC-CN, LC-Si, and LC-Alumina. Retention of an analyte under normal phase conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface (Zwir-Ferenc and Biziuk, 2006).

Ion exchange SPE separate analytes based on electrostatic interactions between the analyte of interest and the positively charged groups on the stationary phase. For ion exchange to occur, both the stationary phase and sample must be at a pH where both are charged (Bulletin, 1998). The sorbents can be classified as both anionic and cationic. Anionic (negatively charged) compounds can be isolated on an aliphatic quaternary amine group that is bonded to the silica surface, such as LC-SAX or LC-NH_2. Cationic (positively charged) compounds are isolated by using the silica with aliphatic sulfonic acid groups that are bonded to the surface, such as LC-SCX or LC-WCX (Zwir-Ferenc and Biziuk, 2006).

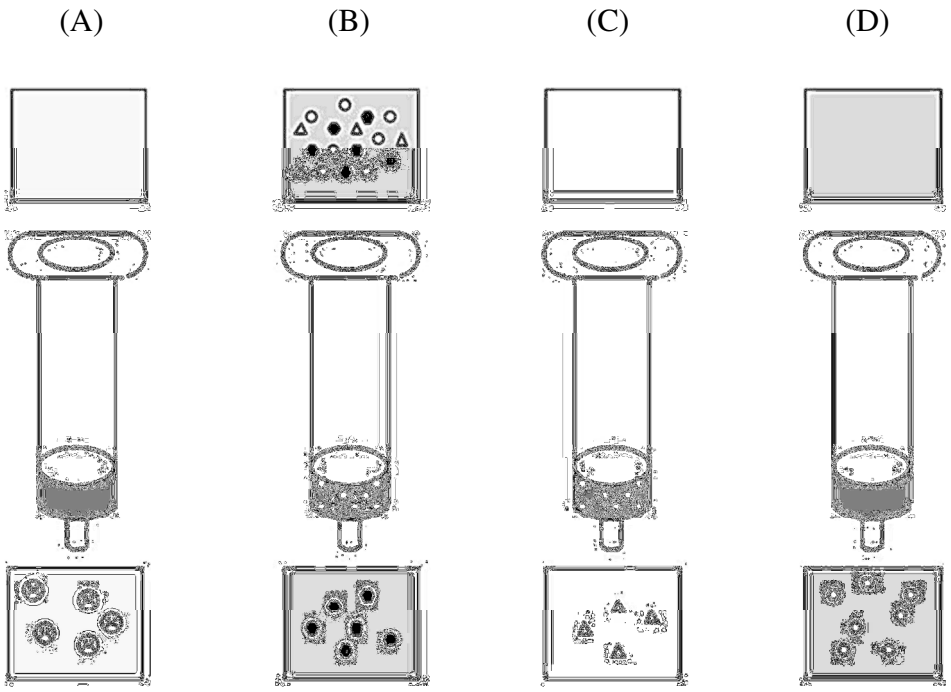


Figure 2.3 The procedures of the SPE consists of four basic steps; (A) Conditioning, (B) Loading, (C) Washing, (D) Elution (Camel, 2003)

A number of SPE methods have been developed to measure CF and its metabolite in biological samples. The extraction mostly used silica gels modified by hydrophobic alkyl groups, mainly C_{18}. Caubet and colleagues (2002) used SPE to extract CF and 14 of its metabolites from human urine. Urine sample was acidified with 1N hydrochloric acid. The solution was loaded onto a SPE column Oasis C18 which had been previously conditioned with 2x1 mL of methanol, 0.02 N hydrochloric acid, and water, respectively. The SPE column was dried under vacuum for 10 min and then washed with 0.1 M sodium acetate in methanol:acetonitrile (80:20 v/v) adjusted to pH 7.5 with glacial acetic acid. The eluate was collected and dried in an evaporator at 45 °C under vacuum. Analytes were determination by HPLC coupled with mass spectrometry. Recoveries ranged between 83-99%. The limit of detection (LOD) was evaluated at 0.003 µg/mL for 1X, TB, 37U, and 13U and 0.002 µg/mL for CF and the other metabolites.

Atia and colleagues (2009) used the ODS C18 cartridge (1 mL/100 mg) for extraction CF and its nine metabolites from human urine samples. The urine samples were acidified to pH 4 by the addition of 10 mM sodium acetate buffer. The urine matrix interferences were removed by washing with water. The CF and its metabolites were eluted with the elution mixture; chloroform:methanol (80:20% v/v). The eluate was collected and dried under a nitrogen stream at 40ºC for 7 min. The analytes were injected into the HPLC system with UV detection. The extraction was achieved high recoveries ranging from 82.06-98.34%. The LOD of analytical method were range from 0.005-0.014 µg/mL.

For a study of rat plasma and urine sample, Abu-Qare and coworkers (2001) used C_{18} Sep-Pak 3cc (500 mg) cartridges to extract CF and analyze by HPLC with UV detection. The cartridges were conditioned with acetonitrile, and water. Those samples were acidified with 1 N acetic acid (pH 5.0). The supernatant was loaded, then washed with water. CF was eluted with methanol. Average percentage recovery was 83.9% and 83.2% in plasma and urine samples respectively. The LOD (signal-to-noise ratio of 3:1) of the assay was 0.1µg/mL.

Zhang and coworkers (2005) used SPE cartridge columns C18 (250 mg) to extract CF and TP. Serum sample was diluted with water and was loaded onto SPE columns. The SPE columns were washed with 0.01 M hydrochloric acid and water. The analytes were eluted with 1 mL methanol and the extract was then dried under a stream of nitrogen at 40 ºC. The analytical method showed recovery ranging from 86.4-107.6% and 80.1-108.3% for CF and TP, respectively.

Cartridges filled by polymeric sorbents have also used. Georga and coworkers (2001) used SPE to extract CF and its ten metabolites from human serum and urine. Three different sorbents were assayed for the extraction: Oasis HLB (60 mg), C_{18} (500 mg), and Nexus (30 mg). The sample was precipitate proteins with ACN. The cartridges were conditioned with methanol and water. CF and its metabolites were eluted using methanol:acetate buffer pH 3.5 (50:50% v /v). High extraction recoveries from both serum and urine ranging from 84.4-100.8%, 87.0-95.6%, and 83.5-89.8%, were achieved using Oasis HLB, C_{18}, and Nexus SPE cartridges, respectively.

Kawahara and colleagues (2004) used Oasis HLB (30 mg/1 cc) cartridge to extract CF in human serum. Sample was diluted with water and applied to cartridge. The cartridge was washed with methanol in water and eluted with methanol. The LOD of this method was 0.1 µg/mL for CF and 0.2 µg/mL for metabolites: TB, TP, and PX.

Saka and coworkers (2007) used Oasis HLB (60 mg/1 cc) to extract TP in human serum for determined by GC with mass spectrometry. The SPE cartridge was precondition with methanol followed water. The cartridge was washed with water and 5% methanol in water. TP were eluted with ethyl acetate. Eluate was evaporated to dryness under a stream of nitrogen at 45 °C. LOD (signal-to-noise ratio of 3:1) of TP was 0.001µg/mL.

Arinobu and colleagues (2009) used Oasis HLB cartridge for extraction and determination of CF and TP from human serum with HPLC-MS. The recoveries of CF and TP ranging from 80.6-108% and 75.0-116.0%, respectively were achieved. The LOD (signal-to-noise ratio of 3:1) of analytical method for CF and TP were 0.5 and 1.0 µg/mL, respectively.

The weak cation-exchange monolithic column was used as an SPE column (Zhu and Row, 2009). Sample was loaded into the SPE column and washed with 10 mL water. Elution was performed with methanol:water (30:70 v/v). Eluates were analyzed by HPLC with UV detection. Extraction recoveries were 77.4%-82.3% and LOD (signal-to-noise ratio of 3:1) was 0.04 µg/mL.

2) Analytical Methods

Several analytical methods have been used for determination of CF and its metabolites in different biological samples including GC, HPLC, and LC-MS. These methods need extensive sample pretreatment or do not allow the complete separation and quantitation of CF and its metabolites present in a same sample

- **High performance liquid chromatography (HPLC)**

Reversed phase HPLC with UV detection is used most often for separation, identification and determination of CF and its metabolites in a wide range of samples such as beverages (Bispo *et al.*, 2002), foods (Thomas *et al.*, 2004) and biological fluids: urine (Atia *et al.*, 2009), serum (Georga *et al.*, 2001), plasma (Perera *et al.*, 2010), and saliva (Bozikas *et al.*, 2004).

In human study, Grant and colleagues (1983) separated and identify CF and its fifteen metabolites (including of xanthines and urates metabolites) in human urine. Samples were prepared by using LLE method and separate by Ultrasphere ODS reversed phase columns (250x4.6 mm i.d., 5 µM particle size) with UV detection at 280 nm. The mobile phase consisted of isocratic program with 0.05% acetic acid:methanol (88:12 v/v, for xanthines and urates; 85: 15 v/v, for AFMU) at a flow rate of 1.2 mL/min. The LODs were between 0.3 and 0.5 mg/L for all metabolites. Recoveries ranged between 90-100%.

Begas and colleagues (2007) developed an HPLC-UV method for the determination of CF and fourteen metabolites in human urine. Urine samples were extracted with chloroform:isopropanol (85:15 v/v) and separated on a Kromasil 100 C_{18} column (250x4.6 mm i.d., 5 µM particle size) by an isocratic program consisting of two solvents: solvent A, mixture 0.1% acetic acid:methanol:acetonitrile (92:4:5 v/v) and solvent B, mixture of 0.1% acetic acid:methanol (60:40 v/v) at a flow rate of 1.2 mL/min, and detected at 280 nm. The limit of quantitation (LOQ) was 5µM for five metabolites quantified.

Atia and coworkers (2009) developed a new rapid, sensitive, and validated an HPLC-UV assay for the simultaneous determination of CF and eight metabolites in human urine. Samples were treated using SPE procedure on ODS C18 cartridge. Extracts were analyzed on Chromolith Performance PR-18e (10x64.6 cm)

The mobile phase was consisted of a mixture 10 mM potassium dihydrogen phosphate buffer:methanol (87.5:12.5 v/v), at a flow rate 1 mL/min. Detection was set at 274 nm. The LOQ and LOD for all the compounds ranged from 0.014-0.0410 and 0.005-0.014 µg/mL, respectively.

Schrader and colleagues (1999) developed an HPLC-UV method for the analysis of CF and its eight major metabolites in Gunn rat urine. Sample was acidified with acetic acid. Separation was performed on a Merck LiChrosphere 100 RP-18 endcapped (5mm) column and achieved by gradient elution: eluent A; 10 mM sodium acetate buffer (pH 5.5):methanol:dimethylformamide (99.0:0.5:0.5 v/v/v) and eluent B; methanol:eluent A (50:50 v/v). The flow rate was 1.5 mL/min. The sample wavelength was 280 nm. The LOD of all analytes were 1 pmoL/mL.

Abu-Qare and Abou-Donia (2001) reported the HPLC-UV method for the separation and quantification of CF in Sprague Dawley rat plasma and urine. The samples were extracted using SPE procedure on C_{18} Sep-Pak^{R} cartridges. A reversed phase C_{18} column µBondapak™ C_{18} (3.9x300 mm i.d., 10 µm particle size) was used for the separation. It was separated using gradient mode of 1-85% acetonitrile in water (pH 3.0) at a flow rate 1 and 1.5 mL/min, and detected at 280 nm. The LOD for both samples was 0.1µg/mL. The LOQs were 0.1µg/mL and 0.2µg/mL, for plasma and urine.

In another work, Novitskaya and colleagues (2013) developed an HPLC-UV technique for determination CF and its four metabolites in rat plasma. Sample was precipitate by adding acetonitrile. HPLC analyses were performed on Phenomenex Luna C18 (250x4.6 mm, i.d.,5 µm particle size) with mobile phase consisting of water (pH 4.0):acetonitrile:methanol (80:8:14 v/v/v), at flow rate 1.5 mL/min. The UV detection was set at 273 nm. The LOQ of this method was 0.01µg/mL for CF, TP, PX, and TB and 0.025µg/mL for 137U.

Caubet and coworkers (2002) developed an HPLC-DAD method for the determination of CF and its fourteen metabolites in urine. Samples were separated by SPE procedures on Oasis C18 column and performed on an Eclipse XDB-C18 reversed phase column (250×4.6 mm i.d., 5 µm particle size). The mobile phase was a mixture of water:acetic acid:tetrahydrofuran (996.5:1:2.5 v/v/v) and acetonitrile in

gradient elution. The detection was wavelength 270 nm for xanthines metabolites and 290 nm for uric acids metabolites. The LOD of the method was calculated at 0.003 µg/mL for 1X, TB, 37U and 13U and 0.002 µg/mL for CF and the other metabolites. LOQ was 0.005 µg/mL.

HPLC-MS was used for measuring CF and its metabolites in biological fluid. Arinobu and colleagues (2009) used an HPLC-MS method for determination of CF and TP in the human serum samples. Samples were analyzed using Oasis HLB cartridge. A Chromolith Performance RP-18e column (100x4.6 mm i.d.) with positive mode MS detection was used for the separation. The LOD of CF and TP was 0.5 and 1.0 µg/mL, respectively.

Schneider and coworkers (2003) developed an HPLC-MS/MS method for the rapid detection of CF and eleven metabolites from human urine. Samples were diluted with an aqueous solution of acetic acid (0.05% v /v) and methanol (10% v /v). The compounds were separated on an Ultrasphere ODS column (250x34.6 mm, 5 µm) with gradient elution of the mobile phase. Elution used three solvents: solvent A; 0.05% acetic acid with 3% methanol and 1.5% isopropanol, solvent B; methanol, and solvent C; 0.05% acetic acid. The flow rate was 0.6 mL/min. The LOQ of the method was lower than 0.05 µM.

- Gas chromatography (GC)

GC-MS detection is the most widely used determination of CF and its. Sheehan and coworkers (1977) documented for determination of TP in human whole blood. The glass column (180 cm X 0.2 cm i.d.) was packed with 3% OV-17. Carrier gas flow rate was 30, 20, and 300 mL/min of nitrogen, hydrogen, and air, respectively. Sample was extracted using chloroform. TP were determination within the calibration mode of the ion intensities at *m/e* 68, 123, 150, 180, 194, 219, and 236. Saks and coworkers (2007) measure TP in serum sample and using SPE technique for this extraction. TP was separated on a J&W DB-5MS capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness) and using helium as carrier gas at a flow rate of 1.0 mL/min. The MS were determination TP set to *m/z* 180.0, 95.0, 68.0, and *m/z* 50.0-400.0 for operated in selected ion monitoring (SIM) and scan mode, respectively. LOD of the assay (signal-to-noise ratio of 3:1) was 0.001 µg/mL. In another work, Rajabi Khorrami and Rashidpur (2012) describes the GC-MS method for determination CF, TP, and TP in human serum. Sample was extracted by solid phase microextraction (SPME) and separated on a J&W DB-1 fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). Helium was used as carrier gas. MS was performed by using SIM with the characteristic ion at 194.1 *m/z*. The LOD and LOQ of CF were 0.1 and 0.33 µg/mL, respectively.

Table 2.3 Methods use for determination of CF and its metabolites

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
1977	GC-MS	TP	LLE	whole blood	OV-17 (180 x 0.2 cm i.d.)	-	-	86%	-	(Sheehan *et al.*)
1983	HPLC-UV	CF and metabolites	LLE	urine	Ultrasphere ODS (250x4.6 mm i.d) 5 µm particle size	0.05% acetic acid: methanol (xanthines; 88:12 v/v and urates; 85: 15 v/v) & 1.2 mL/min	280	90-100%	-	(Grant *et al.*)
1992	HPLC-UV	CF, TB, PX, TP	LLE	plasma	Reversed-phase TSKgel ODS-80TM (150x4.6 mm i.d.) 5 µm particle size	methanol:0.1 M sodium phosphate (30:70 v/v) & 0.8 mL/min	274	-	CF, TB 0.005 PX, TP 0.01	(Tanaka)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
1996	HPLC-UV	TP, 1MU, 3MX, 13DMU	LLE	plasma & urine	Beckman ultrasphere ODS (250×4.6 mm i.d.) 5 µm particle size	0.01 M acetate buffer (pH 4.0): methanol (93:7 v/v; urine & 91:9 v/v; plasma) & 1mL/min	273	86-95%	-	(Rasmussen and Brosen)
1996	HPLC-UV	CF, TP	protein precipitant	plasma	Reverse phase ODS2 (150x4.6 mm i.d.) 5 µm particle size	acetonitrile: tetrahydrofuran: acetic acid: distilled water (20:20:5:955 v/v/v/v) & 1mL/min	273	-	-	(Schreiber-Deturmeny and Brugueroll e)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
1998	HPLC-UV	CF, PX, 17U, 1X, 1U, AFMU	LLE	urine	Spherisorb S5 ODS2 (250x34.6 mm i.d.) 5 µm particle size	acetic acid (33%): tetrahydrofuran: acetonitrile: distilled water (1:2.5:44:925.5, v /v) & 1 mL/min	280	-	-	(Krul and Hageman)
1998	HPLC-UV	CF, PX	LLE	plasma	Spherisorb ODS-2 (250x4 mm id.) 5 µm particle size	0.05% acetic acid: acetonitrile: methanol (81.5:5.5:10 v/v) & 0.7 mL/min	282	96-108%	0.1 umol/L	(Ou-Yang *et al.*)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
1999	HPLC-UV	CF and 8 metabolites	protein precipitant	urine	C_{18} reversed-phase (250x4.6 mm i.d.) 5 µm particle size	Eluent A; 10 mM sodium acetate buffer (pH 5.5) methanol: dimethylformamide (99.0:0.5:0.5 v/v/v) Eluent B; methanol: Eluent A (50:50 v/v) & 1.5 mL/min	280	>70%	-	(Schrader *et al.*)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2000	HPLC-UV	CF and 10 metabolites	LLE	urine	Rexchrom S5-100-ODS (250x34.6 mm i.d.) 5 µm particle size	Elute A; 0.05% acetic acid: methylalcohol (92.5:7.5 v/v) and Elute B; 0.05% acetic acid: methylalcohol (60:40 v /v) & 0.5 and 1.0 mL/min	280	>70% for all, except for 1U 50%	-	(Bendriss *et al.*)
2001	HPLC	CF	SPE	plasma urine	RP C18 column µBondapak (3.9×300 mm i.d.) 10 µm particle size	1-85% acetonitrile in water (pH 3.0) & 1 and 1.5 ml/min	280	83.2% in urine 83.9% in plasma	0.1	(Abu-Qare and Abou-Donia)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2001	HPLC-UV	CF and 10 metabolites	SPE	serum urine	MZ Kromasil C_4 (250x4 mm i.d.) 5 µm particle size	acetate buffer (pH3.5): methanol (97:3 v /v) & 1 mL/min	275	84.6-103.0%	0.0003	(Georga *et al.*)
2002	HPLC-DAD	CF and 14 metabolites	SPE	urine	Eclipse XDBC18 reversed phase (250×4.6 mm i.d) 5 um particle size	Elute A; water: acetic acid: tetrahydrofuran (996.5:1:2.5 v/v/v) and Elute B; acetonitrile	270	83-99%	TB, 1X, 37U,13U 0.003 CF and other metabo-lites 0.002	(Caubet *et al.*)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2002	HPLC-UV	CF	protein precipitant	plasma	Phenomenex C_{18} (150×4.6 mm i.d)	acetonitrile: tetrahydrofuran: acetic acid: distilled water (50:30:5:915 v/v/v/v) & 1.0 mL/min	274	-	-	(Kamimori *et al.*)
2003	HPLC-MS/MS	CF and 11 metabolites	protein precipitant	urine	Ultrasphere ODS column (250x34.6 mmmi.d.) 5 µm particle size	Eluent A: 0.05% acetic acid with 3% methanol and 1.5% isopropanol, Eluent B: methanol, and Eluent C: 0.05% acetic acid & 0.6 mL/min	-	90-110%	-	(Schneider *et al.*)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2003	HPLC	CF	LLE	urine	Ultrasphere ODS C18 (75x4.6 mm i.d.) 3µm particle size	distilled water: acetonitrile (90:10 v/v) & 1.0 mL/min	280	81.4	0.7	(Ventura *et al.*)
2004	HPLC-UV	CF, TB, PX, TP	SPE	serun	Shimpack CLC-ODS (150x6 mm i.d.)	0.1M Sodium dihydrogen phosphate in 30% methanol & 1 mL/min	274	-	CF 1.0 TB, PX, TP 0.2	(Kawahara *et al.*)
2005	HPLC-MS/MS	CF, AAMU,AFMU , 1U, 1X, 17U	protein precipitant	urine	YMC-Pack C30 (500x2.1 mm i.d.) 3µm particle size	Eluent A: 0.5% acetic acid Eluent B: acetonitrile & 200 µL/min	-	-	-	(Weimann *et al.*)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2007	HPLC-UV	AFMU, 1U, 1X, PX,17X	LLE	urine	Kromasil 100 C_{18} (250×4.6 mm i.d.) 5 µm particle size	Elute A: 0.1% acetic acid: methanol: acetonitrile (92:4:5 v/v) Elute B: 0.1% acetic acid: methanol (60:40 v/v) & 0.7 and 1.1 mL/min	280	AFMU 73.6, 1U 65.2, 1X 94.3, PX 91.7, 17X 97.5	AFMU 0.08, 1U 0.03, 1X 0.09, PX 0.10 17x 0.17 µM	(Begas *et al.*)
2007	GC-MS	TP	SPE	serum	J&W DB-5MS capillary column (3 m×0.25 mm i.d.) 0.25 µm film thickness	high purity helium (99.9999%) & 1.0 mL/min	-	-	0.001	(Saka *et al.*)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2008	GC- IT–MS/MS	CF	SPE	surface marine and freshwater	CP-SIL 8CB-MS (30 m×0.25 mm i.d.) 0.25 µm film thickness	-	-	-	0.001-0.002	(Verenitch and Mazumder)
2009	HPLC-MS	CF, TP	SPE	serum	Chromolith Performance RP-18e (100×4.6 mm i.d.)	0.1% formic acid in acetonitrile: 0.1% formic acid in distilled water (20:80 v/v) & 1.0 mL/min	-	CF 80.6-10.8 TP 75.4-116	CF 0.5 TP 1.0	(Arinobu *et al.*)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2009	HPLC-UV	CF and 8 metabolites	SPE	urine	Chromolith Performance PR-18e (10x64.6 cm)	10 mM potassium dihydrogen phosphate buffer: methanol (87.5:12.5 v/v) & 1.0 mL/min	274	82.06-98.34	0.005-0.014	(Atia *et al.*)
2008	HPLC-UV	CF, TP	SPE	urine	OptimaPak C_{18} (150x94.6 mm i.d.) 5 µm particle size	methanol:water (30:70 v/v) & 0.5 mL/min	274	77.4-82.3	0.04	(Zhu and Row)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2010	HPLC-UV	CF, PX	LLE	plasma saliva	Ultrasphere ODS C18 (250x4.6 mm i.d.) 5 µm particle size	acetonitrile (99.9%): acetic acid: distilled water (100:1:899 v/v/v) & 1.5 mL/min	280	CF 72 PX 73.2	0.005	(Perera *et al.*)
2011	HPLC-DAD	CF	protein precipitant	plasma	RP Atlantis C18 (4.6×150 mm i.d.) 5 µm particle size	15 mM potassium phosphate (pH 3.50): acetonitrile (83:17 v/v) & 1.0 mL/min	274	91	-	(Alvi and Hammami)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2011	HPLC-MS\MS	CF,TB,TP	protein precipitant	plasma	Zorbax SB C_{18} (210×150 mm i.d.) 2.5 µm particle size	acetonitrile: 0.05% acetic acid in distilled water (15:85 v/v) & 0.25 mL/min	-	-	-	(Noh *et al.*)
2012	GC-MS	CF, TB, TP	SPME	serum	DB-1 (30 m x 0.25 mm i.d.) 0.25 µm film thickness	-	-	CF 88, TB 54, TP 41	0.1	(Rajabi Khorrami and Rashidpur)
2012	HPLC-UV	CF, PX	LLE	plasma	Ultrasphere ODS (46x75 mm x 3 µL)	acetonitrile: 0.05% acetic acid (99:1 v/v) & 1 mL/min	274	-	-	(Yubero-Lahoz *et al.*)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (ug/mL)	Ref.
2013	HPLC-UV	CF, TB, PX, TP, 137U	protein precipitant	plasma	Phenomenex Luna C18 (250x4.6 mm i.d.) 5 µm particle size	water (pH 4.0):acetonitrile: methanol (80:8:14 v/v/v) & 1.5 mL/min	273	CF 89.4 TP 87.8 PX 85.9 TB 88.2 137U 80.1	CF, TB, PX, TP, 0.01 137U 0.025	(Novitskaya *et al.*)

2.2 Watercress

Watercress (*Nasturtium officinale* R. Br.; Figure 2.4) is a member of the family Brassicaceae (or Cruciferae), which includes broccoli, cabbage, cauliflower, kale, bok choy, turnips, radish, and onion. This family consists of about 338 genera and over 3,709 species (Lysak *et al.*, 2009). It is an aquatic or semi-aquatic perennial herb and is a native herb in the eastern Mediterranean and adjoining areas of Asia (Goncalves *et al.*, 2009). Watercress grows in and around water. It has succulent and smooth stems, creeping or freely floating, and deep green and small leaves. It is commonly used as fresh in salad, soups, sandwiches, and steamed or boiled vegetable. Watercress in Thailand is called Betong watercress or Phak Num Betong. It cultivated mainly in the South of Thailand at the Betong District, Yala Province.

Figure 2.4 Watercress

(Photographed By Chunhajan Aksornthong at 21/06/13)

2.2.1 Taxonomy

Watercress can be classified scientifically as follows:

Kingdom: Plantae – Plants

Subkingdom: Tracheobionta – Vascular plants

Superdivision: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliopsida – Dicotyledons

Subclass: Dilleniidae

Order: Capparales

Family: Brassicaceae – Mustard family

Genus: *Nasturtium* R. Br.

Species: *Nasturtium officinale*

Binomial name: *Nasturtium officinale* W.T. Aiton

Synonyms: *Nasturtium nasturtium-aquaticum* (L.) Karsten,

Rorippa nasturtium-aquaticum (L.) Hayek,

Sisymbrium nasturtium-aquaticum L.

(USDA, 2014)

2.2.2 Phytochemicals

Watercress is very nutritious. It includes vitamins (A, C, D, E, and K), proteins (arginine, glycine, lysine, and tryptophan), and minerals (folic acid, calcium, iron, magnesium, phosphorous, potassium, and sodium) (Costain, 2007; Herbsarespecial, 2009). It is low in calories because of the high water content (93%). It also contains a high concentration of glucosinolates (gluconasturtiin) precursor. Glucosinolates are sulphur containing and water-soluble. This precursor is hydrolysed by myrosinase to isothiocyanates (ITCs), mostly phenethyl isothiocyanate (PEITC; Figure 2.5) after chewing and chopping.

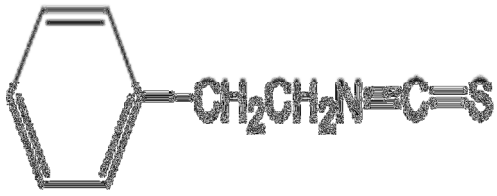


Figure 2.5 The chemical structure of PEITC
($C_6H_5CH_2CH_2NCS$; molecular weight 163.24) (Ji *et al.*, 2005)

The glucosinolates are hydrolysed by myrosinase enzyme from both plant cells and microflora in human intestine (Cheng *et al.*, 2004). ITCs are reactive compounds with the structure R-N=C=S, which R is an alkyl or aryl group (Wu *et al.*, 2009). Jiao and colleagues (1998) reported that watercress contained the highest level of ITCs than other cruciferous vegetables such as broccoli, cauliflower, choi sum, kai choi, kai lan, bok choi, and wong nga pak. Gill and colleagues (2007) reported amounts of phenolics, glucosinolates (GLS), and hydroxylated cinamic acid (HCA) derivatives in watercress leaves consumed of subjests (Table 2.4).

Table 2.4 Average concentration of phenolics, GLS, and HCA derivatives in watercress leaves (Gill *et al.*, 2007)

Phenolic or glucosinolate	**fresh weight**	**dry weight**
	μmol/g	
Q-3-*O*-Sophoroside, 7-*O*-Glucoside	0.09	1.0
Q-3-*O*-Glc-(6´´-Malonyl-Glc)	0.13	1.43
Q-3-*O*-Sophoroside	0.05	0.59
Q-3-*O*-Rutinoside (Rutin)	0.05	0.60
7-Methylsulphinylheptyl	0.1	1.07
8-Methylsulphinylheptyl	0.06	0.68
3-Indolylmethyl	0.04	0.43
2-Phenylethyl-GLS	1.53	17.98
4-Methoxy-3-Indolylmethyl	0.065	0.791
Total HCA derivatives	9.40	109.03

2.2.3 Health benefit and chemoprotection in human and rats

Watercress and PEITC have many health benefits. The chemopreventive effect has been demonstrated to inhibit rat and human CYP isoforms, involving in bioactivation of pro-carcinogens. Nakajima and coworkers (2001) reported the inhibitory effect of PEITC on human CYPs activity by using microsomes from baculovirus-infected insect cells expressing specific human CYPs. The study showed specific CYPs inhibition such as competitive inhibition: CYP1A2 and 2A6, noncompetitive inhibiton: 2B6 and 2C9, and a mechanism-based inactivation: 3A4. Similarly, *in vitro* incubation of rat liver microsomes with PEITC showed a dose dependent inhibition of CYP1A1, 1A2, and 2B1 (Thapliyal and Maru, 2001). CYP1A1 and 1A2 play the most important role in the bioactivation of many carcinogens.

The effects of watercress consumption on drug and carcinogen metabolism in humans have been documented. A single ingestion of watercress homogenate (50 g) in healthy volunteers inhibits CYP2E1-mediated metabolism of acetaminophen (Chen *et al.*, 1996) and chlorzoxazone (Leclercq *et al.*, 1998).

In rat model, Janchawee and colleagues (2014) documented that Betong watercress (800 mg/kg) and PEITC (10 and 20 mg/kg) inhibit N-demethylation of CF in male Wistar rats by using CMRs as a probe. CMRs are decreased after treatment with them. The effect showed dose- and time-independent inhibition. The results indicated that they have inhibitory activity on CYP1A2 and CYP2C.

Cell culture and animal studies showed that PEITC has chemopreventive effects, which occur during the initiation and post- initiation stages of carcinogenesis. PEITC is an effective inhibitor of lung tumorigenesis induced in rats and mice by tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Hecht and colleagues (2000) reported that PEITC inhibit NNK-induced lung multiplicity in A/J mice. The mice were maintained on AIN-93G diet and treated weekly for 8 weeks by gavage with NNK (3 mmol) and benzo[*a*]pyrene (BaP; 3 mmol), in cottonseed oil (0.1 mL). PEITC (3, 1, or 0.3 mmol/g diet) was administered 1 week before carcinogen treatment until 1 week after the last carcinogen treatment. Significant reductions (31-74%) were obtained at all dose of PEITC. However, PEITC had no significant effect on lung tumor multiplicity in the mice treated with BaP. These results indicate that the inhibitory effect of PEITC on lung tumor induction by NNK.

Stoner and colleagues (1998) reported that PEITC inhibit the esophageal metabolism of *N'*-nitrosonornicotine (NNN) in F344 rats. Following feeding of PEITC for 2 weeks, rats were killed and the esophagi were incubated *in vitro* with [5-^{3}H]NNN. PEITC decreased NNN metabolism, yielding inhibition ranging from 55-91% of the control production of various NNN metabolites.

Futakuchi and colleagues (1998) documented that 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis in female Sprague-Dawley rats. Rats received an intra-gastric of DMBA at dose 50 mg/kg, BW. Experiment, starting 1 week thereafter received powdered diet containing 1.0% 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ), 1.0% 3-*O*-ethylascorbic acid (EAsA), 1.0% 3-*O*-dodecylcarbomethyl ascorbic acid (DAsA), 0.1% PEITC or a basal diet alone for 35 weeks. The final incidences of mammary adenocarcinomas did not significantly differ among the DMBA-treated groups, multiplicities were significantly lowered in the EAsA (1.6+/-1.6 per rat, $P < 0.01$) and HTHQ (2.6+/-1.9 per rat, $P < 0.05$) animals as compared with the basal diet case (4.1+/-2.9 per rat). The average carcinoma volumes were also significantly smaller in rats given EAsA (2.1+/-3.8 cm^3, $P < 0.05$), DAsA (2.5 +/- 5.3 cm^3, $P < 0.05$), or PEITC (2.4+/-5.9 cm^3, $P < 0.05$) than in those receiving DMBA alone (4.9+/-9.2 cm^3). These results indicate that

HTHQ, EAsA and PEITC all exert chemopreventive influence on the promotion/progression stage of DMBA-induced rat mammary carcinogenesis, with EAsA being particularly effective.

In addition, Phase I and Phase II clinical trials conducted by the National Cancer Institute show that PEITC may be effective preventing lung cancer in smokers (NCI, 2013).

2.3 Objectives

2.3.1 To develop and validate SPE-HPLC method for quantification of CF and its metabolites; TB, PX, TP, and 137U in rat urine

2.3.2 To determine urinary CF metabolic ratio in rats receiving a single oral dose of CF

2.3.3 To determine urinary CF metabolic ratio in rats given a single oral dose of fresh Betong watercress juice

1) To evaluate effect of a single oral dose of PEITC on CF metabolic ratio

2) To compare effects of PEITC verses fresh Betong watercress juice on CF metabolic ratio

CHAPTER 3

Methodology

3.1 Overview of the study

The scope of this study was shown in Figure 3.1. The SPE method was developed to simultaneously determine CF and its metabolites in rat urine by using HPLC with UV detection. The urine samples were collected while each rat is in a metabolic cage, and then were prepared by using SPE technique (Oasis® HLB cartridges). Fresh Betong watercress in a form of juice and PEITC were orally administered to the rats. Then, urine CF and its metabolites were measured for calculation of amount excreted and metabolic ratio.

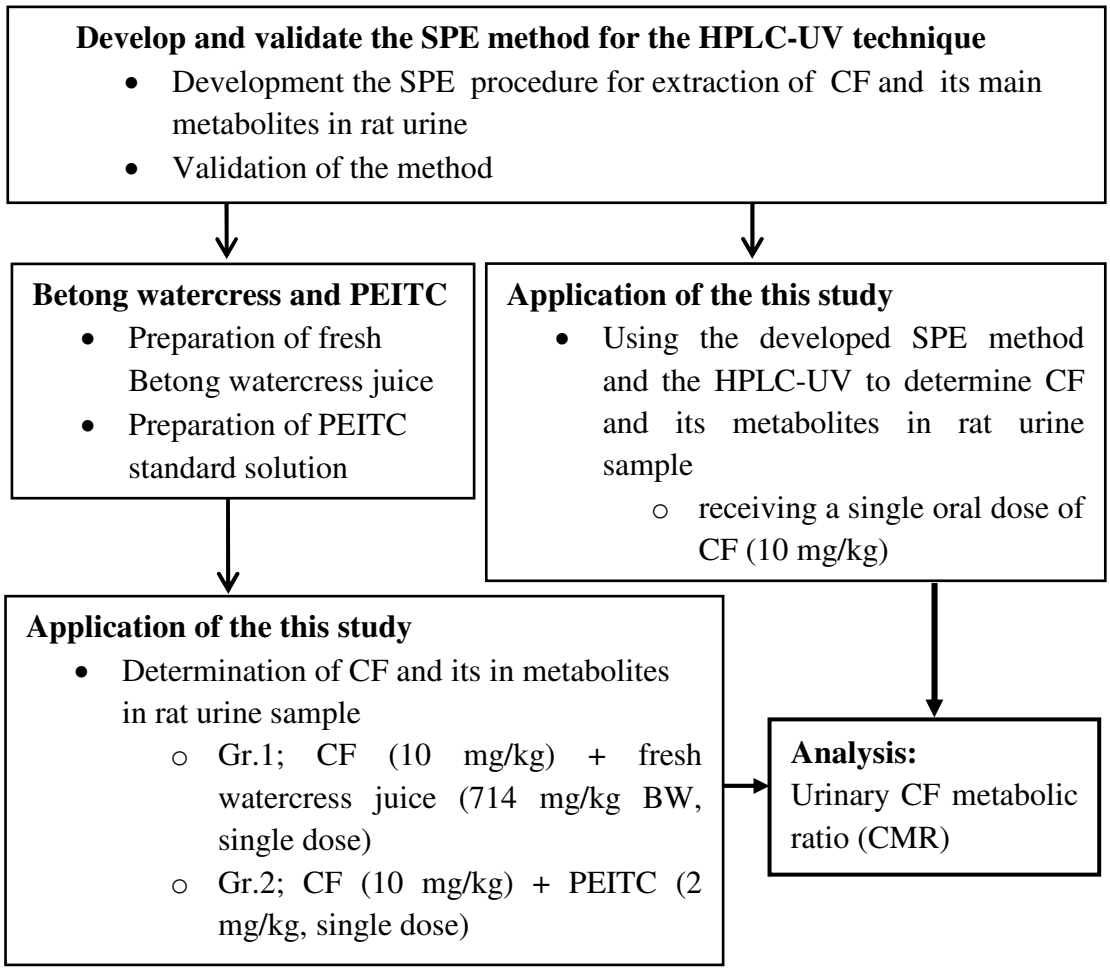


Figure 3.1 Scope of the study

3.2 Chemicals, materials and instruments

Highly pure (>98%) reference standards of CF ($C_8H_{10}N_2$), TB ($C_7H_8N_4O_2$), PX ($C_7H_8N_4O_2$), TP ($C_7H_8N_4O_2$), and PEITC were purchased from Sigma-Aldrich (MO, USA). 137U was obtained from Suzhou Yacoo Chemical Reagent Corporation (Shanghai, China). Methanol and acetonitrile HPLC grade, ammonium hydroxide (NH_4OH) and hydrochloric acid (HCl) AR grade were purchased from Millinckrodt Baker Inc. (USA). Tetrahydrofuran (THF) HPLC grade was purchased from Fisher Scientific UK Limited (Leicestershire, UK). Acetic acid (CH_3COOH) and sodium hydroxide (NaOH) AR grade were obtained from Merck (Frankfurter, Germany). Phosphoric acid (H_3PO_4) AR grade was obtained from VWR International Limited (Poole, England).

Oasis® HLB cartridges (30 mg, 1cc), Symmetry® C18 column (250 x 4.6 mm i.d., 5 µm particle size), Symmetry® C18 guard column (20 x 3.9 mm., 5 µm particle size), Nova-Pak® C18 column (150 x 3.9 mm i.d., 4 µm particle size), Waters 2695 Separation Module, and a Waters 5487 Dual λ Absorbance detector were obtained from Waters Corporation (Massachusetts, USA.). VertiPure™ nylon syringe filters (0.2 µm pore size) with 4 mm and 13 mm diameter membranes were purchased from Vertical Chromatography Co. Ltd. (Nonthaburi, Thailand).

3.3 Development of SPE method for analysis of CF and its metabolites in rat urine

This study demonstrated the development of SPE method for extraction of CF and its metabolites; TB, PX, TP, and 137U in rat urine. Urine samples were subjected to SPE method using the Oasis® HLB cartridges (30 mg, 1cc). The method employed a generic protocol; precondition, loading, washing, and eluting. It was necessary to dilute urine sample before loading to improve efficiency of extraction. The cartridges were previously conditioned with 1×1 mL of water and methanol, respectively prior to loading the samples. Then the analytes were washed and eluted. The eluate was collected in a clean glass tube and evaporated to dryness under nitrogen stream at room temperature. The residue was reconstituted with mobile phase (water: acetic acid: tetrahydrofuran; 996.5:1:2.5 v/v/v), vortex mixed and filtered with a 0.22 µm nylon membrane. An aliquot was injected into the HPLC system for analysis. The method was developed for removed interferences and improves the presented recovery of the extraction. Procedure was developed as follows sample loading, washing, eluting, reconstitution and injection volume into the HPLC system. The details of the SPE procedures which were tried in this study were shown in Table 3.1.

Table 3.1 SPE development method for the extraction of CF and its metabolites in rat urine

SPE method No.	Sample loading			Washing						Eluting		Reconstitution volume (µL)	Injection volume (µL)
	Urine	Dilution solvent		Wash 1 (1x1mL)		Wash 2 (1x1mL)		Wash 3 (1x1mL)		Elute (1mL)			
	Volume (µL)	Solvent	Volume (µL)	Solvent	Time	Solvent	Time	Solvent	Time	Solvent	Time		
1	250	0.1N HCL	250	H_2O	2	5%MeOH-H_2O	1	-	-	MeOH	1	250	20
2	250	0.1N HCL	250	H_2O	1	3% MeOH-H_2O	1	-	-	MeOH	1	250	20
3	250	0.1N HCL	250	H_2O	1	3% MeOH-H_2O	2	-	-	MeOH	1	250	20
4	250	0.1N HCL	250	H_2O	1	CH_3COOH: 3% MeOH-H_2O (ratio 1:99)	1	-	-	MeOH	1	250	20
5	250	0.1N HCL	250	H_2O	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	-	-	MeOH	1	250	20
6	250	0.1N HCL	250	H_2O	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	-	-	MeOH :ACN (80:20 v/v)	1	250	20
7	250	0.1N HCL	250	H_2O	1	3% MeOH-H_2O	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	MeOH	1	250	20
8	250	0.1N HCL	250	H_2O	1	CH_3COOH: 3% MeOH-H_2O (ratio 1:99)	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	MeOH	1	250	20

Table 3.1 SPE development method for the extraction of CF and its metabolites in rat urine (Cont.)

SPE method No.	Sample loading			Washing						Eluting		Reconstitution volume (µL)	Injection volume (µL)
	Urine	Dilution solvent		Wash 1 (1x1mL)		Wash 2 (1x1mL)		Wash 3 (1x1mL)		Elute (1mL)			
	Volume (µL)	Solvent	Volume (µL)	Solvent	Time	Solvent	Time	Solvent	Time	Solvent	Time		
9	250	0.1N HCL	250	CH_3COOH: 3% MeOH-H_2O (ratio1:99.5)	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	-	-	MeOH	1	250	20
10	250	0.1N HCL	250	H_2O	1	CH_3COOH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	MeOH	1	250	20
11	250	0.1N HCL	250	H_2O	1	1%CH_3COOH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	MeOH	1	250	20
12	250	4% H_3PO_4	250	H_2O	1	1%CH_3COOH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	MeOH	1	250	10
13	250	4% H_3PO_4	250	H_2O	1	1%CH_3COOH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	MeOH : ACN (80:20 v/v)	1	250	10
14	200	4% H_3PO_4	800	H_2O	1	1%CH_3COOH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	1% NH_4OH: 3% MeOH-H_2O (ratio 0.5:99.5)	1	MeOH : ACN (80:20 v/v)	1	200	10

3.4 Animals

Male Wistar rats weighing between 200 and 220 g were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The experimental design was approved by the Ethics Committee for Experimental Animals (Ref no. 09/55), Prince of Songkla University. During the experimental period, the animals were maintained under a controlled environment (temperature of 25±2 ºC, light/dark cycle of 12/12h) with food and water ad libitum.

3.5 Sample extraction

Urine samples were extracted using the optimum SPE procedure. The details were shown in Figure 3.2. An aliquot of urine sample (200 µL) was diluted with 800 µL of a 4% phosphoric acid. The Oasis® HLB cartridges (30 mg, 1cc) were previously conditioned with 1×1 mL of water and methanol, respectively, prior to loading the samples. The cartridges were washed sequentially with 1×1 mL of water, 1% acetic acid:3% methanol-water (0.5:99.5 v/v) and 1% ammonium hydroxide:3% methanol-water (0.5:99.5 v/v). The analytes were eluted with 1×1 mL of methanol:acetronitrile (80:20 v/v). The eluate was collected in a clean glass tube and evaporated to dryness under nitrogen stream at room temperature. The residues were reconstituted in 200 µL of mobile phase (water:acetic acid:tetrahydrofuran; 996.5:1:2.5 v/v/v) and a 10 µL aliquot was injected into the HPLC system.

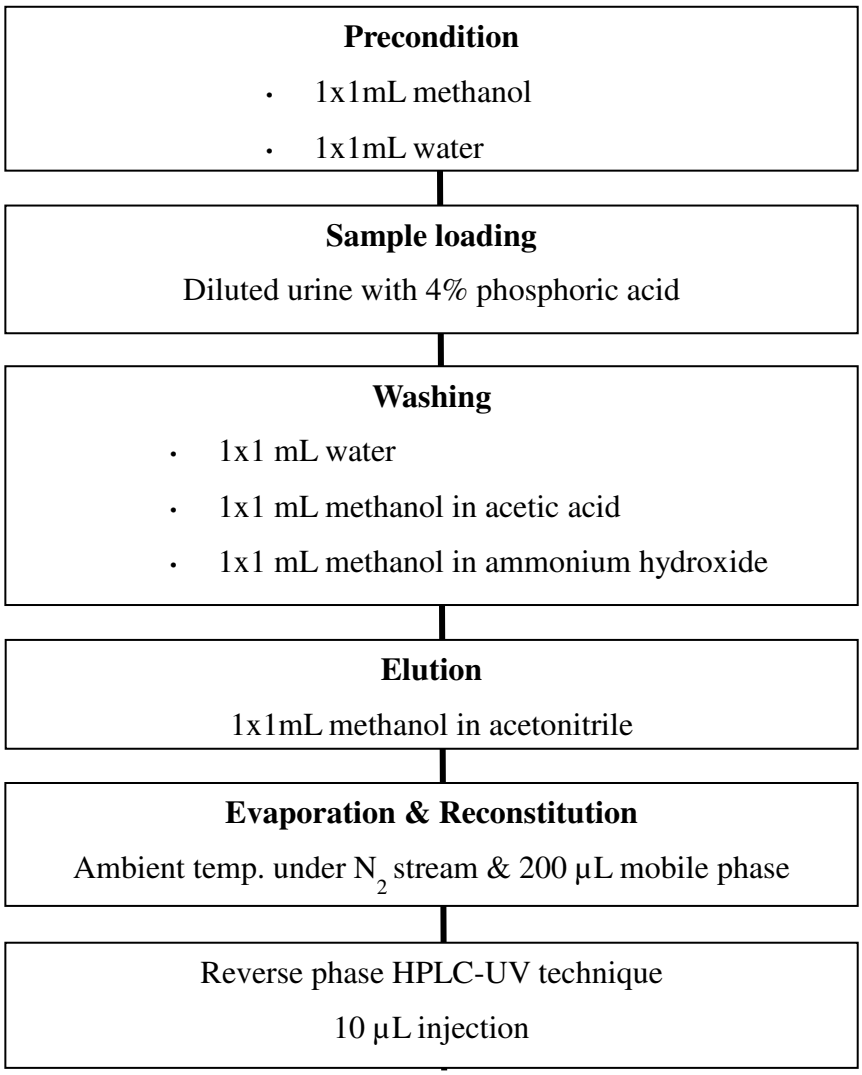


Figure 3.2 SPE protocol for urine sample preparation

3.6 Chromatographic instruments and condition

The Chromatographic system consisted of a Waters 2695 Separation Module and a Waters 5487 Dual λ Absorbance detector (Massachusetts, USA.). Data were collected and processed using the Empower™ Software System.

Urinary concentrations of CF and its metabolites were determined using the HPLC method described by Caubet and colleagues (2002) with some modifications. A HPLC column was reverse-phase Symmetry® C18 (250 x 4.6 mm., 5 µm particle size) connected to Symmetry® C18 guard column (20 x 3.9 mm., 5 µm particle size) from Waters (Massachusetts, USA). The mobile phase consisted of a mixture of water: acetic acid: tetrahydrofuran (996.5:1:2.5, v/v/v) as a solvent A and acetronitrile as a solvent B. Separation was performed using the following gradient: B was set at 6% for 9 min, then increased to 15% from 9 to 10 min under curve 10 and then decrease to 6% in 1 min under curve 6; with a post run of 13 min in order to equilibrate the column between injection. The mobile phase was freshly prepared each day and it was filtered through 0.22 µm nylon filtered paper and degassed in sonicator for 30 min. The mobile phase constantly flowed at 1.3 mL/min. The temperature of column was controlled at 32ºC. Detection was made at a wavelength of 274 nm.

3.7 Method validation

The method was validated for linearity, intra-day and inter-day precision, accuracy, recovery and lower limit of quantification (LLOQ). The method of validation was in accordance with United States Food and Drug Administration guidance for the validation of bioanalytical methods (FDA, 2001)

3.7.1 Linearity and range

The linearity was determined by constructing calibration curves (n=3) between different concentrations of analytes against the peak area. The concentrations were 0.25, 1, 2.5, 5, 7.5, and 10 µg/mL for CF and TP; 0.5, 1, 2.5, 5, 7.5, and 10 µg/mL for TB and 137U; and 0.1, 1, 2.5, 5, 7.5, and 10 µg/mL for PX. Correlation coefficient (*r*) and the calibration equation were determined by linear regression analysis for each calibration curve.

3.7.2 Precision

The precision was evaluated by analyzing four quality controls (QC) samples obtained by preparing of mixture of CF and its metabolites; 0.25, 1, 5, and 10 µg/mL for CF and TP; 0.5, 1, 5, and 10 µg/mL for TB and 137U; and 0.1, 1, 5, 10 µg/mL for PX; five replicates of each concentration in urine blank were used. The intra-day precision of the method was performed using five samples of each concentration on the same day under the same experimental condition, while the inter-day precision was evaluated by assaying of the samples for five consecutive days. The precision was expressed as the relative standard deviation (% RSD) and calculated by the formula:

$$\text{RSD}(\%) = \frac{\text{standard deviation}}{\text{mean}} \text{x} 100.$$

The level of acceptance for precision is within 15% RSD except at LLOQ, where 20% RSD is accepted.

3.7.3 Accuracy

The accuracy was evaluated by analyzing four QC samples obtained by preparing of mixture of CF and its metabolites; 0.25, 1, 5, and 10 µg/mL for CF and TP; 0.5, 1, 5, and 10 µg/mL for TB and 137U; and 0.1, 1, 5, 10 µg/mL for PX; five replicates of each concentration in urine blank were used. The intra-day accuracy of the method was performed using five samples of each concentration on the same day under the same experimental condition, while the inter-day accuracy was evaluated by assaying of the samples for five consecutive days. The precision was expressed as the deviation (% DEV) and calculated by the formula:

$$\text{DEV}(\%) = \frac{\text{measured concentration-nominal concentration}}{\text{nominal concentration}} \text{x}100.$$

The level of acceptance for accuracy is within ±15% DEV except at LLOQ where ±20% DEV is accepted.

3.7.4 Recovery

The recovery of extraction for CF and its metabolites in urine sample was determined by comparing the peak areas of extracted sample with those of direct injected sample (n=5) at LLOQ (0.1 µg/mL for PX, 0.25 µg/mL for TP and CF, and 0.5 µg/mL for TB and 137U), low (1 µg/mL), medium (5 µg/mL), and high (10 µg/mL) concentration. The recovery was derived from the expression:

$$\text{Recovery}(\%) = \frac{\text{response after extraction}}{\text{response after direct injection}} \text{ x}100.$$

3.7.5 Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) was defined as the lowest concentration of the analyte producing a signal-to-noise ratio (S/N) of 5:1 or the precision of <20% RSD, and the accuracy of <±20% DEV.

3.8 Preparation of stock and standard solutions

3.8.1 Stock solution

Individual stock solutions (1000 µg/mL) of CF and its metabolites were prepared in deionized water (CF), 0.1 M sodium hydroxide (TB and 137U), and 0.1 M ammonium hydroxide (PX and TP).

3.8.2 Working standard solutions

Working standard mixtures of CF, TB, PX, TP, and 137U were prepared by diluting the stock solutions with deionized water to different concentrations of 5, 12.5, 25, 50, 125, 250, 375, and 500 µg/mL.

3.8.3 Calibration standard solutions

Calibration standard mixtures at the concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, and 10 µg/mL were prepared by adding an appropriate concentration of working standard solutions (20 µL) into rat urine blank samples (final volume of 1 mL). Similarly, QC samples at concentrations of 0.25, 1, 5, and 10 µg/mL for CF and TP, 0.5, 1, 5, and 10 µg/mL for TB and 137U, and 0.1, 1, 5, and 10 µg/mL for PX, respectively, were prepared**.**

3.9 Determination of urinary CF metabolic ratio in rats given a single oral dose of CF

The animals were housed individually in a metabolic cage. One day before the experiment, urine samples were collected during 0-1 (T_1), 1-3 (T_3), 3-6 (T_6) and 6-9 (T_9) h intervals (Figure 3.3). Then the animals were fasted overnight with free access to water. On the day of the experiment, they received a single dose of 10 mg/kg CF (dissolved in water) by gavage. Urine samples during the same interval were collected. The samples were centrifuged at 6000 rpm (equivalent to 3,461 xg) for 10 min at room temperature (ca. 25ºC). The supernatant was collected and filtered through a 0.22 µm nylon filter and kept at -20 ºC until analysis.

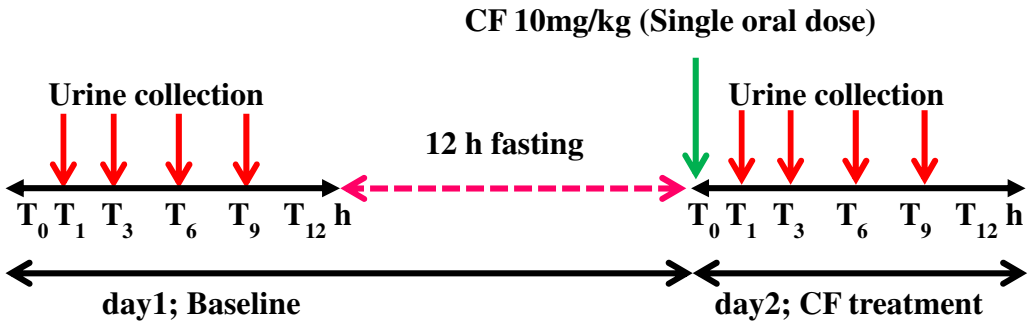


Figure 3.3 The experimental design of animal treatment in rats receiving a single oral dose of CF alone

3.10 Determination of urinary caffeine metabolic ratio in rats given a single oral dose of PEITC and fresh Betong watercress juice

3.10.1 Preparation of fresh Betong watercress juice

Fresh Betong watercress (*Nasturtium officinale* R.Br.) was obtained from a local farm in Betong distict, Yala province. It was cleaned thoroughly, air-dried and divided into portions. A portion of vegetable (150 g) was chopped and mixed with 100 mL of deionized water. The mixture was blended for 1 min using a kitchen miniblender. The homogenate was filtered with cheesecloth and squeezed to release the juice into a clean container (Jiao *et al.*, 1998). The fresh juice was immediately kept at -80 ºC**.**

3.10.2 Animal treatment and sample collection

The animals were divided into two groups (n=3) based on pre-treatments; group 1, a single oral dose of Betong watercress juice (714 mg/kg BW); group 2, a single oral dose of PEITC (2 mg/kg). PEITC was dissolved in corn oil. The experiment for each group consisted of two phases, i.e. phase I and phase II (Figure 3.4). In order to collect urine, the rats were housed individually in a metabolic cage. Phase I; the rats were fasted for 12 h with free access to water and then they were received a single dose of 10 mg/kg CF (dissolved in water) by gavage. Urine samples were collected at 0-1 (T_1), 1-3 (T_3), 3-6 (T_6) and 6-9 (T_9) h intervals before drug administration and at the same intervals after drug administration. CF wash-out period was two days. For Phase II, the same animal was fasted for 12 h with free access to water and pre-treated with either PEITC or watercress juice. Then after pre-treated 30 min, they received the same dose of CF as in phase I and collected urine at the same intervals. The samples were centrifuged at 6,000 rpm (3,461 xg) for 10 min at room temperature (ca. 25ºC). The supernatant was filtered through a 0.22 µm nylon filter and kept at -20 ºC The samples were centrifuged at 6,000 rpm (3,461 xg) for 10 min at room temperature (ca. 25ºC). The supernatant was filtered through a 0.22 µm nylon filter and kept at -20 ºC until analysis.

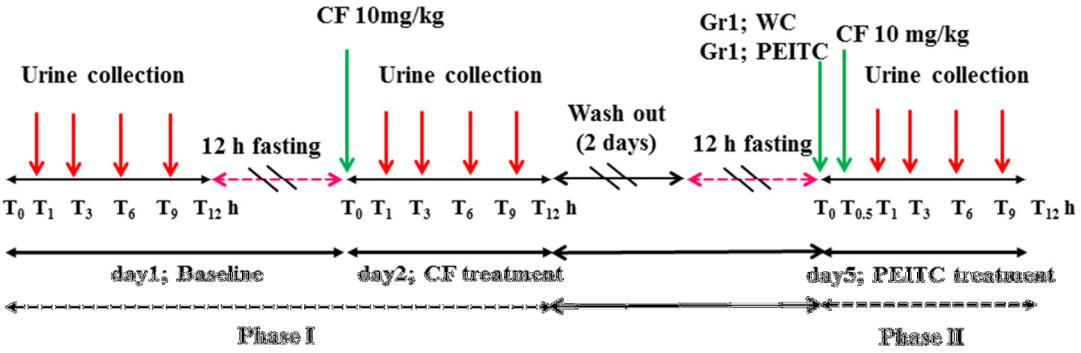


Figure 3.4 The experimental design of animal treatment in rats receiving a single oral dose of PEITC and fresh Betong watercress juice

CHAPTER 4

Results

4.1 Development of SPE method for analysis of CF and metabolites in rat urine

This study demonstrated the development of SPE method for extraction of CF and its metabolites: TB, PX, TP, and 137U in rat urine. Urine samples were extracted by the SPE procedure with modification of the generic protocol including precondition, loading, washing, eluting, and other. For SPE development, we have developed with fourteen methods. The recoveries achieved using standards solution and spiked blank urine samples were comparable (Table 4.1). The recoveries indicate the suitability of the SPE method development. Table 4.2 summarized the chromatographic separation of each extraction method at different concentrations and blank urine.

Table 4.1 Summarize extraction recovery for determination of TB, PX, TP, 137U, and CF at different concentration in rat urine

SPE No	Concentrations (µg/mL)	Recovery (%)					*Residue clearness
		TB	PX	TP	137U	CF	
1	10	0	0	0	0	0	+
2	10	87.91	96.56	98.36	102.28	90.28	++++
3	10	59.06	93.13	99.66	81.70	92.85	+++
4	10	68.63	93.50	93.21	70.09	93.55	++++
5	10	96.66	98.42	98.71	86.71	90.74	++++
6	10	-	-	-	-	-	+
7	10	67.41	93.88	98.88	80.88	96.48	+++
8	1	39.32	103.71	101.14	160.02	89.21	++
9	10	39.41	88.44	94.87	104.81	95.58	+++
10	10	44.71	90.94	96.21	103.68	95.15	++
11	1	60.02	97.04	101.61	154.75	104.12	++
12	1	64.06	96.30	110.23	197.52	90.33	++
13	1	62.54	91.79	117.85	80.79	92.06	++
14	1	78.93	98.54	98.76	99.33	99.43	++
14	10	79.35	99.26	99.17	98.25	99.04	++

* Residue clearness

++++; very good

+++; good

++; fair

+; poor

Table 4.2 Chromatographic separation of blank and sample spiked with a standard mixture of CF, TB, PX, TP, and 137U to achieve the different concentrations in rat urine

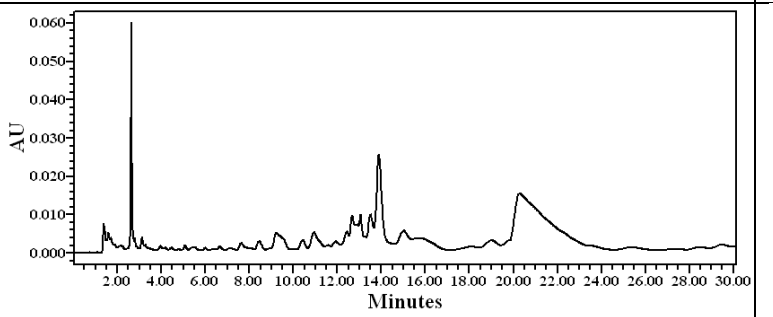
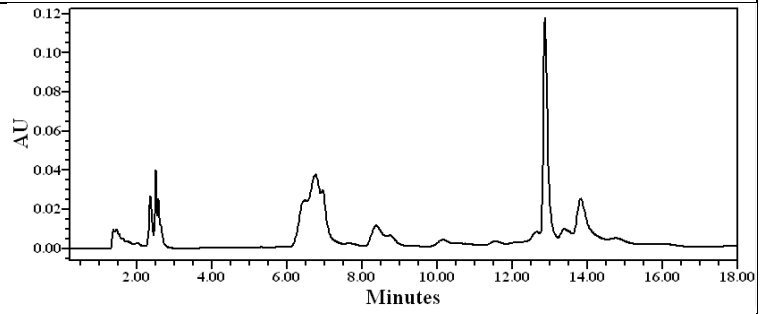
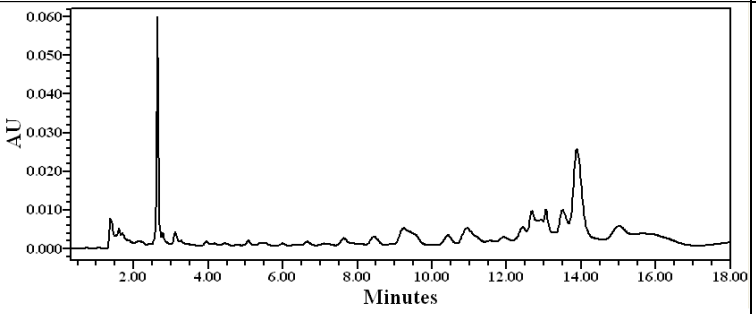
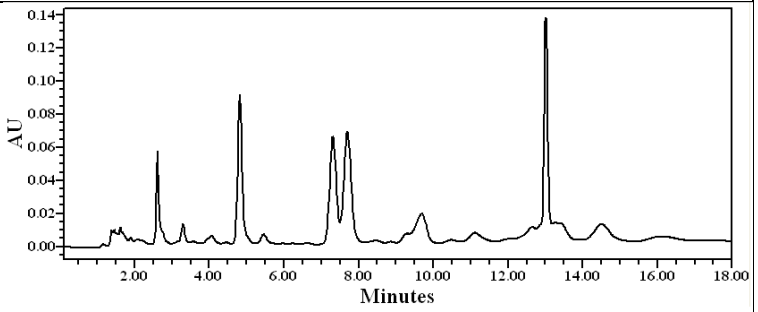
SPE No.	Conc. (µg/mL)	Blank chromatogram	Spike sample chromatograms
1	10		
2	10		

Table 4.2 Chromatographic separation of blank and sample spiked with a standard mixture of CF, TB, PX, TP, and 137U to achieve the different concentrations in rat urine (cont.)

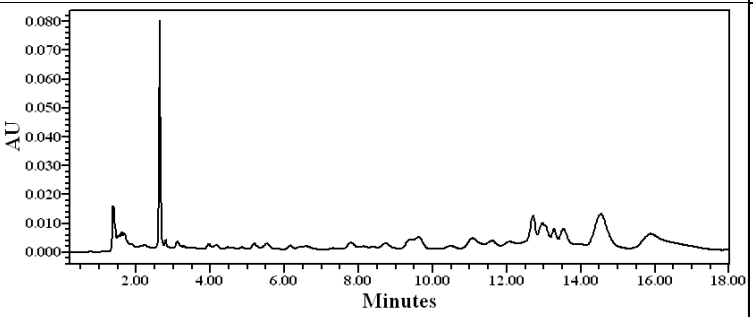
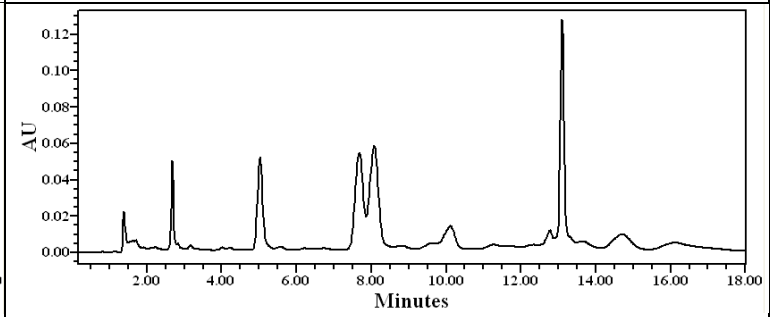
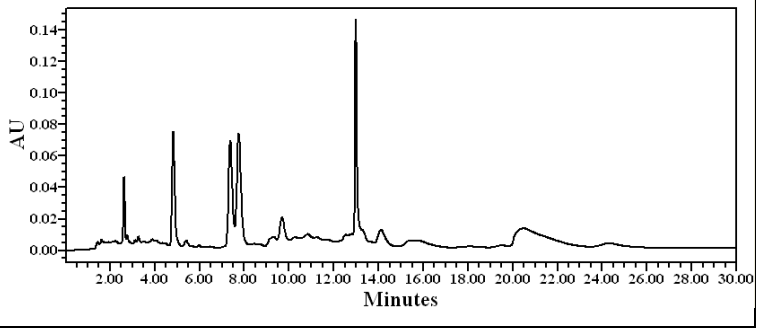
SPE No.	Conc. (µg/mL)	Blank chromatogram	Spike sample chromatograms
3	10		
4	10	**Not injected**	

Table 4.2 Chromatographic separation of blank and sample spiked with a standard mixture of CF, TB, PX, TP, and 137U to achieve the different concentrations in rat urine (cont.)

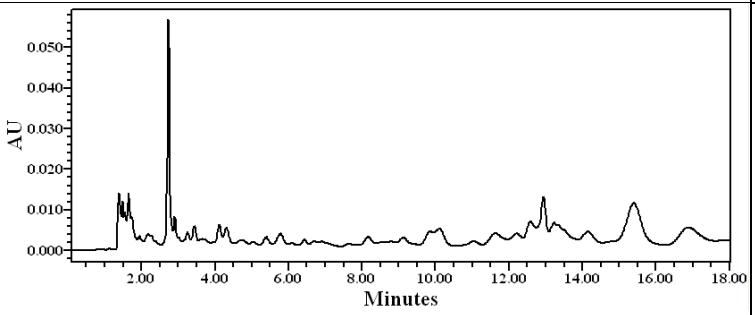
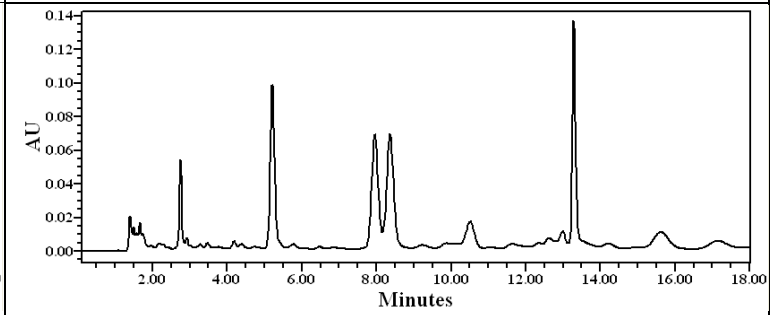
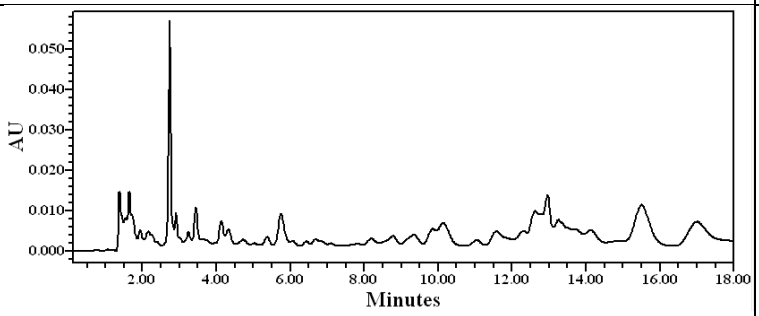
SPE No.	Conc. (µg/mL)	Blank chromatogram	Spike sample chromatograms
5	10		
6	10		**(Eluted = methanol:acetronitrile (80:20 v/v))** Not injected

Table 4.2 Chromatographic separation of blank and sample spiked with a standard mixture of CF, TB, PX, TP, and 137U to achieve the different concentrations in rat urine (cont.)

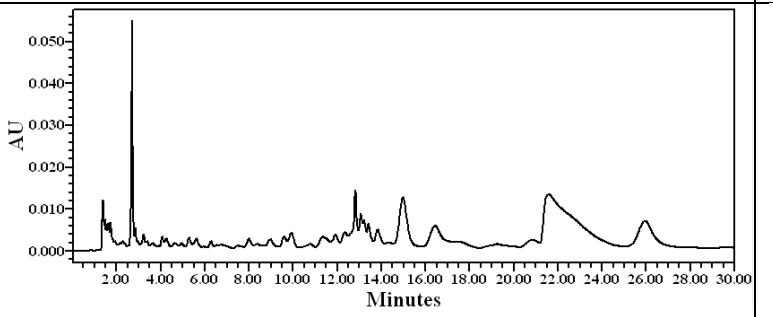
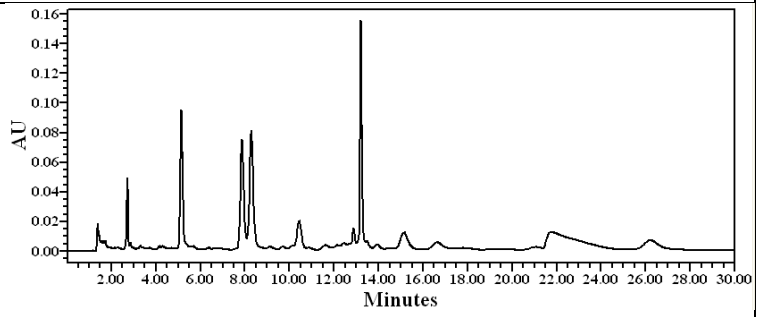
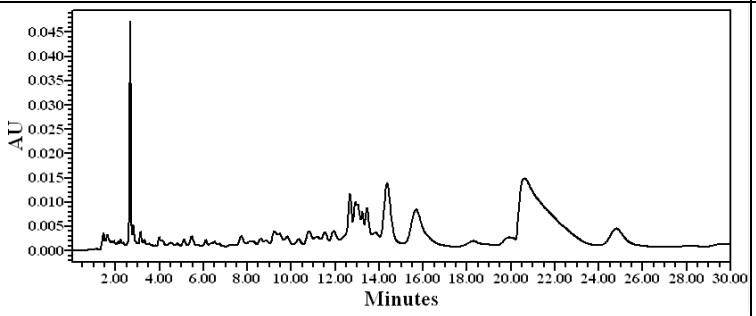
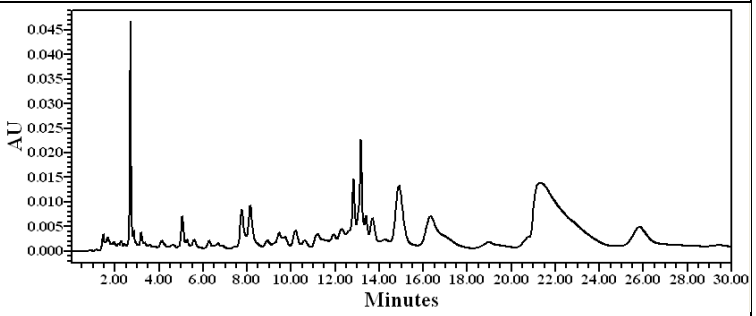
SPE No.	Conc. (µg/mL)	Blank chromatogram	Spike sample chromatograms
7	10		
8	1		

Table 4.2 Chromatographic separation of blank and sample spiked with a standard mixture of CF, TB, PX, TP, and 137U to achieve the different concentrations in rat urine (cont.)

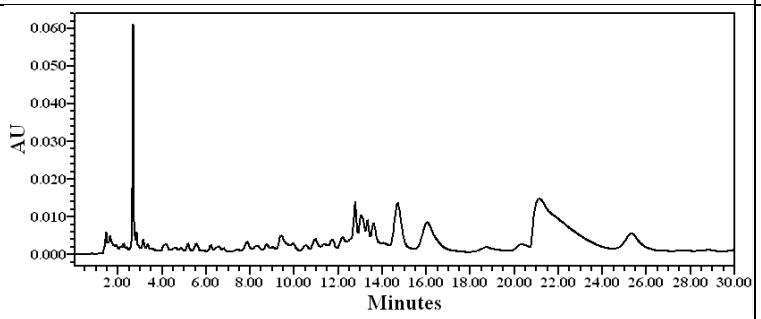
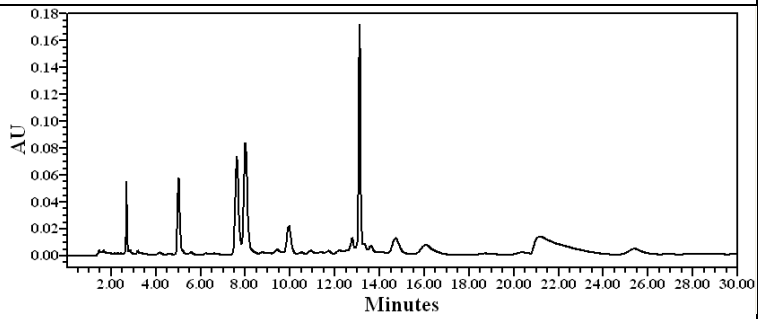
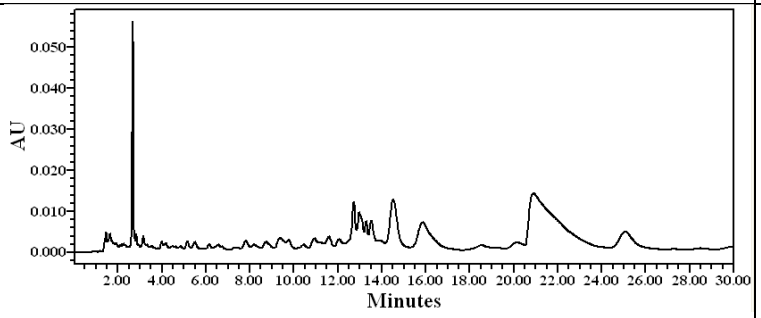
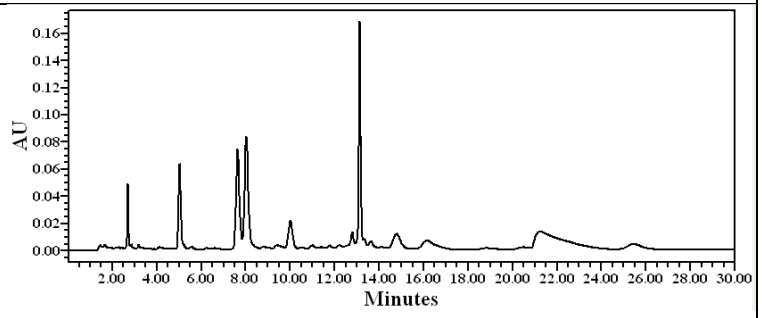
SPE No.	Conc. (µg/mL)	Blank chromatogram	Spike sample chromatograms
9	10		
10	10		

Table 4.2 Chromatographic separation of blank and sample spiked with a standard mixture of CF, TB, PX, TP, and 137U to achieve the different concentrations in rat urine (cont.)

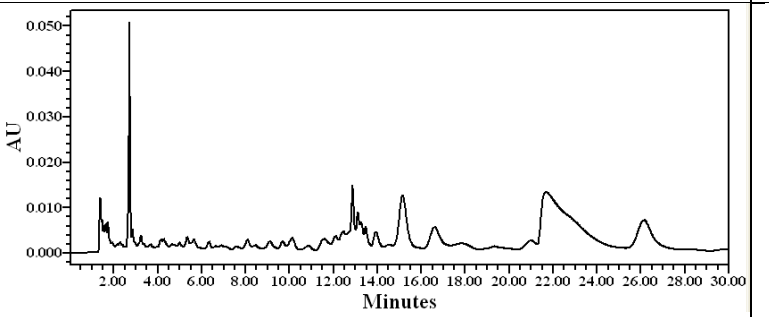
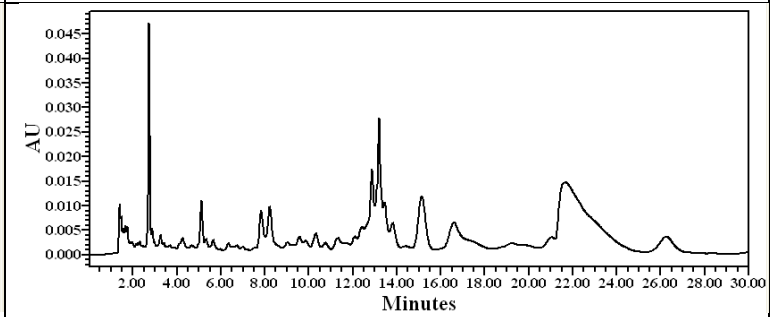
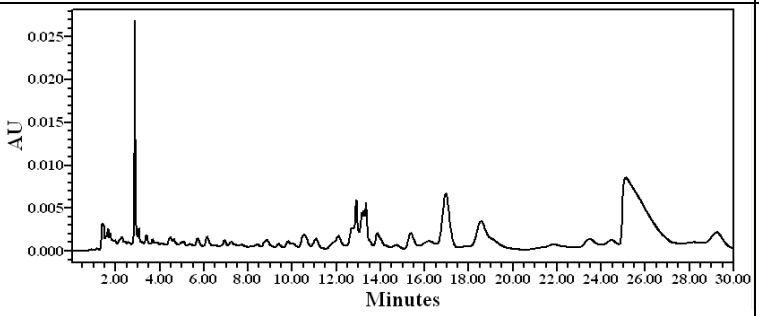
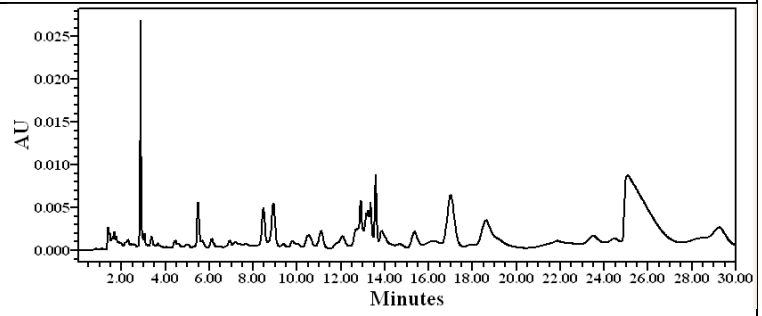
SPE No.	Conc. (µg/mL)	Blank chromatogram	Spike sample chromatograms
11	11		
12	1		

Table 4.2 Chromatographic separation of blank and sample spiked with a standard mixture of CF, TB, PX, TP, and 137U to achieve the different concentrations in rat urine (cont.)

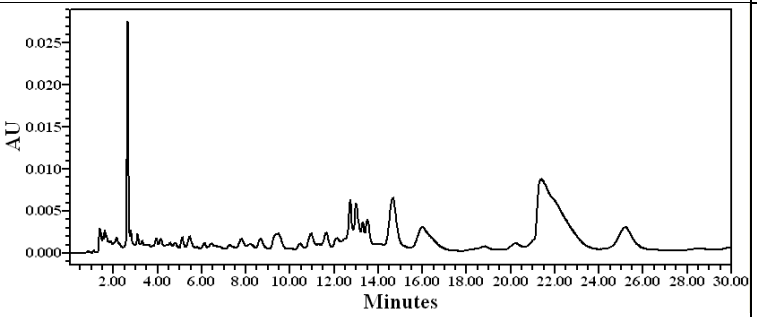
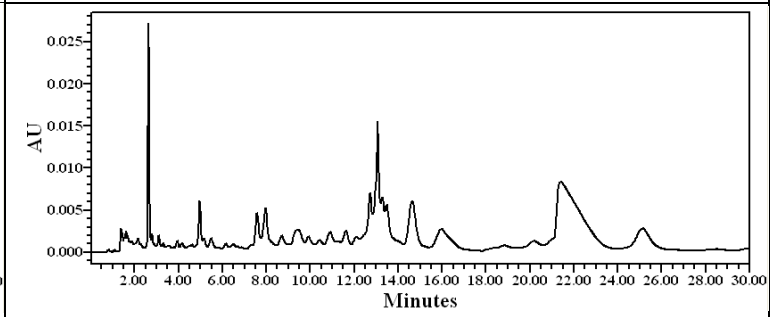
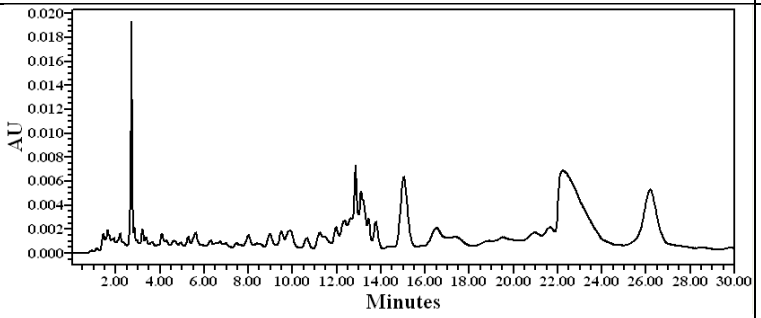
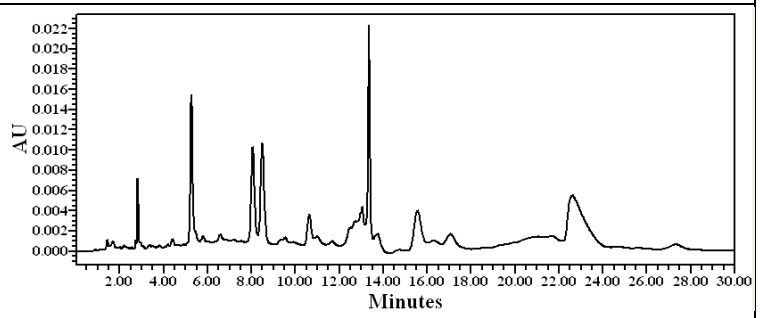
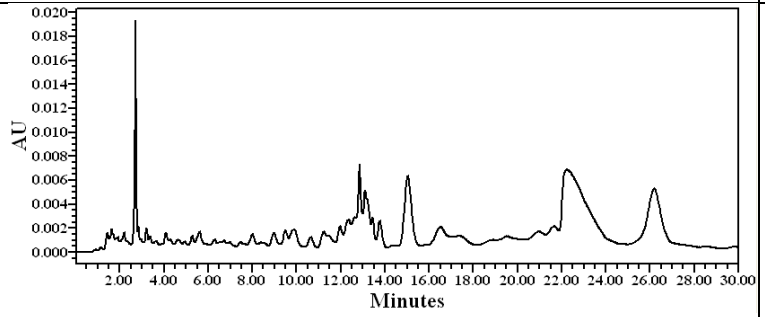
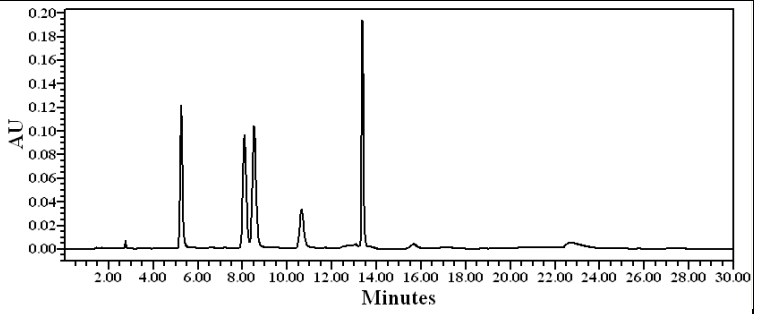
SPE No.	Conc. (µg/mL)	Blank chromatogram	Spike sample chromatograms
13	1		
14	1		

Table 4.2 Chromatographic separation of blank and sample spiked with a standard mixture of CF, TB, PX, TP, and 137U to achieve the different concentrations in rat urine (cont.)

SPE No.	Conc. (µg/mL)	Blank chromatogram	Spike sample chromatograms
14	10		

4.2 Method validation

4.2.1 Linearity and range

For the analysis of CF TB, PX, TP, and 137U in rat urine samples, regression analysis results showed that the calibration curves were linear over the concentration ranges of 0.25-10 µg/mL for CF and TP, 0.5-10 µg/mL for TB and 137U and 0.1-10 µg/mL for PX (Figure 4.1) (three replicates of each concentration).

The regression equations as follows:

CF; y = (20439±822.8)x-(1895±383.1) (r = 0.9999)

TB; y = (16881±370.8)x+(964.5±410.1) (r = 0.9997)

PX; y = (16199±370.8)x-(566.56±117.4) (r = 0.9997)

TP; y = (18383±105.8)x+(108.0±150.8) (r = 0.9999)

137U; y = (7689.6±37.6)x-(1277.2±613.7) (r = 0.9997), where $\varkappa$ is concentration and y is the peak area (Table 4.3).

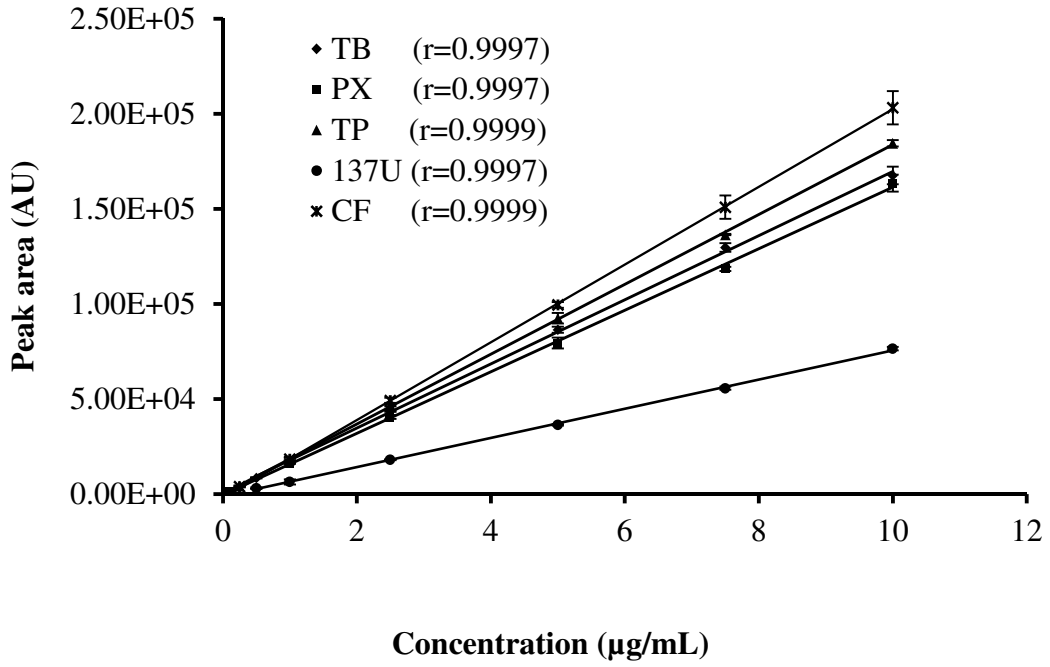


Figure 4.1 Linearity plots of mean peak area ratio ±SD (AU) against different concentrations of CF, TB, PX, TP and 137U spike in rat urine (n=3); correlation coefficients (r) = 0.9997 for TB and 137U and 0.9999 for CF, PX and TP

Table 4.3 Linear regression analyses for CF, TB, PX, TP, and 137U

Analytes	Range of linearity (µg/mL)	Calibration equation[a]	Correlation coefficient (*r*)
CF	0.25 - 10	(20439±822.75)x - (1895±383.10)	0.9999
TB	0.5 - 10	(16881±370.80)x + (964.49±410.08)	0.9999
RX	0.1 - 10	(16199±370.80)x - (566.56±117.39)	0.9997
TP	0.25 - 10	(18383±105.75)x + (107.98±150.79)	0.9997
137U	0.5 - 10	(7689.6±37.58)x - (1277.2±613.66)	0.9997

[a]Mean±SD

4.2.2 Precision

Method reproducibility was determined by measuring repeatability of intra- and inter-day precisions by using four different concentrations of QC samples of CF (0.25, 1, 5 and 10 µg/mL), TB (0.5, 1, 5 and 10 µg/mL), PX (0.1, 1, 5 and 10 µg/mL), TP (0.25, 1, 5 and 10 µg/mL) and 137U (0.5, 1, 5 and 10 µg/mL) (five replicates of each concentration) in rat urine blank. Intra- and inter-day precision of the method are shown in Table 4.4. The % RSD values ranged from 3.01-19.06 for CF, 2.36-4.7 for TB, 5.52-12.54 for PX, 4.29-10.51 for TP and 1.03-17.58 for 137U. Both parameters for all analytes were found to be within the acceptable value (15% RSD and ±20% RSD for the concentration at LLOQ) (FDA, 2001).

4.2.3 Accuracy

Intra- and inter-day accuracies were determined using four QC of CF, TB, PX, TP and 137U in rat urine blank with same concentrations for precision. The intra-day and inter-day accuracies (% DEV) ranged from (-) 0.43 to (+) 14.96 for CF, (-) 12.70 to (+) 0.94 for TB, (-) 12.13 to (+) 15.99 for PX, (-) 13.82 to (+) 2.48 for TP, and (-) 1.08 to (+) 14.32 for 137U (Table 4.4). The accuracy (% DEV) for determination of all analytes ranged between ±15% and ±20% for the concentration at LLOQ (FDA, 2001).

Table 4.4 Precision and accuracy of the method for determination of CF, TB, PX, TP, and 137U in rat urine (n=5)

Analytes	Concentration (µg/mL)	Precision (% RSD)		Accuracy (% DEV)	
		Intra-day	Inter-day	Intra-day	Inter-day
TB	0.5	4.14	4.71	-12.70	-6.04
	1	3.31	2.36	-5.09	-2.59
	5	3.00	3.13	0.94	0.90
	10	4.23	3.35	-0.78	-0.15
PX	0.1	12.54	8.82	15.99	-12.13
	1	6.10	5.52	1.87	1.15
	5	8.58	6.39	-0.55	0.33
	10	6.64	5.96	0.74	0.80
TP	0.25	10.51	6.57	-13.82	2.48
	1	6.36	4.29	-1.78	1.25
	5	6.70	4.50	0.37	0.23
	10	6.27	4.99	0.22	0.31
137U	0.5	12.31	17.58	14.32	5.05
	1	16.03	10.53	2.11	2.03
	5	1.03	5.00	-1.08	0.50
	10	2.20	6.28	0.60	0.30
CF	0.25	19.06	13.92	14.96	4.58
	1	8.82	5.34	0.95	0.49
	5	3.75	3.34	-0.43	-1.00
	10	4.02	3.01	0.19	-0.78

4.2.4 Recovery

The SPE method developed in this work produced good recovery of extraction of CF and its metabolites presented in rat urine (Table 4.5). The mean percentages of recovery were ranged from 93.86-97.44% for CF, 75.88-76.76% for TB, 90.63-95.80% for PX, 92.89-98.51% for TP, and 93.74-95.80% for 137U.

Table 4.5 Extraction recovery for determination of CF, TB, PX, TP, and 137U in rat urine (n=5)

Analytes	Concentration (µg/mL)	Mean peak area ratio		Recovery (%)[a]
		Direct injection	After extraction	
TB	0.5	11411.80	8661.03	75.88±2.98
	1	22715.20	17370.21	76.47±1.57
	5	114287.60	87733.61	76.76±2.23
	10	227673.00	173131.87	76.06±2.80
PX	0.1	1576.80	1427.45	90.63±4.22
	1	18351.80	17489.91	95.40±3.37
	5	91449.60	87181.77	95.34±3.02
	10	182860.40	175218.56	95.80±2.55
TP	0.25	4906.40	4560.84	92.89±2.98
	1	20300.40	19694.19	97.02±2.34
	5	101712.20	99722.15	98.03±2.15
	10	203239.40	200229.99	98.51±1.20
137U	0.5	3285.40	3085.77	93.74±3.14
	1	6951.40	6639.23	95.80±6.75
	5	36151.60	35447.72	98.10±1.36
	10	72814.00	71526.07	98.28±1.05
CF	0.25	4940.60	4648.35	93.86±6.79
	1	20725.80	20078.92	96.86±3.19
	5	105786.20	102048.75	96.48±2.37
	10	210798.60	205373.84	97.44±1.99

[a]Mean (±SD)

4.2.5 Lower limit of quantification

For rat urine samples, the LLOQ was determined by using three calibration curves of standard CF, TB, PX, TP and 137U spiked in urine samples at concentration 1, 5 and 10 µg/mL. The LLOQs were 0.25 µg/mL for CF and TP; 0.5 µg/mL for TB and 137U; and 0.1 µg/mL for PX.

4.3 Determination of urinary CF in rats receiving a single oral dose of CF (10 mg/kg)

4.3.1 Chromatographic profile

The developed SPE-HPLC method was employed to determine CF and its metabolites in urine of rats receiving a single oral dose (10 mg/kg) of CF. Chromatograms of separation of CF and its metabolites in rat urine samples were shown in Figure 4.2. Peaks of CF and its metabolites were well separated from other urine components. The retention time of TB, PX, TP, 137U, and CF were 4.93, 7.51, 7.90, 9.81, and 13.10 min, respectively. The elution time for each sample was approximately 30 min.

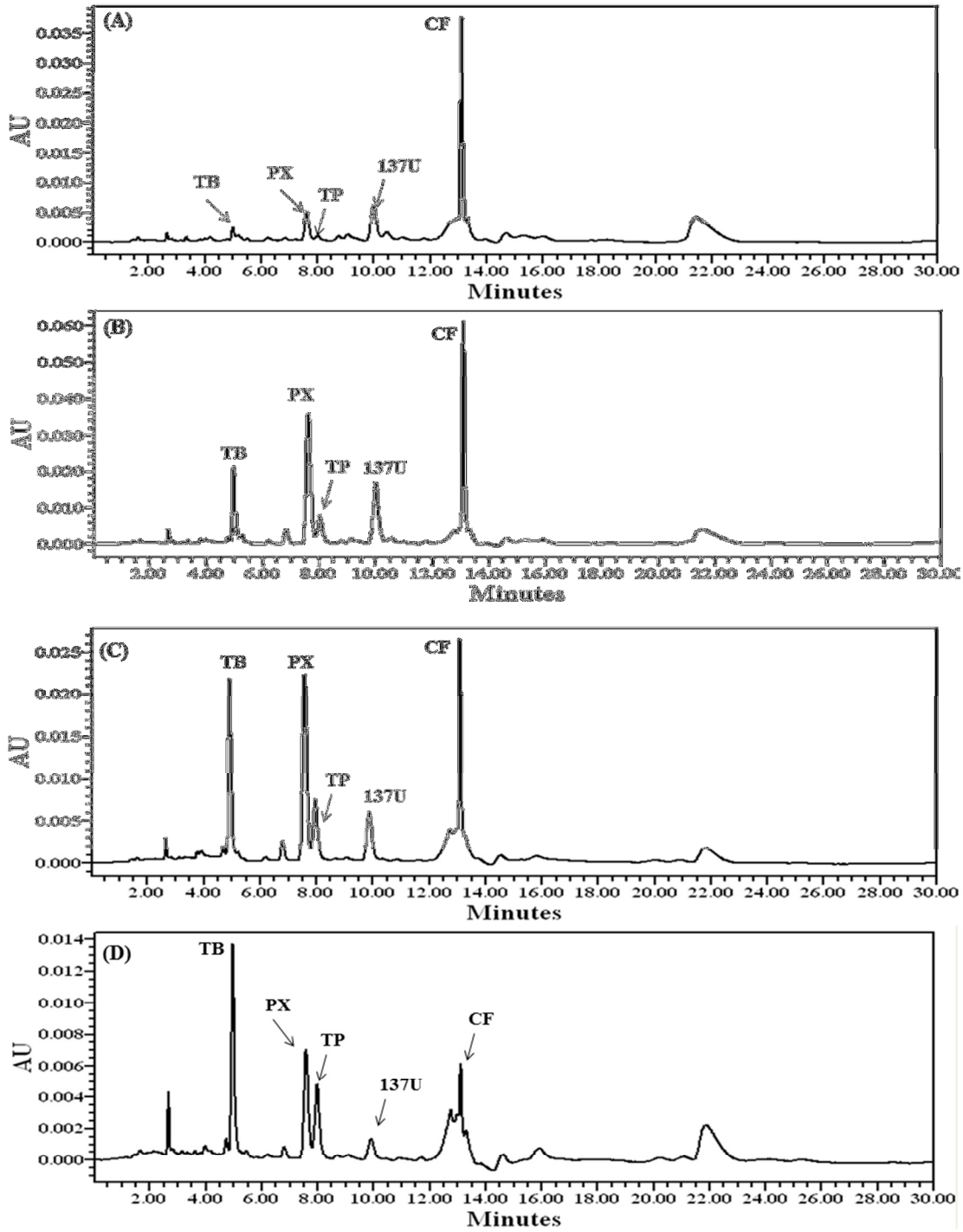


Figure 4.2 Representative chromatograms for CF, TB, PX, TP and 137U in urine of rats following an oral administration of CF (10 mg/kg) on various time intervals: (A); T_1 (0-1 h.), (B); T_3 (1-3 h.), (C); T_6 (3-6 h.), and (D); T_9 (6-9 h.)

4.3.2 Urinary excretory profile

Figure 4.3 (A) shows the mean urinary concentration-time profile and (B) shows the mean amount excreted of CF and its metabolites; TB, PX, TP, and 137U and total metabolite; TB+PX+TP+137U in rats receiving a single oral dose of 10 mg/kg of CF for all intervals of collection. The concentration of these compounds were gradually increasing and were highest at 3-6 h.

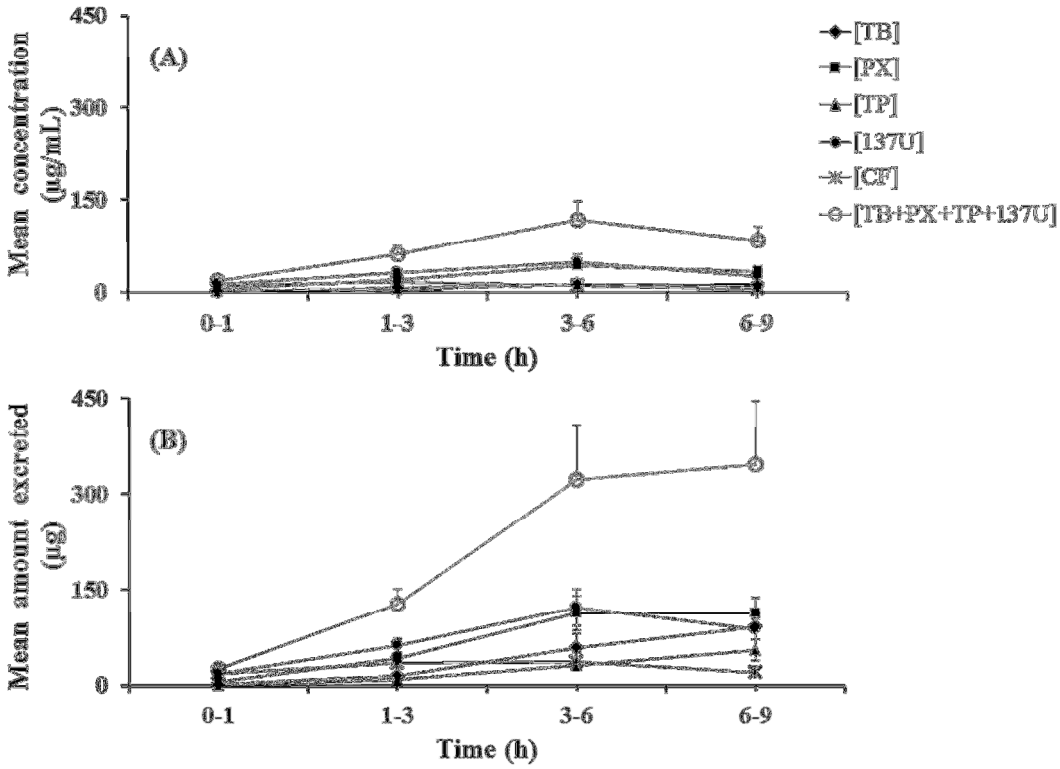


Figure 4.3 Urinary excretory profile (mean ±SEM) of CF and its metabolites in rats (n=6) after a single oral dose of CF (10 mg/kg): (A); concentrations profile and (B); amount excreted profile

4.3.3 CF metabolic ratio

The mean metabolic ratios of CF were shown in Figure 4.4. Urinary metabolic ratios were calculated as the ratio of eithers the concentration or the amount excreted. The mean metabolic ratios of each and total metabolites of CF in each rats were calculated as the ratio of the concentration (Figure 4.5). As seen in Figure 4.4, urinary metabolic ratios of each and total metabolites of CF were gradually increasing and were highest at 6-9 h interval both for the metabolic ratios derived from the concentration and the amount excreted. Similar findings were also observed in metabolic ratios of each and total metabolites in each rat.

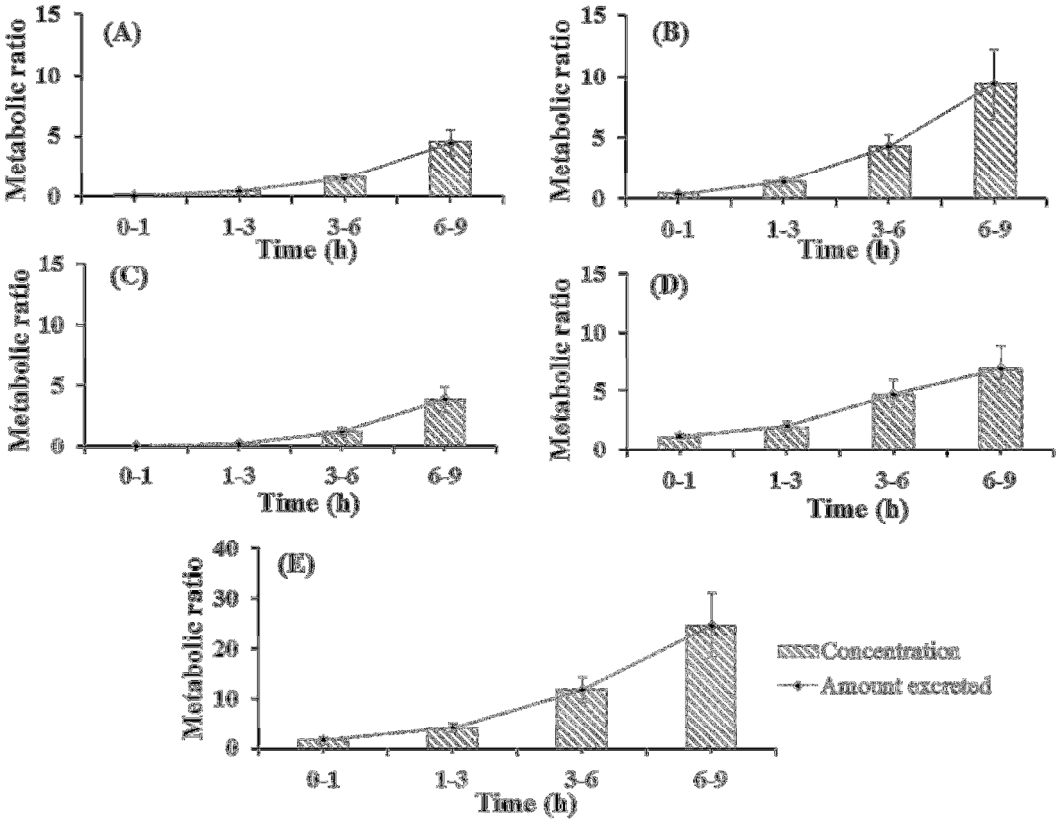


Figure 4.4 Metabolic ratios (mean ±SEM) of CF in rats (n=6) after a single oral dose of CF (10 mg/kg) derived from the concentration and the amount excreted: (A); TB/CF, (B); PX/CF, (C); TP/CF, (D); 137U/CF and (E); TB+PX+TP+137U/CF

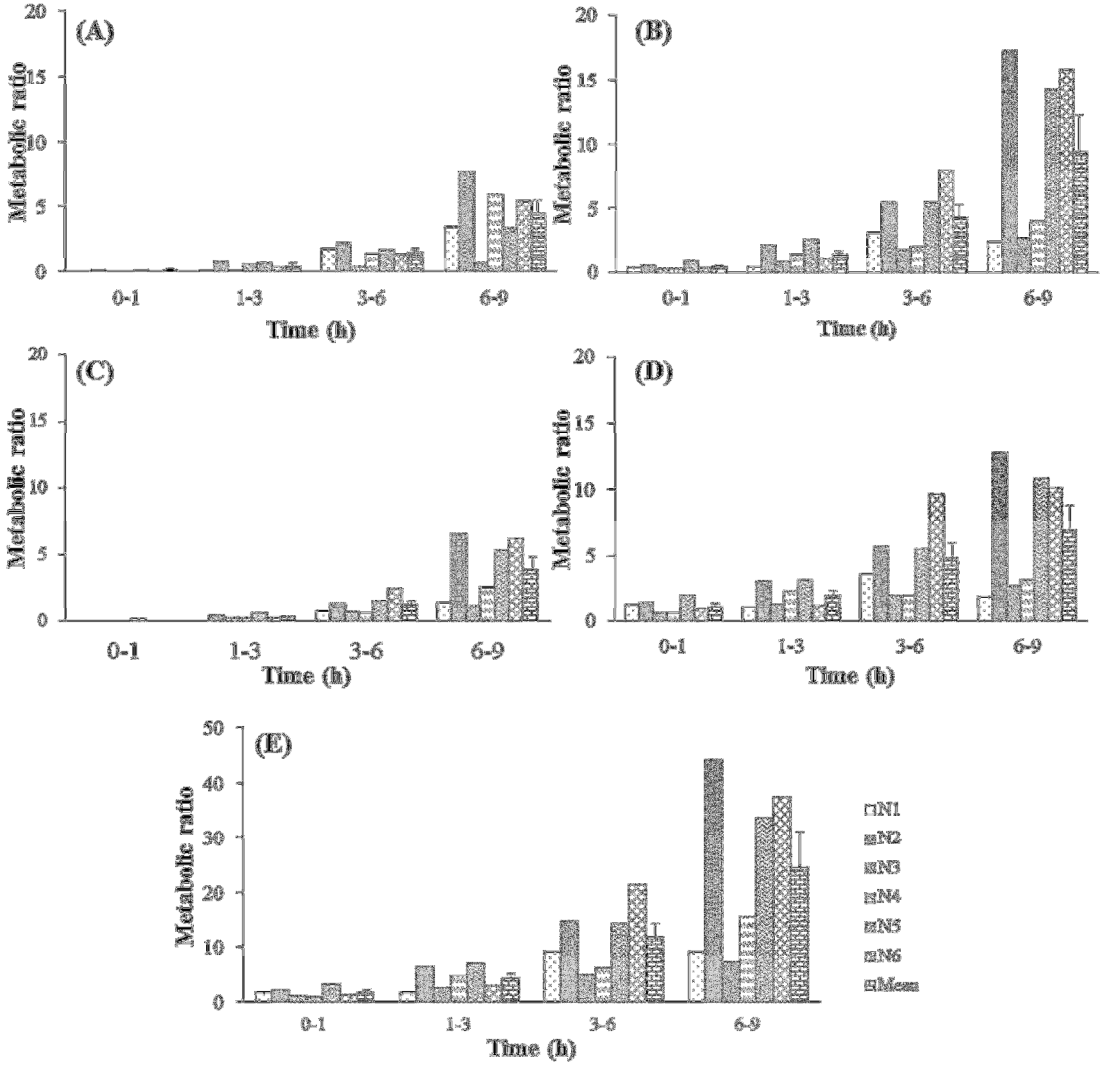


Figure 4.5 Metabolic ratios (mean ±SEM) of CF in rats (n=6) after a single oral dose of CF (10 mg/kg): (A); TB/CF, (B); PX/CF, (C); TP/CF, (D); 137U/CF and (E); TB+PX+TP+137U/CF

4.4 Determination of urinary CF in rats before (Phase I) and after (Phase II) pretreatment with a single dose of PEITC and fresh Betong watercress juice

4.4.1 Area under the curve (AUC)

The area under the curve value was calculated based on the urinary concentration-time curve of CF and its metabolites; TB, PX, TP, 137U, and total metabolites from 0-9 h (AUC_{0-9}) urine before pretreatment with PEITC (10 mg/kg) and Betong watercress (714 mg/kg BW). The AUC_{0-9} in phase I and phase II of pretreatment with PEITC and Betong watercress were shown in Figure 4.6 (A) and (B), respectively. The AUC_{0-9} of CF and its metabolites, except TB, alter were increased pretreatment with PEITC and Betong watercress.

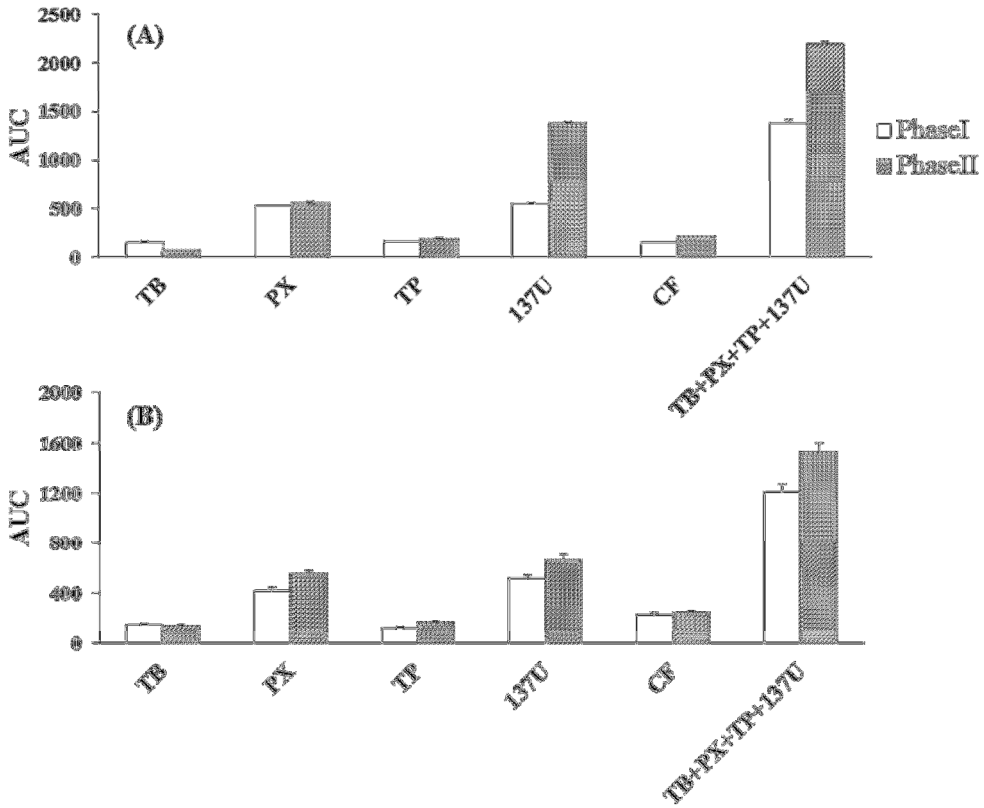


Figure 4.6 AUC_{0-9} (mean ±SEM) of CF and its metabolites in rats (n=3) after a single oral dose of CF (10 mg/kg): (A); PEITC (2 mg/kg, p.o.) and (B); Betong watercress (714 mg/kg BW, p.o.)

4.4.2 Metabolic ratio

Urinary metabolic ratios of CF at 0-9 h in phase I and phase II in rats receiving a single dose of PEITC (2 mg/kg) and Betong watercress (714 mg/kg BW) were shown in Figure 4.7 (A) and (B), respectively. Pretreatment of PEITC and Betong watercress before CF administration caused an increase in all metabolic ratios, except for TB. Urinary metabolic ratios for each rats (n=3) of each and total metabolites of CF in phase I and phase II in rats receiving a single dose of PEITC (2 mg/kg) and Betong watercress (714 mg/kg BW) were shown in Figure 4.8 and 4.9, respectively. The CF metabolic ratios of each rat when pretreatment with PEITC and Betong watercress were much varied.

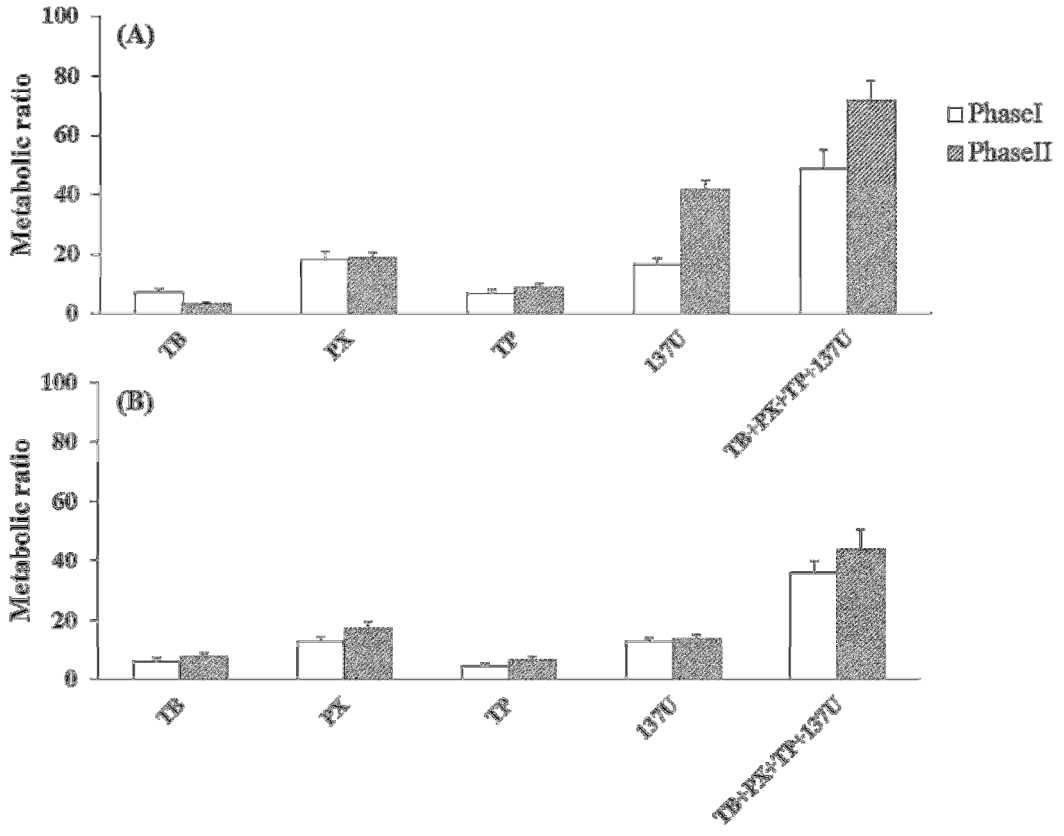


Figure 4.7 Metabolic ratios (mean ±SEM) of CF in rats (n=3) at 0-9 h after a single dose of CF (10 mg/kg, p.o.) alone (phaseI) and pretretmant with (A); PEITC (2 mg/kg, p.o.) and (B); Betong watercress (714 mg/kg BW, p.o.)

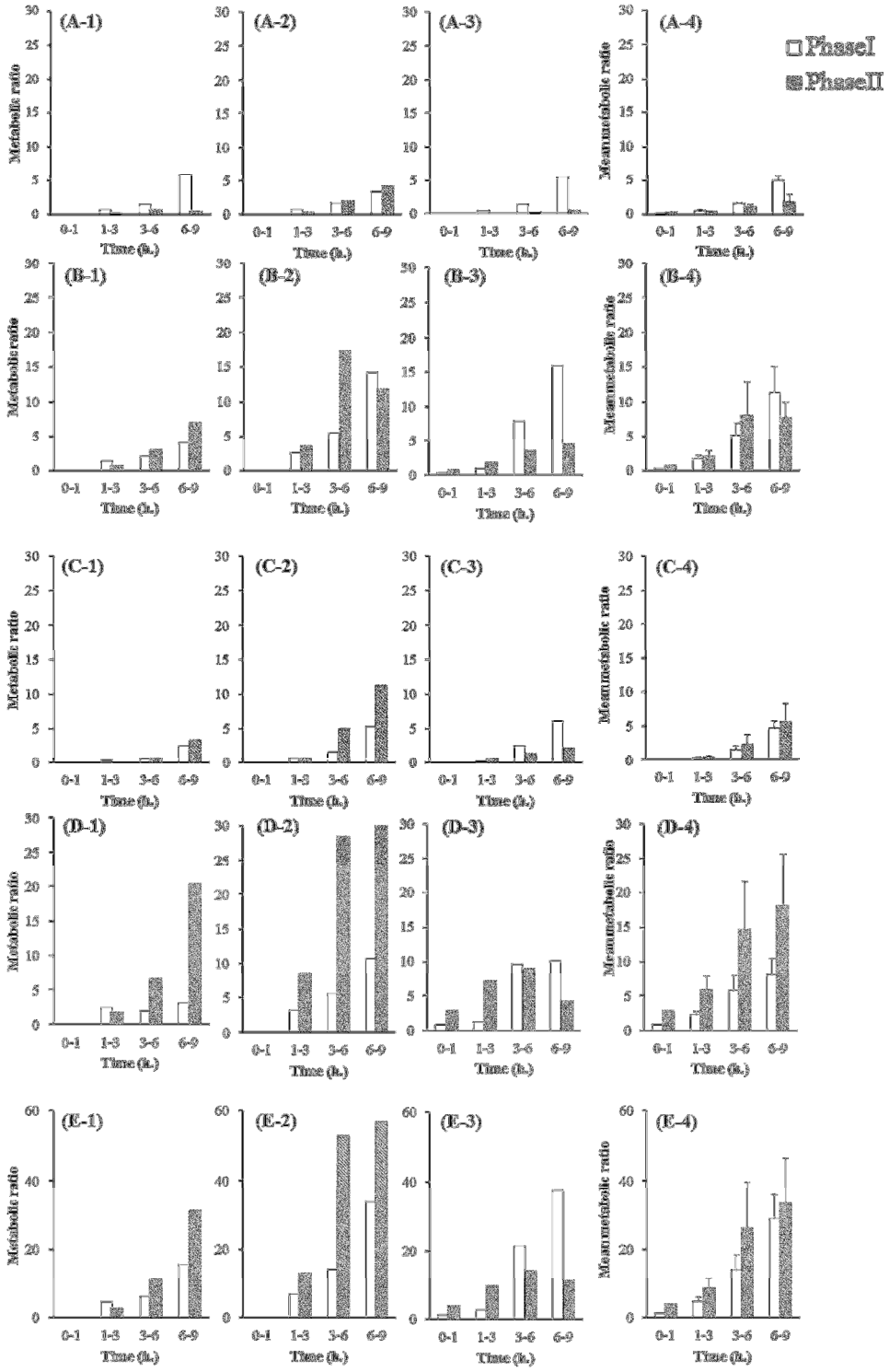


Figure 4.8 Metabolic ratios of CF metabolites in rats (n=3) after a single oral dose of CF (10 mg/kg) and pretreatment with PEITC (2 mg/kg)

: (A); TB/CF, (B); PX/CF, (C); TP/CF, (D); 137U/CF and (E); TB+PX+TP+137U/CF

: (-1); N1, (-2); N2, (-3); N3, and (-4); Mean (±SEM)

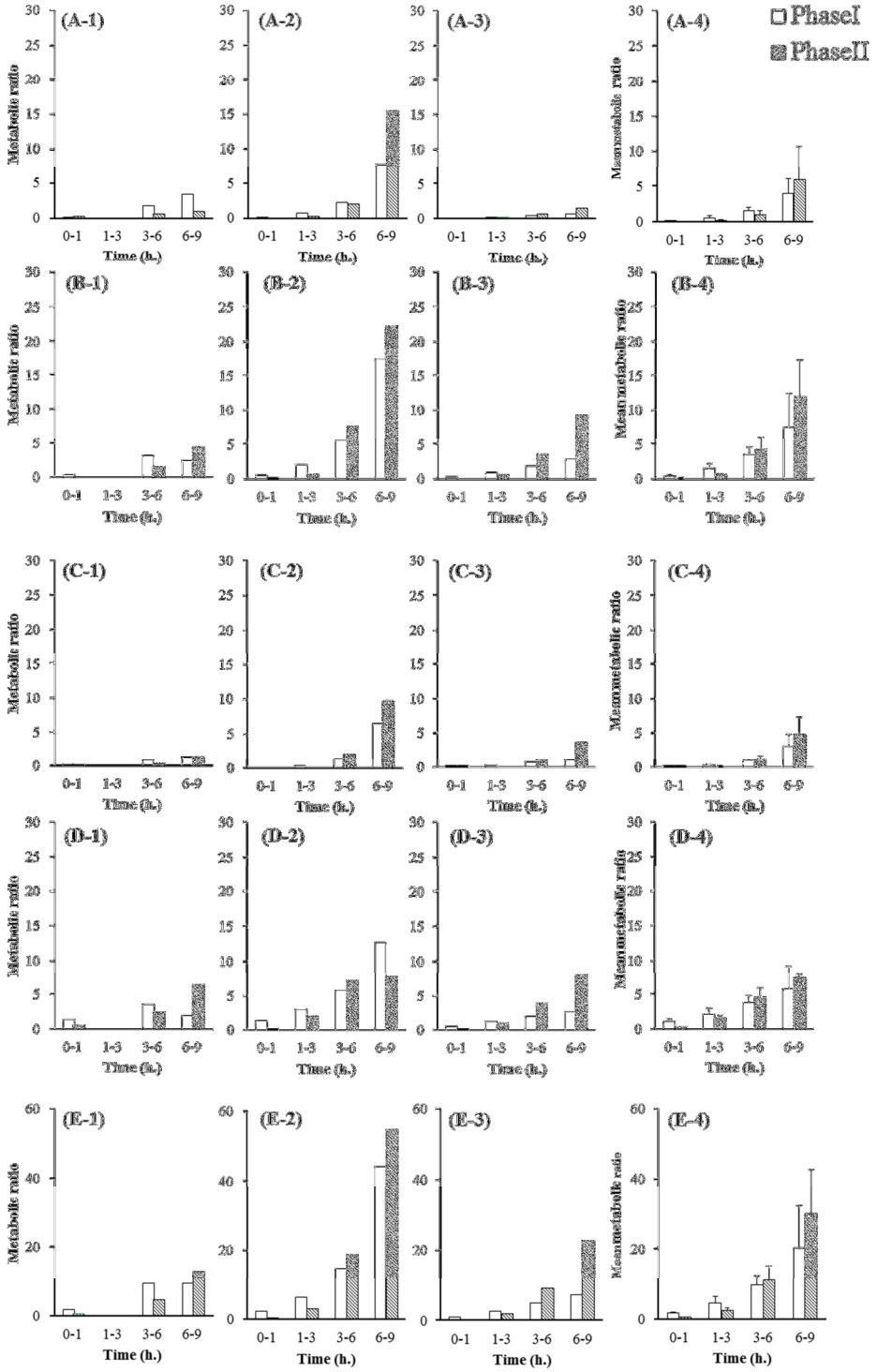


Figure 4.9 Metabolic ratios of CF metabolites in rats (n=3) after a single oral dose of CF (10 mg/kg) and pretreatment with Betong watercress (714 mg/kg BW)
: (A); TB/CF, (B); PX/CF, (C); TP/CF, (D); 137U/CF and (E); TB+PX+TP+137U/CF
: (-1); N1, (-2); N2, (-3); N3, and (-4); Mean (±SEM)

CHAPTER 5

Discussion and Conclusion

The present study use SPE technique for preparing urine sample. The SPE is a rapid and simple technique for extracting, concentrating, and cleaning up the sample. The SPE column is available in a variety of chemistries, adsorbents, and sizes. Selecting the sorbent and solvents for loading, washing, and eluting steps is important to result in effective of extraction. For many years that SPE reversed-phase (RP) silica columns have been use. One of limitation is that it is able to extract either acidic or basic or neutral compounds. The Oasis® HLB sorbent is a macroporous copolymer of two monomers: the lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone (Blahova and Brandsteterova, 2004). Therefore, samples with a mixture of acidic, basic, and neutral properties can be isolated on it. Since CF, TB, PX, and TP are basic compounds but 137U is acidic compounds, the present study therefore used the Oasis® HLB sorbent.

The SPE procedure employed a generic protocol: precondition, loading, washing, and eluting. It was necessary to dilute urine sample before loading to improve efficiency of extraction. This study found that urine diluted with 4% phosphoric acid was better than urine diluted with 0.1N hydrochloric acid. For the generic SPE protocol development, only water is usually used in a single washing step to remove highly polar interferences. However, the most important is the solvent and the ratio of solvent mixtures in the washing step because when the ratio of the washing solution was inappropriate, the analytes may be eluted from the column during this step. This development found that a mixture of 1% acetic acid:3% methanol-water (0.5:99.5 v/v) and 1% ammonium hydroxide:3% methanol-water (0.5:99.5 v/v) are the most efficiency in washing step. The mixtures were able to increase efficiency of cleaning up the urine sample. In this case, acetic acid removed neutral and basic polar interferences and ammonium hydroxide removed acidic polar interferences. Therefore, the chromatograms of rat urine samples observed in this

study showed less interference. In eluting step, a mixture of methanol:acetronitrile (80:20 v/v) was more beneficial than pure methanol. Such solvent mixture resulted higher recovery for all analytes (72.9-98.9%) when compared with the previous method, i.e. more than 70% for all analytes in rat urine (Schrader *et al.*, 1999), 83.9% and 83.2% for CF in rat plasma and urine sample, respectively (Abu-Qare and Abou-Donia, 2001).

The study used SPE method number 14 and HPLC-UV for analysis of CF and its metabolites. Chromatogram of standard mixture (10 µg/mL) of CF and its main metabolites spiked in urine sample is presented in Table 3.4 (SPE No. 14). All analytes were well separated within 14.0 min. The retention times were about 5.07, 7.75, 8.16, 10.18, and 13.17 min for TB, PX, TP, 137U and CF, respectively. The retention times of all analytes obtained from this study were shorter than those reported by Caubet and colleagues (2002), which were 14.12, 18.99, 19.31, 20.73 and 23.08 min, respectively, due to the difference in the column temperature and flow rate. The column temperature and flow rates used in this study were 32 ºC and 1.3 mL/min, respectively, compared to those reported by Caubet and colleagues (2002), i.e. 23 ºC and 1mL/min, respectively. In addition, the number of analytes of interest in this study was less than those in the previous study.

The SPE-HPLC analytical method was validated in terms of linearity, precision, accuracy, recovery, and LLOD using spiked urine sample from normal rats. For linearity, calibration curve prepared by different concentration in urine gave good values of the correlation coefficients (r) of > 0.999. Intra- and inter-day precision of the method for all analytes was found to be within the acceptable value of 15% RSD and ±20% RSD for the concentration at LLOQ. Similary, Intra- and inter-day accuracy for determination of all analytes ranged between ±15% and ±20% for the concentration at LLOQ. The results indicated that this method of analysis was precise and accurate. The SPE method developed in this work produced good recovery and was found to be in acceptable (80-120%), except TB. The mean percentages of recovery were ranged from 83.04-94.90% for CF, 72.91-75.81% for TB, 94.27-98.87% for PX, 90.54- 98.59% for TP, and 90.28- 97.15% for 137U. This SPE procedure is highly efficient for determining CF and its main metabolites in rat urine. In addition, the LLOQs of all analytes ranged from 0.1-0.5 µg/mL. The LLOQ values

are much higher than those in the previous report, i.e. which, 5ng/mL for all analytes in human urine because those the methods use diode array detection (Caubet *et al.,* 2002). In comparison to other reports which used SPE method, those values are higher than those previously reported, i.e. 24 ng/mL for CF in human milk (Aresta *et al.*, 2005), 14-41 ng/mL for all analytes in human urine (Atia *et al.*, 2009), 0.08 µg/mL for CF and TP in human urine (Zhu and Row, 2009). This may be due to difference in components of biological matrices and detection.

The proposed SPE-HPLC method was applied for determining the levels of CF and its metabolites excreted to urine of rats receiving a single oral dose (10 mg/kg) of CF. All compounds were well separated from each other with less interferences when compared to the separation from the previous reports (Khanna *et al.*, 1972; Schrader *et al.*, 1999). This may be due to the difference in the method of extraction.

The urinary concentration and the amount-time profiles of CF and its metabolites during 0-9 h were shown in the present work. The urinary concentration profiles correlated with the excreted amount. All compounds were detectable in all intervals and were the highest during 3-6 h. The concentrations of all analytes were above the LLOQ. 137U was found the highest in both concentration and amount excretion when compared with other compounds. That may be due to it is the main metabolite in rats (Kot and Daniel, 2008). The metabolic ratios calculated form the concentration correlated with the amount excreted. During phase I, the CF metabolic ratios were much varied. This may be due to interindividual variation in CF metabolism. Indeed, the interindividual variability depends on both intrinsic and extrinsic factor. The intrinsic factors include genetics, age, sex, and disease states. The intrinsic factors include diet, chemical exposures from the environment, and even sunlight (Thummel and Lin, 2014).

The interindividual variation in CF metabolism, primarily due to variations in CYP1A2 enzyme activity and one is the pharmacogenetic aspect. It has been reported that CYP1A2 has genetic polymorphism. CYP1A2 164C→A (CYP1A2*1F) is the most common allele for CYP1A2 polymorphism in humans and the C variant is associated with a slower CF metabolism. It causes a decrease in inducibility of tobacco in smokers (Sachse *et al.*, 1999). The less common alleles include CYP1A2 3858G→A (CYP1A2*1C) and CYP1A2 1545T→C (CYP1A2*1B) (Nakajima *et al.*, 1999; Sachse *et al.*, 2003).

Regarding to the values of metabolic ratios for each rat, their large variation can lead to subdivision of rats into two group i.e. high and low metabolic ratio. Rats with high metabolic ratios refer to fast metabolizer, which is N2 (rat 2; results in Figure 4.5). Those with low metabolic ratios may refer to slow metabolizer, which is N3 (rat 3; results in Figure 4.5). The observation may suggest that there is more than one phenotype of CYP1A2 in rats. While the study about polymorphism of CYP1A2 in rat is still lacking, and the urinary CF metabolic ratio is acceptable for determining CYP1A2 phenotype (Butler *et al.*, 1992), the study with increased sample size should be performed.

In vivo, CF is a probe that has been widely used to assess the activity of CYP1A2 in humans (Krul and Hageman, 1998; Weimann *et al.*, 2005; Perera *et al.*, 2013; Tanaka *et al.*, 2014). On the other hand, the scarcity of *in vivo* studies in rat urine samples is due to their complex process metabolism and its composition contain many compounds more than in human urine (Fort *et al.*, 1998). Jorritsma and colleagues (2000) recommended 1U+1X/PX as a simple measurement of CYP1A2 activity in Gunn rat urine.

This study estimated parameters probably related to CYP1A2 activity in rats administered PEITC and Betong watercress. Those included the $AUC_{0\text{-}9}$ and metabolic ratios calculated from urinary concentration of CF and its metabolites. The $AUC_{0\text{-}9}$ and metabolic ratios were increased by PEITC and Betong watercress. The induction effect of PEITC was stronger than Betong watercress. These results suggested that PEITC and Betong watercress may induced N-demethylation and C-8 hydroxylation of CF via induction of CYP1A2 in rats. However it is still controversy that PEITC and fresh Betong watercress induce CYP1A2 activity since some factors

may interfere the result of this study such as interindividual variability as described above and the sample size.

PEITC has been reported that it inhibited NNK metabolism, mediated by CYP1A2 (Smith *et al.,* 1990). The authors suggested that PEITC itself is a potent inhibitor of CYP1A2. Nakajima and colleagues (2001) using microsomes from baculovirus-infected insect cells expressing specific human CYP isoforms reported that PEITC competitively inhibited CYP1A2 with K_i value of 4.5 μM. In addition, Thapliyal and Maru (2001) reported that PEITC showed a dose-dependent inhibition of CYP1A2 activity using rat liver microsomes. In recent *in vivo* report, Janchawee and colleagues (2014) have reported that Betong watercress (800 mg/kg) and PEITC (10 and 20 mg/kg) inhibited N-demethylation of CF by determining plasma metabolic ratio. The authors suggested the PEITC and Betong watercress have inhibitory activity on CYP1A2 and CYP2C mediated N-demethylation of CF.

On the other hand, activities of CYPs have been shown to be induced by PEITC (Gross-Steinmeyer *et al.*, 2004). In cultured human primary hepatocytes, it was shown that PEITC dose dependently upregulated the expression of carcinogen-activating enzymes CYP1A1 and CYP1A2 using quantitative polymerase chain reaction analyses. The findings of the present study correspond with that from Gross-Steinmeyer and colleagues (2004)

Urinary CF metabolic ratio are frequently used to assess the activity of CYP1A2 in humans (Butler *et al.,* 1992; Campbell *et al.,* 1987; Caubet *et al.,* 2004; Begas *et al.,* 2007). Using CF metabolic ratio in rat plasma or serum has been reported (Noh *et al.,* 2011; Janchawee *et al.,* 2014) but that in urine of rats has less reported. However, this study provided prelimination results about effects of PEITC and Betong watercress on CF metabolism that related to CYP1A2 activity. Increase in number of samples should be included in the further study.

Conclusion

The SPE-HPLC-UV method presented in this study was simple, sensitive, precise and accurate to simultaneously determine CF and its methylxanthines (TB, PX, and TP) and methyluric acid (137U) metabolites in rat urine. The SPE procedure was highly efficient to clean up the sample with high recovery of extraction. The developed method was applicable to quantify all analytes in real sample. It appeared to be beneficial for studying effects of xenobiotics on CF metabolism by using urinary CF metabolic ratio.

In addition, the present study described the prelimination effects of PEITC and Betong watercress on CF metabolism that related to CYP1A2 activity. They may induce N-demethylation and C-8 hydroxylation of CF in rats because the $AUC_{0\text{-}9}$ and the CF metabolic ratios tended to increase. These effects indicate that they have induced activity on CYP1A2 in rats. CYP1A2 involves in bioactivation of procarcinogens to carcinogens. Hence, they may affect on CYP1A2 activity and may be benefit in chemoprevention.

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Appendix-1 Data of method validation for CF and metabolites

Table A1.1 Data for determination of linearity and lower limit of quantification of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=3)

Concentration (µg/ml)	**Peak area (AU)**						**Measured concentration (µg/mL)**		
TB	**n_1**	**n_2**	**n_3**	**Mean**	**S.D.**	**% RSD**	**Mean**	**S.D.**	**% RSD**
0.5	8082.60	8780.80	8649.00	8504.13	370.96	4.36	0.41	0.03	7.66
1	16719.60	17668.40	17140.80	17176.27	475.39	2.77	0.93	0.03	3.11
2.5	43529.20	44150.20	43308.40	43662.60	436.47	1.00	2.51	0.02	0.63
5	85357.00	88316.20	85694.40	86455.87	1619.90	1.87	5.05	0.02	0.48
7.5	129265.80	132284.40	127706.60	129752.27	2327.35	1.79	7.63	0.05	0.64
10	165460.60	172923.40	164382.20	167588.73	4651.32	2.78	9.88	0.04	0.40
PX									
0.1	1223.40	1381.00	1309.60	1304.67	78.92	6.05	0.13	0.02	12.67
1	15853.60	16240.60	16224.80	16106.33	219.02	1.60	1.04	0.01	1.32
2.5	39484.20	40856.80	41271.40	40537.47	935.42	6.76	2.54	0.01	0.20
5	76180.40	80854.40	81253.00	79429.27	2820.65	1.36	4.94	0.06	1.30
7.5	117173.40	118968.00	119097.60	118413.00	1075.48	2.31	7.35	0.10	1.39
10	158456.00	166516.00	165865.20	163612.40	4477.41	3.55	10.13	0.05	0.46

Table A1.1 Data for determination of linearity and lower limit of quantification of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=3) (cont.)

Concentration (µg/ml)	Peak area (AU)						Measured concentration (µg/mL)		
TP	**n_1**	**n_2**	**n_3**	**Mean**	**S.D.**	**% RSD**	**Mean**	**S.D.**	**% RSD**
0.25	3883.40	4202.00	4392.20	4159.20	257.09	6.18	0.20	0.04	19.82
1	17738.20	18767.00	18672.40	18392.53	568.64	3.09	0.98	0.03	2.57
2.5	46020.20	47537.80	47635.00	47064.33	905.55	1.92	2.54	0.01	0.42
5	89225.80	94293.60	93950.40	92489.93	2832.03	3.06	5.02	0.09	1.74
7.5	136148.20	136495.80	136914.00	136519.33	383.44	0.28	7.42	0.06	0.83
10	182755.20	186127.20	184823.20	184568.53	1700.36	0.92	10.04	0.02	0.17
137U									
0.5	2662.40	3419.80	3316.40	3132.87	410.70	13.11	0.62	0.04	7.13
1	4831.00	7033.60	7180.40	6348.33	1316.10	20.73	1.03	0.07	7.18
2.5	17814.00	18259.80	18180.40	18084.73	237.80	1.31	2.55	0.07	2.56
5	35929.00	36484.80	36701.00	36371.60	398.25	1.09	4.91	0.04	0.87
7.5	54757.20	55767.20	56459.20	55661.20	855.94	1.54	7.40	0.02	0.26
10	75545.40	76740.20	77249.60	76511.73	874.77	1.14	10.10	0.02	0.22

Table A1.1 Data for determination of linearity and lower limit of quantification of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=3) (cont.)

Concentration (µg/ml)	**Peak area (AU)**						**Measured concentration (µg/mL)**		
CF	**n_1**	**n_2**	**n_3**	**Mean**	**S.D.**	**% RSD**	**Mean**	**S.D.**	**% RSD**
0.25	4453.40	3563.20	3662.80	3893.13	487.75	12.53	0.30	0.05	16.20
1	19710.40	17785.20	17723.00	18406.20	1129.90	6.14	1.01	0.05	4.98
2.5	50785.60	48531.60	48196.20	49171.13	1408.19	2.86	2.51	0.01	0.35
5	101291.60	98838.00	98438.00	99522.53	1545.06	1.55	4.97	0.10	1.95
7.5	157927.60	146518.00	148266.80	150904.13	6145.03	4.07	7.48	0.06	0.76
10	213326.60	199311.40	197155.80	203264.60	8780.35	4.32	10.03	0.05	0.49

Table A1.2 Data for determination of intra- and inter-day precision of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5)

Concentration (µg/ml)	Intra-day precision					Inter-day precision				
TB	**n**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**	**Day**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**
0.5	n_1	8082.60	8681.64	359.57	4.14	**1**	8504.13	8661.03	407.51	4.71
	n_2	8780.80				**2**	8232.60			
	n_3	8649.00				**3**	9198.00			
	n_4	8894.80				**4**	8973.80			
	n_5	9001.00				**5**	8396.60			
1	n_1	16719.60	17521.76	580.68	3.31	**1**	17176.27	17370.21	410.69	2.36
	n_2	17668.40				**2**	16947.40			
	n_3	17140.80				**3**	17730.60			
	n_4	18067.40				**4**	17882.80			
	n_5	18012.60				**5**	17114.00			
5	n_1	85357.00	88193.56	2648.67	3.00	**1**	86455.87	87733.61	2746.65	3.13
	n_2	88316.20				**2**	85525.40			
	n_3	85694.40				**3**	91017.60			
	n_4	90511.40				**4**	90370.60			
	n_5	91088.80				**5**	85298.60			
10	n_1	165460.60	172340.56	7293.55	4.23	**1**	167588.73	173131.87	5805.09	3.35
	n_2	172923.40				**2**	170214.40			
	n_3	164382.20				**3**	179615.20			
	n_4	179181.00				**4**	179198.40			
	n_5	179755.60				**5**	169042.60			

Table A1.2 Data for determination of intra- and inter-day precision of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5) (cont.)

Concentration (µg/ml)	Intra-day precision					Inter-day precision				
PX	**n**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**	**Day**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**
0.1	n_1	1223.40	1429.00	179.16	12.54	**1**	1304.67	1427.45	125.93	8.82
	n_2	1381.00				**2**	1496.40			
	n_3	1309.60				**3**	1361.40			
	n_4	1616.40				**4**	1614.00			
	n_5	1614.60				**5**	1360.80			
1	n_1	15853.60	16844.08	1027.41	6.10	**1**	16106.33	17489.91	965.32	5.52
	n_2	16240.60				**2**	17166.80			
	n_3	16224.80				**3**	18688.40			
	n_4	18099.60				**4**	18011.60			
	n_5	17801.80				**5**	17476.40			
5	n_1	76180.40	84507.60	7248.21	8.58	**1**	79429.27	87181.77	5570.23	6.39
	n_2	80854.40				**2**	84113.60			
	n_3	81253.00				**3**	93394.80			
	n_4	92762.60				**4**	91125.40			
	n_5	91487.60				**5**	87845.80			
10	n_1	158456.00	171599.52	11385.98	6.64	**1**	163612.40	175218.56	10437.27	5.96
	n_2	166516.00				**2**	167049.60			
	n_3	165865.20				**3**	188205.40			
	n_4	183657.60				**4**	183228.60			
	n_5	183502.80				**5**	173996.80			

Table A1.2 Data for determination of intra- and inter-day precision of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5) (cont.)

Concentration (µg/ml)	Intra-day precision					Inter-day precision				
TP	**n**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**	**Day**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**
0.25	n_1	3883.40	4475.64	470.30	10.51	**1**	4159.20	4560.84	299.61	6.57
	n_2	4202.00				**2**	4815.80			
	n_3	4392.20				**3**	4894.60			
	n_4	4978.00				**4**	4511.20			
	n_5	4922.60				**5**	4423.40			
1	n_1	17738.20	19235.84	1222.79	6.36	**1**	18392.53	19694.19	845.22	4.29
	n_2	18767.00				**2**	19358.40			
	n_3	18672.40				**3**	20424.40			
	n_4	20515.20				**4**	20377.80			
	n_5	20486.40				**5**	19917.80			
5	n_1	89225.80	97004.96	6499.36	6.70	**1**	92489.93	99722.15	4490.35	4.50
	n_2	94293.60				**2**	98192.60			
	n_3	93950.40				**3**	102711.00			
	n_4	103645.60				**4**	103062.60			
	n_5	103909.40				**5**	102154.60			
10	n_1	182755.20	193372.68	12115.41	6.27	**1**	184568.53	200229.99	9991.74	4.99
	n_2	186127.20				**2**	198150.80			
	n_3	184823.20				**3**	210833.40			
	n_4	206541.40				**4**	206231.00			
	n_5	206616.40				**5**	201366.20			

Table A1.2 Data for determination of intra- and inter-day precision of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5) (cont.)

Concentration (µg/ml)	Intra-day precision					Inter-day precision				
137	**n**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**	**Day**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**
0.5	n_1	2662.40	3346.28	411.99	12.31	**1**	3132.87	3085.77	542.561	17.58
	n_2	3419.80				**2**	2569.20			
	n_3	3316.40				**3**	2496.80			
	n_4	3667.20				**4**	3588.80			
	n_5	3665.60				**5**	3641.20			
1	n_1	4831.00	6751.76	1082.38	16.03	**1**	6348.33	6639.23	699.006	10.53
	n_2	7033.60				**2**	5954.60			
	n_3	7180.40				**3**	6115.00			
	n_4	7382.20				**4**	7360.20			
	n_5	7331.60				**5**	7418.00			
5	n_1	35929.00	36544.92	374.88	1.03	**1**	36371.60	35447.72	1772.304	5.00
	n_2	36484.80				**2**	32860.60			
	n_3	36701.00				**3**	34349.80			
	n_4	36904.00				**4**	36834.20			
	n_5	36705.80				**5**	36822.40			
10	n_1	75545.40	75399.40	1658.98	2.20	**1**	76511.73	71526.07	4493.709	6.28
	n_2	76740.20				**2**	65869.60			
	n_3	77249.60				**3**	67829.00			
	n_4	73415.60				**4**	73112.60			
	n_5	74046.20				**5**	74307.40			

Table A1.2 Data for determination of intra- and inter-day precision of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5) (cont.)

Concentration (µg/ml)	Intra-day precision					Inter-day precision				
CF	**n**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**	**Day**	**Peak area (AU)**	**Mean**	**S.D.**	**% RSD**
0.25	n_1	4453.40	4460.56	850.32	19.06	**1**	3893.13	4648.35	646.92	13.92
	n_2	3563.20				**2**	4134.00			
	n_3	3662.80				**3**	4659.80			
	n_4	5283.80				**4**	5431.60			
	n_5	5339.60				**5**	5123.20			
1	n_1	19710.40	19517.24	1720.47	8.82	**1**	18406.20	20078.92	1072.56	5.34
	n_2	17785.20				**2**	19829.60			
	n_3	17723.00				**3**	20471.60			
	n_4	21303.40				**4**	21300.60			
	n_5	21064.20				**5**	20386.60			
5	n_1	101291.60	102190.80	3828.62	3.75	**1**	99522.53	102048.75	3410.57	3.34
	n_2	98838.00				**2**	98503.00			
	n_3	98438.00				**3**	102090.00			
	n_4	106673.60				**4**	107230.60			
	n_5	105712.80				**5**	102897.60			
10	n_1	213326.60	207285.44	8341.41	4.02	**1**	203264.60	205373.84	6180.11	3.01
	n_2	199311.40				**2**	198341.20			
	n_3	197155.80				**3**	204032.60			
	n_4	214515.40				**4**	215204.60			
	n_5	212118.00				**5**	206026.20			

Table A1.3 Data for determination of intra- and inter-day accuracies of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5) (cont.)

Concentration (µg/ml)	Intra-day accuracy					Inter-day accuracy				
TB	**n**	**Measured concentration (µg/mL)**	**Mean**	**S.D.**	**% DEV**	**Day**	**Measured concentration (µg/mL)**	**Mean**	**S.D.**	**% DEV**
0.5	n_1	0.39	0.44	0.04	-12.70	**1**	0.42	0.47	0.03	-6.04
	n_2	0.45				**2**	0.48			
	n_3	0.40				**3**	0.50			
	n_4	0.47				**4**	0.49			
	n_5	0.47				**5**	0.47			
1	n_1	0.91	0.95	0.03	-5.09	**1**	0.94	0.97	0.02	-2.59
	n_2	0.96				**2**	0.99			
	n_3	0.91				**3**	0.97			
	n_4	0.98				**4**	0.98			
	n_5	0.98				**5**	0.99			
5	n_1	5.04	5.05	0.02	0.94	**1**	5.10	5.05	0.03	0.90
	n_2	5.04				**2**	5.02			
	n_3	5.08				**3**	5.05			
	n_4	5.03				**4**	5.03			
	n_5	5.04				**5**	5.03			
10	n_1	9.85	9.92	0.06	-0.78	**1**	9.98	9.99	0.01	-0.15
	n_2	9.93				**2**	9.99			
	n_3	9.87				**3**	9.98			
	n_4	9.99				**4**	9.99			
	n_5	9.98				**5**	9.99			

Table A1.3 Data for determination of intra- and inter-day accuracies of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5) (cont.)

Concentration (µg/ml)	Intra-day accuracy					Inter-day accuracy				
PX	**n**	**Measured concentration (µg/mL)**	**Mean**	**S.D.**	**% DEV**	**Day**	**Measured concentration (µg/mL)**	**Mean**	**S.D.**	**% DEV**
0.1	n_1	0.12	0.12	0.02	15.99	**1**	0.13	0.09	0.03	-12.13
	n_2	0.14				**2**	0.05			
	n_3	0.11				**3**	0.09			
	n_4	0.08				**4**	0.11			
	n_5	0.12				**5**	0.06			
1	n_1	1.05	1.02	0.03	1.87	**1**	1.06	1.01	0.03	1.15
	n_2	1.05				**2**	0.99			
	n_3	1.02				**3**	1.01			
	n_4	0.98				**4**	1.01			
	n_5	1.00				**5**	0.98			
5	n_1	4.87	4.97	0.06	-0.55	**1**	5.07	5.02	0.04	0.33
	n_2	4.97				**2**	5.01			
	n_3	4.99				**3**	4.98			
	n_4	5.04				**4**	4.99			
	n_5	5.00				**5**	5.03			
10	n_1	10.08	10.07	0.08	0.74	**1**	10.40	10.08	0.18	0.80
	n_2	10.17				**2**	9.99			
	n_3	10.15				**3**	10.01			
	n_4	9.98				**4**	10.01			
	n_5	10.00				**5**	9.99			

Table A1.3 Data for determination of intra- and inter-day accuracies of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5) (cont.)

Concentration (µg/ml)	Intra-day accuracy					Inter-day accuracy				
TP	**n**	**Measured concentration (µg/mL)**	**Mean**	**S.D.**	**% DEV**	**Day**	**Measured concentration (µg/mL)**	**Mean**	**S.D.**	**% DEV**
0.25	n_1	0.25	0.22	0.03	-13.82	**1**	0.26	0.26	0.04	2.48
	n_2	0.18				**2**	0.28			
	n_3	0.18				**3**	0.31			
	n_4	0.24				**4**	0.23			
	n_5	0.23				**5**	0.20			
1	n_1	1.01	0.98	0.02	-1.78	**1**	1.04	1.01	0.03	1.25
	n_2	0.97				**2**	1.01			
	n_3	0.96				**3**	1.04			
	n_4	0.99				**4**	1.00			
	n_5	0.99				**5**	0.97			
5	n_1	4.92	5.02	0.06	0.37	**1**	5.10	5.01	0.07	0.23
	n_2	5.07				**2**	4.98			
	n_3	5.07				**3**	4.93			
	n_4	5.01				**4**	5.00			
	n_5	5.02				**5**	5.05			
10	n_1	10.04	10.02	0.03	0.22	**1**	10.14	10.03	0.06	0.31
	n_2	10.06				**2**	10.01			
	n_3	10.03				**3**	10.03			
	n_4	9.99				**4**	10.00			
	n_5	9.99				**5**	9.98			

Table A1.3 Data for determination of intra- and inter-day accuracies of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5) (cont.)

Concentration (µg/ml)	Intra-day accuracy					Inter-day accuracy				
137U	n	Measured concentration (µg/mL)	Mean	S.D.	% DEV	Day	Measured concentration (µg/mL)	Mean	S.D.	% DEV
0.5	n_1	0.67	0.57	0.07	14.32	**1**	0.73	0.53	0.12	5.05
	n_2	0.60				**2**	0.48			
	n_3	0.59				**3**	0.43			
	n_4	0.48				**4**	0.48			
	n_5	0.52				**5**	0.51			
1	n_1	0.95	1.02	0.06	2.11	**1**	1.14	1.02	0.07	2.03
	n_2	1.07				**2**	0.98			
	n_3	1.08				**3**	0.96			
	n_4	0.99				**4**	1.00			
	n_5	1.01				**5**	1.02			
5	n_1	4.96	4.95	0.06	-1.08	**1**	5.02	5.02	0.04	0.50
	n_2	4.89				**2**	5.03			
	n_3	4.88				**3**	5.08			
	n_4	5.02				**4**	5.03			
	n_5	4.98				**5**	4.97			
10	n_1	10.07	10.06	0.06	0.60	**1**	10.20	10.03	0.10	0.30
	n_2	10.12				**2**	9.99			
	n_3	10.10				**3**	9.97			
	n_4	9.99				**4**	9.99			
	n_5	10.01				**5**	10.01			

Table A1.3 Data for determination of intra- and inter-day accuracies of the method for analyzing TB, PX, TP, 137U, and CF in rat urine (n=5) (cont.)

Concentration (µg/ml)	Intra-day accuracy					Inter-day accuracy				
CF	n	Measured concentration (µg/mL)	Mean	S.D.	% DEV	Day	Measured concentration (µg/mL)	Mean	S.D.	% DEV
0.25	n_1	0.36	0.29	0.04	14.96	**1**	0.33	0.26	0.05	4.58
	n_2	0.28				**2**	0.22			
	n_3	0.27				**3**	0.22			
	n_4	0.26				**4**	0.27			
	n_5	0.26				**5**	0.26			
1	n_1	1.07	1.01	0.04	0.95	**1**	1.01	1.00	0.01	0.49
	n_2	0.99				**2**	1.01			
	n_3	0.98				**3**	1.00			
	n_4	1.01				**4**	1.00			
	n_5	1.00				**5**	1.00			
5	n_1	4.86	4.98	0.07	-0.43	**1**	4.78	4.95	0.10	-1.00
	n_2	5.03				**2**	4.98			
	n_3	5.02				**3**	5.00			
	n_4	4.98				**4**	4.99			
	n_5	4.99				**5**	5.00			
10	n_1	10.07	10.02	0.04	0.19	**1**	9.60	9.92	0.18	-0.78
	n_2	10.05				**2**	10.01			
	n_3	9.97				**3**	10.00			
	n_4	10.01				**4**	10.00			
	n_5	10.00				**5**	10.00			

Table A1.4 Data for determination of recoveries of extraction of TB, PX, TP, 137U, and CF in rat urine (n=5)

Concentration (µg/ml) TB	n	Peak area (AU)		% Recovery		
		Direct injection	After extraction	% Recovery	Mean	S.D.
0.5	n_1	11430	8504	74.40	75.88	2.98
	n_2	11214	8233	73.41		
	n_3	11602	9198	79.28		
	n_4	11369	8974	78.93		
	n_5	11444	8397	73.37		
1	n_1	22658	17177	75.81	76.47	1.57
	n_2	22459	16948	75.46		
	n_3	22464	17731	78.93		
	n_4	23193	17883	77.10		
	n_5	22802	17114	75.05		
5	n_1	114868	86456	75.27	76.76	2.23
	n_2	112943	85525	75.72		
	n_3	114734	91018	79.33		
	n_4	114413	90371	78.99		
	n_5	114480	85299	74.51		
10	n_1	229863	167589	72.91	76.06	2.80
	n_2	222929	170214	76.35		
	n_3	226358	179615	79.35		
	n_4	229363	179198	78.13		
	n_5	229852	169043	73.54		

Table A1.4 Data for determination of recoveries of extraction of TB, PX, TP, 137U, and CF in rat urine (n=5)

Concentration (µg/ml) PX	n	Peak area (AU)		% Recovery		
		Direct injection	After extraction	% Recovery	Mean	S.D.
0.1	n_1	1384	1305	94.27		
	n_2	1637	1496	91.41		
	n_3	1633	1361	83.37		
	n_4	1743	1614	92.60		
	n_5	1487	1361	91.51	90.63	4.22
1	n_1	16291	16106	98.87		
	n_2	18901	17167	90.82		
	n_3	18965	18688	98.54		
	n_4	19197	18012	93.83		
	n_5	18405	17476	94.95	95.40	3.37
5	n_1	82407	79429	96.39		
	n_2	92876	84114	90.57		
	n_3	94486	93395	98.85		
	n_4	95163	91125	95.76		
	n_5	92316	87846	95.16	95.34	3.02
10	n_1	169546	163612	96.50		
	n_2	181201	167050	92.19		
	n_3	189612	188205	99.26		
	n_4	190906	183229	95.98		
	n_5	183037	173997	95.06	95.80	2.55

Table A1.4 Data for determination of recoveries of extraction of TB, PX, TP, 137U, and CF in rat urine (n=5)

Concentration (µg/ml) TP	n	Peak area (AU)		% Recovery		
		Direct injection	After extraction	% Recovery	Mean	S.D.
0.25	n_1	4594	4159	90.54	92.89	2.98
	n_2	5113	4816	94.19		
	n_3	5022	4895	97.46		
	n_4	4907	4511	91.93		
	n_5	4896	4423	90.35		
1	n_1	18960	18393	97.01	97.02	2.34
	n_2	20789	19359	93.12		
	n_3	20681	20424	98.76		
	n_4	20599	20378	98.93		
	n_5	20473	19918	97.29		
5	n_1	95615	92490	96.73	98.03	2.15
	n_2	103483	98193	94.89		
	n_3	103681	102711	99.06		
	n_4	103532	103063	99.55		
	n_5	102250	102155	99.91		
10	n_1	187207	184569	98.59	98.51	1.20
	n_2	204937	198151	96.69		
	n_3	212602	210833	99.17		
	n_4	206451	206231	99.89		
	n_5	205000	201366	98.23		

Table A1.4 Data for determination of recoveries of extraction of TB, PX, TP, 137U, and CF in rat urine (n=5)

Concentration (µg/ml) 137U	n	Peak area (AU)		% Recovery		
		Direct injection	After extraction	% Recovery	Mean	S.D.
0.5	n_1	3470	3133	90.284	93.74	3.14
	n_2	2827	2569	90.88		
	n_3	2655	2497	94.04		
	n_4	3679	3589	97.55		
	n_5	3796	3641	95.92		
1	n_1	7580	6348	83.751	95.80	6.75
	n_2	6027	5955	98.80		
	n_3	6156	6115	99.33		
	n_4	7504	7360	98.08		
	n_5	7490	7418	99.04		
5	n_1	37863	36372	96.061	98.10	1.36
	n_2	33232	32861	98.88		
	n_3	34526	34350	99.49		
	n_4	37355	36834	98.61		
	n_5	37782	36822	97.46		
10	n_1	78759	76512	97.147	98.28	1.05
	n_2	66007	65870	99.79		
	n_3	69034	67829	98.25		
	n_4	74997	73113	97.49		
	n_5	75273	74307	98.72		

Table A1.4 Data for determination of recoveries of extraction of TB, PX, TP, 137U, and CF in rat urine (n=5)

Concentration (µg/ml) CF	n	Peak area (AU)		% Recovery		
		Direct injection	After extraction	% Recovery	Mean	S.D.
0.25	n_1	4688	3893	83.04	93.86	6.79
	n_2	4480	4134	92.28		
	n_3	4692	4660	99.31		
	n_4	5449	5432	99.68		
	n_5	5394	5123	94.98		
1	n_1	20001	18406	92.03	96.86	3.19
	n_2	20035	19830	98.97		
	n_3	20589	20472	99.43		
	n_4	21580	21301	98.71		
	n_5	21424	20387	95.16		
5	n_1	106650	99523	93.32	96.48	2.37
	n_2	101628	98503	96.93		
	n_3	103997	102090	98.17		
	n_4	108205	107231	99.10		
	n_5	108451	102898	94.88		
10	n_1	214185	203265	94.90	97.44	1.99
	n_2	202023	198341	98.18		
	n_3	206007	204033	99.04		
	n_4	216661	215205	99.33		
	n_5	215117	206026	95.77		