DETERMINATION OF PHENYLALANINE AND TYROSINE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

BY

MR. WORAPHOT WANICHALANANT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE (CHEMISTRY)

DEPARTMENT OF CHEMISTRY

FACULTY OF SCIENCE AND TECHNOLOGY

THAMMASAT UNIVERSITY

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was approved as partial fulfillment of the requirements for the degree of Master of Science (Chemistry)

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Thesis Title	DETERMINATION OF PHENYLALANINE AND TYROSINE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
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ABSTRACT

In this research, hydrophilic interaction liquid chromatography (HILIC) with diode array detection was developed for the simultaneous determination of polar compounds namely phenylalanine (Phe), tyrosine (Tyr) and creatinine (Cre). Chromatographic separation was carried out using a HILIC column (dihydroxypropyl phase, 3.0 mm x 150 mm x 3 µm) with three detection wavelengths (210 nm for Phe and α-methyl phenylalanine (internal standard), 225 nm for Tyr and 234 nm for Cre). The mobile phase consisted of 84:16 v/v of acetonitrile and 50 mM ammonium formate pH 3.0 with isocratic elution at a flow rate of 0.8 mL min^{-1}. The injection volume was 4 µL.

The proposed method was applied to determine Phe, Tyr and Cre in urine for an alternative diagnosis and monitoring of phenylketonuria (PKU). The spot urine collected sample was diluted 10 times with 0.1 M hydrochloric acid. One milliliter of the diluted urine was introduced to condition strong cation exchange solid-phase extraction cartridge and then washed by 4.00 mL of methanol in order to elimination the matrices. The analytes were eluted with 4.00 mL of 0.5 M ammonia in ethanol. The eluate was evaporated to dryness under nitrogen at 60°C and reconstituted with

100 µL of mobile phase prior to injection into the HILIC system. The analytical performance was successfully validated. The calibration curves with the coefficient of determination higher than 0.999 ranged from 1 to 400 mg L^{-1}, 0.5 to 200 mg L^{-1} and 3 to 3000 mg L^{-1} for Phe, Tyr and Cre, respectively. The proposed method provided satisfactory precision (%RSD < 5.58) and recoveries (88 – 108%). This method was applied to determine Phe, Tyr and Cre in human urine with various ages and genders.

Additionally, this proposed HILIC method was also applied to determine Phe and Tyr in dietary supplements that necessary for PKU or tyrosinemia patient. The homogeneous powders of the dietary supplement were accurately weighed of 0.0250 g and extracted with 25.00 mL of 0.1 M hydrochloric acid by vortex for 3 minutes. One milliliter of the extracted solution was diluted to 10.00 mL with acetonitrile prior to injection into the HILIC system. The validation of the proposed HILIC and sample extraction method was achieved. The calibration curves of Phe and Tyr ranged from 1 to 500 mg L^{-1} with the coefficient of determination at 0.9999. The proposed method was optimized to obtain satisfactory precision (%RSD < 1.76) and recoveries (95 – 102%). The developed method was applied to simultaneously determine Phe and Tyr in single and multi-nutrients dietary supplements.

Keywords: Hydrophilic interaction liquid chromatography, Phenylalanine, Tyrosine, Creatinine, Solid-phase extraction, Urine, Dietary supplement

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Woraphot Wanichalanant

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
ºC	Degree celsius
%RSD	Relative standard deviation percentage
µL	Microlitre
µm	Micrometre
µmol/day	Micromole per day
µmol L^{-1}, µM	Micromolar
AAs	Amino acids
Cre	Creatinine
Crn	Creatine
CSF	Cerebrospinal fluid
DAD	Diode array detection
DBS	Dried blood spot
Em	Emission
ESI	Electrospray ionization
et al.	Et Alli (Latin), and others
Ex	Excitation
FLD	Fluorescence detection
FMOC	9-fluorenylmethyl chloroformate
g L^{-1}	Gram per litre
HILIC	Hydrophilic interaction liquid chromatography

His	Histidine
HPLC	High performance liquid chromatography
I.S.	Internal standard
IC	Ion chromatography
LC	Liquid chromatography
LLE	Liquid-liquid extraction
M	Molar
Met	Methionine
mg	Miligram
mg dL^{-1}	Miligram per decilitre
mg L^{-1}	Miligram per litre
mg/24 hours	Miligram per 24 hours
min	Minute
mL	Millilitre
mL min^{-1}	Millilitre per minute
mmol L^{-1}, mM	Millimolar
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MΩcm^{-1}	Megaohms per centimetre
n.d.	Non-detectable
NDA	Naphthalenedicarboxaldehyde
NFPA	Nonafluoropentanoic acid

nm	Nanometre
NP-LC	Normal-phase liquid chromatography
OPA	*o*-phthalaldehyde
PDA	Photodiode array detection
Phe	Phenylalanine
PITC	Phenylisothiocyanate
PKU	Phenylketonuria
PS-DVB	Polystyrene-divinylbenzene
psi	Pound per square inch
RP-LC	Reversed-phase liquid chromatography
SCX	Strong cation exchange
SD	Standard deviation
SPE	Solid-phase extraction
TDFHA	Tridecafluoroheptanoic acid
TOF	Time-of-flight
Trp	Tryptophan
Tyr	Tyrosine
UHPLC	Ultra high performance liquid chromatography
UV	Ultraviolet
v/v	Volume by volume
Vis	Visible
w/w	Weight by weight

CHAPTER 1
INTRODUCTION

1.1 High performance liquid chromatography (HPLC)

The acronym high performance liquid chromatography or HPLC was created by the late Prof. Csaba Horváth for his paper in 1970. The original term of HPLC has indicated the fact that liquid chromatography required high pressure for generating the flow in packed columns. In the starting point, the capability of pumps only had a pressure of 500 psi. High pressure liquid chromatography or HPLC was termed by this component of the instrument. New HPLC instruments were developed a pressure capability up to 6,000 psi with improving columns, injectors and detectors in the early 1970s. HPLC literally started to take hold in the Mid-Late 1970s [1]. During this time, the performance of this instrument continued advances. However, the acronym HPLC withstood, the definition of HPLC was reformed to high performance liquid chromatography.

Nowadays, one of the most effective separation methods in analytical chemistry is HPLC because of its capability to separate, identify, and determine the analytes dissolving in the liquid sample. Nowadays, trace concentrations of the analytes can efficiently be determined. HPLC can be implemented to various samples, such as food, pharmaceuticals, dietary supplements, cosmetics, industrial chemicals, biological samples and environmental matrices.

The conventional HPLC technique is normal-phase liquid chromatography (NP-LC) or reversed-phase liquid chromatography (RP-LC). A stationary phase or functional group of NP-LC is polar more than a mobile phase. The more polarity of the mobile phase diminishes the more retention increases resulting in the analytes with the polar functional group are more retained than nonpolar analytes vice versa in RP-LC [2, 3]. In NP-LC, the solvent of the sample is a non-polar solvent. This technique is good for separating hydrophilic analytes. However, the mobile phase of NP-LC is a non-polar organic solvent which is higher costs for the purchase of mobile phase, difficulty

in controlling the strength of the mobile phase and non-environmentally friendly. Later, the RP-LC was developed to separate hydrophobic analytes. The mobile phase of RP-LC was comprised of an aqueous phase such as water and buffer with the polar organic solvent as methanol and acetonitrile which are more environmentally friendly and lower costs. However, the limitation of RP-LC is not appropriate for analyzing high polar compounds because they hardly retain on the hydrophobic stationary phase.

1.2 Hydrophilic interaction liquid chromatography (HILIC)

Hydrophilic interaction liquid chromatography (HILIC) is an alternative technique for separating small polar compounds. Previously, HILIC has been described as a variant of NP-LC, but the HILIC retention mechanism is greater convoluted than NP-LC. Alpert first proposed the acronym HILIC in 1990 [3], many publications on HILIC has extensively increased since 2003.

HILIC utilizes common stationary phases with a polar functional group such as silica, cyano or amino group like NP-LC, however, the composition of the mobile phase is based on the polar solvent relating to RP-LC. HILIC can analyze charged compounds, as in ion chromatography (IC) as shown in **Figure 1.1**.

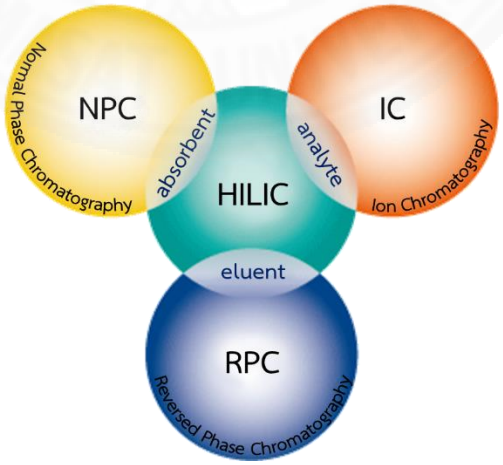

Figure 1.1 The peculiarities of the three major methods in LC combining to be HILIC [2].

The separation mechanism of analyte in HILIC has been proposed in many articles. There are fundamentally three possible approaches to model the mechanism [2]. First, the analyte partition between the polar stationary phase and the mobile phase; the second is the analyte adsorption onto the adsorbent surface; the third assumes the organic solvent adsorption onto the surface of the adsorbent, after that the analyte partitioning into the organic layer. The HILIC retention mechanism is based on different intermolecular forces of the solute with the functional group of the stationary phase, the stationary phase with the mobile phase and the solute with the mobile phase. The elution pattern was similar to non-aqueous NP-LC, therefore the mechanism must be similar too. When using especially low-water mobile phases, hydrogen bonding probably also associates with the retention mechanism in HILIC.

The advantages of HILIC overcome the conventional NP-LC and RP-LC. HILIC is applicable for the separation of polar compounds, which elute near the void in RP-LC and not require ion-pairing reagents. Polar samples can solute in the aqueous phase, that overcomes the NP-LC with the drawbacks of the low solubility. HILIC can easily couple to mass spectrometry (MS). Comparing with RP-LC, a gradient mode of HILIC starts with a small amount of solvent and increases the aqueous content to elutes polar analytes [4]. HILIC is a choice for the separation mode of high polar compounds, which hardly retained in RP-LC such as amphiphilic and uncharged highly hydrophilic compounds, but the charge of these compounds is not sufficient to retain by effective electrostatic interaction in ion-exchange chromatography.

According to the advantages of HILIC, it is appropriate for the separation of polar and hydrophilic compounds. Therefore, we interested in the HILIC method to simultaneous and rapid determine some amino acids in a various samples such as food, dietary supplements or biological samples.

1.3 Amino acids (AAs)

Amino acids are chemical compounds containing functional groups carboxyl (-COOH) and amine (-NH_2) groups with a side chain (R group) which is specified to the particular amino acid. Amino acids are not only the proteins subunit, but also

serve as precursors of many compounds and a source of energy [5]. Many important amino acids have their biological functions. For example, phenylalanine and tyrosine is a precursor for the monoamine neurotransmitters epinephrine (adrenaline), norepinephrine (noradrenaline), dopamine, and the skin pigment melanin.

Phenylalanine (Phe)

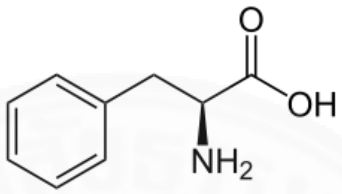

Figure 1.2 The structure of L-Phenylalanine.

L-Phenylalanine is a crucial aromatic amino acid. It is a precursor for amino acid tyrosine and also a precursor for important neurotransmitters such as catecholamine, epinephrine, norepinephrine, tyramine and dopamine. Catecholamine act as adrenalin-like substances. In the human brain and plasma, phenylalanine is highly concentrated. Large amounts of phenylalanine are in many foods with high protein such as wheat, cheese and meat. Phenylalanine is also an ingredient in several psychoactive drugs and dietary supplements. Moreover, the dietary sources of phenylalanine can be found in sugar substitutes such as artificial sweeteners namely aspartame. Therefore, phenylketonurics and pregnant women should avoid aspartame sweetener [6].

Tyrosine (Tyr)

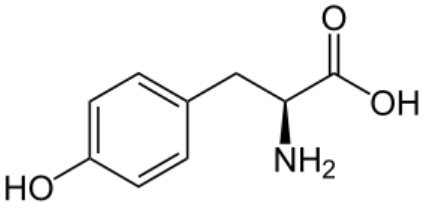

Figure 1.3 The structure of L-Tyrosine.

L-Tyrosine is a crucial aromatic amino acid. It is a precursor for the neurotransmitters in the brain such as epinephrine, norepinephrine and dopamine which are an important chemical of the sympathetic nervous system [7]. The concentrations of these neurotransmitters in the brain and body are precisely related to tyrosine digestion. Tyrosine in the body is found in low concentrations, probably because of rapid metabolism. Tyrosine is also the precursor for hormones, thyroid, catecholestrogens and melanin.

Determination of amino acids is required in many fields such as biomedical research, food science and clinical diagnostics of inborn error metabolism.

1.4 Phenylketonuria (PKU)

Phenylketonuria or PKU is an autosomal recessive disorder caused by an inadequacy of the enzyme namely phenylalanine hydroxylase that results in decreased metabolism of the essential amino acid phenylalanine (Phe) to tyrosine (Tyr) [8].

1.4.1 Phenylketonuria disease

The genotype and the symptoms of the phenylketonuria disease depend on the deficiency of the enzyme as shown in **Figure 1.4**, the different classifications for PKU phenotypes have been set up from different clinical phenotypes [9]. Classification of PKU can be divided into classic and variant PKU including all milder forms of PKU [10] as shown in **Table 1.2**. The PKU definition may be important in providing medical care.

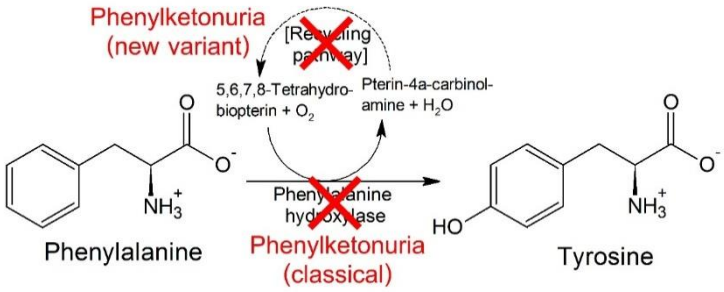

Figure 1.4 The metabolism of the phenylalanine (Phe) to tyrosine (Tyr) [11].

PKU causes severe intellectual impairment. Within the first year of life, developmental delay is obvious and develops to severe intellectual disability (IQ < 50) [12]. Untreated PKU can lead to seizures, mental disorders and behavioral problems such as offensive, tension, short attention and social withdrawal. Other affections may comprise hypopigmentation of the skin, iris and hair because of the reduction of melanin synthesis. The selective restriction of phenylalanine consumption as long as phenylketonurics provide enough tyrosine and nutrients with an additional protein to help regular growth is an approach for treating this disease.

1.4.2 Phenylketonuria treatment

The control of phenylalanine in blood to a normal level for brain development is the goal of PKU treatment. A blood phenylalanine concentration involved dietary consumption of protein and also the remaining of phenylalanine hydroxylase activity. It occasionally has a possibility to increase phenylalanine hydroxylase activity. However, treatment mostly depends on the reduction of phenylalanine intake. Milk, cheese, bread, fish and meat must be refrained [12]. The use of a semi-synthetic and phenylalanine-reduced diet is recommended that consist of:

- Low phenylalanine concentration diet such as vegetables and fruits;
- Weighing quantities of food contained a medium concentration of phenylalanine such as potato and broccoli. The quantity of phenylalanine consumed can be determined by the UK exchange system. This system, 1 exchange equals about 1 g of protein or 50 mg of phenylalanine;

• Amino acid mixtures without phenylalanine for normal total protein consume;

• Minerals, vitamins and trace elements.

Aspartame, the artificial sweetener for dietary food, must be avoided because of the large phenylalanine quantity. Infant formula feeds containing essential fatty acids which are phenylalanine-free are available. Continue breastfeeding is possible even in severe PKU by providing a precise quantity of formula with phenylalanine-free. Therefore, a method for the determination of phenylalanine in food, infant formula, pharmaceutical product and dietary supplement is necessary.

1.4.3 Phenylketonuria diagnosis

Phenylketonuria diagnosis is important for PKU treatment. PKU screening must be done within the days after birth of all infants so as to allow dietary intrusion to protect phenuketonurics from neurological damage. The screening is performed by maternity wards, the blood sample is generally collected between days 2 – 5; The general screening is generally accomplished between 2 – 7 days [9]. The normal range concentration of phenylalanine in blood and urine classified by age as shown in **Table 1.1**.

Table 1.1 The normal phenylalanine concentration in blood and urine [13].

Age	The normal concentration of phenylalanine	
	Blood	Urine
Infants (2 – 5 days)	< 2 mg dL^{-1} (< 120 µmol L^{-1})	
Normal people		5 – 10 mg dL^{-1} 302 – 605 µmol L^{-1} (Negative dipstick)

Table 1.1 The normal phenylalanine concentration in blood and urine [13] (cont.).

Age	The normal concentration of phenylalanine	
	Blood	Urine
		24-hour urine
Infants (to 7 weeks after birth)		1.2 – 1.7 mg/24 hours (7.2 – 10.3 µmol/day)
Children (3 – 12 years old)		4.0 – 17.5 mg/24 hours (24 – 106 µmol/day)
Adults		< 16.5 mg/24 hours (< 100 µmol/day)

PKU may be classified into four different phenotypes and presented by phenylalanine pretreatment levels as shown in **Table 1.2**.

Table 1.2 Classification of phenylketonuria and its variants [14].

Disorder	Blood phenylalanine pretreatment levels on an unrestricted Diet (Units)	
	mg dL^{-1}	µmol L^{-1}
Classical phenylketonuria	> 20	> 1200
Atypical/mild phenylketonuria	10 – 20	600 – 1200
Non-phenylketonuria or mild hyperphenylalaninemia (HPA)	3 – 10	200 – 600
Biopterin deficiency variants	Variable	

PKU can be classified into four subtypes based on phenylalanine pretreatment concentration in blood. PKU is generally correlated with a high chance

of cognitive impairment; Nevertheless, non-PKU or mild HPA is correlated with a lower chance of cognitive impairment [8].

1.4.4 Phenylketonuria screening method in blood

The screening method is made in newborn. The screening is carried out by measurement of increased blood phenylalanine on the newborn heel-prick blood test called the Guthrie test. It was implemented by the inhibition of bacteria in a minimal culture medium by phenylalanine in blood [15]. In order to decrease the number of false negatives (missed patients with PKU), current screening methods measure blood phenylalanine concentration using 4 mg dL^{-1} as a cut off value as shown in **Figure 1.5**.

However, more than 90% of infants with phenylalanine concentration values over 4 mg dL^{-1} were shown to be false positive [16].

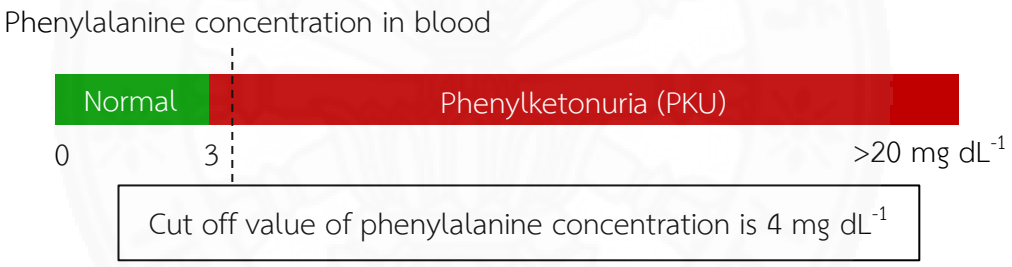

Figure 1.5 The cut off value of phenylalanine concentration in blood for PKU screening.

Although the risk for missing a case can be minimized by the cut off value of phenylalanine concentration, the expense of specificity was required. The determination of Phe and Tyr in order to calculate the phenylalanine to tyrosine ratio (Phe/Tyr ratio) and use of this ratio as a cut off value can support to optimize the clinical specificity and sensitivity of the diagnosis test. Previously, the Phe to Tyr ratio has been used to diagnose phenylketonuric, differentiate between PKU and non-PKU hyperphenylalaninemia [17]. Therefore, the simultaneous determination of phenylalanine and tyrosine concentrations in blood and calculating the Phe/Tyr ratio can reduce the false positive rate in PKU screening [18].

However, Phe determination is generally carried out in blood, the urinary analysis is favored by chemists and adults because urinary analysis is non-invasive method and urine has a high amount of Phe than blood (the range of Phe concentration in urine is 20 to 60 mmol L^{-1} for phenylketonurics compared to 0.6 to 3.8 mmol L^{-1} in blood) [19].

In addition, M. Boulos et al. and U. Langenbeck et al. [20, 21] have been proposed that phenylalanine concentration in blood can be predicted from phenylalanine and creatinine concentration in urine if the patient's age is considered. They evaluate the feasibility of the non-invasive method. Their observation could open the new approach to a diagnosis of phenylketonurics in which painful frequent blood sampling could be replaced by urine testing.

1.5 Phenylalanine and tyrosine in urine

Amino acids about 70 grams are daily filtered by the kidneys and reabsorbed in the proximal renal tubules. Only about three grams of them are regularly excreted, approximately one - third each as small peptides, conjugates of compounds such as benzoic acid and free amino acids. The excretion of free amino acids can be indicated an inborn error of metabolism [22]. As in phenylketonuria, the excretion of significantly larger amounts of free phenylalanine and less amounts of tyrosine in urine could indicate this disease.

The criteria of PKU diagnosis test such as the concentration of phenylalanine and Phe/Tyr ratio was not yet reported in urine. The phenylalanine and tyrosine concentration including Phe/Tyr ratio was surveyed as shown in **Table 1.3**.

Table 1.3 The concentration of phenylalanine and tyrosine in urine and Phe/Tyr ratio.

Author (Year, Country)	Normal			Phenylketonuric		
	Phe concentration (mg L^{-1})	Tyr concentration (mg L^{-1})	Phe/Tyr ratio	Phe concentration (mg L^{-1})	Tyr concentration (mg L^{-1})	Phe/Tyr ratio[a]
Y. Yokoyama et al. (2015, Japan) [23]	4.96 – 26.43	-[b]	-	52.86 – 300.65	-	-
Y. Yokoyama et al. (2014, Japan) [24]	1.49 – 16.02	0.72 – 18.12	0.795 – 4.00	29.73 – 133.80	2.54 – 11.96	6.67 – 41.67
Y. Yokoyama et al. (2005, Japan) [25]	1.65 – 19.00	n.d.	-	32.05 – 159.08	n.d.	-
Y. Yokoyama et al. (2005, Japan) [26]	2.97 – 12.55	4.89 – 34.43	0.22 – 2.08	29.24 – 177.91	8.15 – 37.51	3.30 – 6.65
Y. Yokoyama et al. (1996, Japan) [27]	n.d.	n.d.	n.d.[c]	27.75 – 180.88	n.d. – 5.25	20.73 – 37.76
Y. Yokoyama et al. (1991, Japan) [28]	1.98 – 10.74	1.99 – 18.30	0.54 – 1.73	27.92 – 176.92	2.17 – 3.08	14.08 – 63.00

[a] calculated by this work; [b] - is not reported; [c] n.d. is non-detectable

1.6 Urinary creatinine

Creatinine (presented the structure in **Figure 1.6**) is the waste product from metabolic pathway associating with the conversion of creatine and muscle creatine phosphate, essential molecules for the energy transfer.

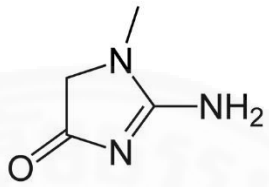

Figure 1.6 The structure of creatinine.

Excretion of creatinine into urine is occurred by kidneys. The excretion of creatinine is approximately constant under normal conditions, the production quantity of creatinine was proportionate to the personal muscle mass. The diagnosis of muscular dysfunction or renal diseases is done by the determination of urinary creatinine which normally indicated as the clearance of the creatinine. In addition, the concentration of urinary creatinine is used as a tool of standardization for the determination of xenobiotics and drugs concentration in urine, so as to prevent the diversity of physiology in urine water content [29]. The creatinine ratio, providing by the correction of the concentration of the analyte with the urinary creatinine concentration, is required for the determination of biological markers concentration in random or spot urine [25]. The normal concentration of creatinine in urine is in the ranges of 2.5 to 23 mM (280 to 2590 mg L^{-1}) as shown in **Table 1.4**, while the forensic toxicology reports that the concentration of creatinine below 0.45 mM (50 mg L^{-1}) was not proper for the analysis [29].

Table 1.4 The normal concentration of creatinine in urine.

Gender	Cre concentration (mg L^{-1})
Male	390 – 2590
Female	280 – 2170

The analytical method for the quantitation of amino acid in urine is carried out by collecting the urine over 24 hours. The amino acid analysis by 24 hours urine collection is an inconvenient task because of many errors including incomplete collections, incorrect timings, bladder emptying and bacterial growth and also the requirement of hospital admission and inconvenience, especially for repeated follow up. As creatinine in urine is constantly excreted and its concentration varies with hydration status. Random or spot urine sample collection is a simple procedure and can be done at any time of the day [30].

Therefore, the analytical method for the simultaneous determination of phenylalanine, tyrosine and creatinine in urine is required. According to the analytes are polar and very water-soluble compounds. In this work, we interested in HILIC technique to simultaneous and rapid determine the analytes in urine for diagnosis and monitoring of PKU. However, urine is a complicated sample, some sample clean-up and preconcentration steps are required.

1.7 Solid-phase extraction (SPE)

Solid-phase extraction (SPE) is a sample preparation technique which commonly used for cleaning matrix complexities and preconcentrating analytes before quantitative analysis [31]. The advantages of SPE over other sample preparation techniques such as liquid-liquid extraction and precipitation are simple and faster procedure, better recoveries, higher enrichment factors, fast phase separation, low cost and the low consumption of organic solvents [32].

A common solid-phase extraction as shown in **Figure 1.7** comprises four processes [33]:

1. Conditioned (equilibrated) the cartridges with a solvent
2. Loaded the sample solution through the SPE sorbent. The analytes and some matrices are absorbed on the SPE sorbent.
3. Washed the SPE to eliminate matrices.
4. Eluted the analytes by eluting solvent and then collected the solution in this step.

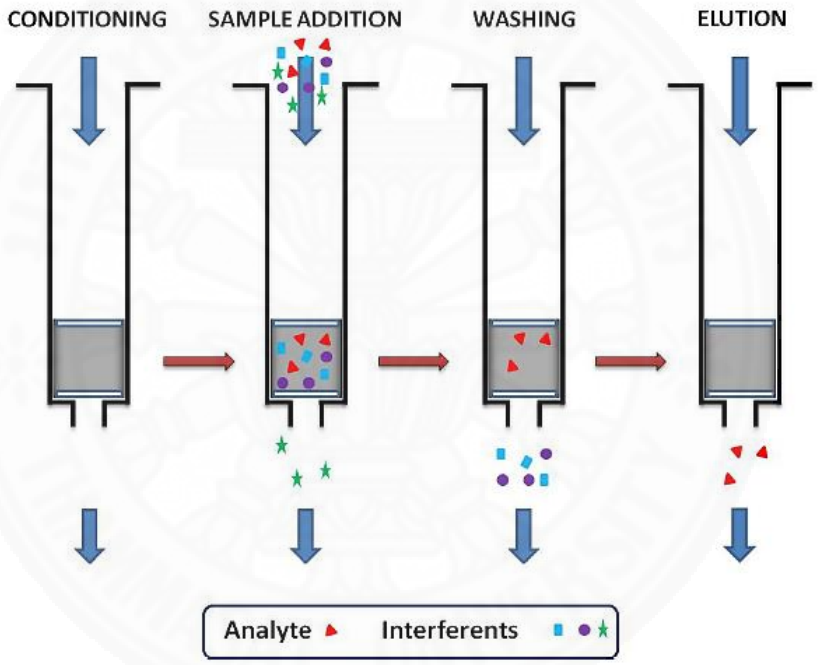

Figure 1.7 Schematic representation of SPE clean-up procedure [34].

Since the analytes in this work; phenylalanine, tyrosine and creatinine, are polar compound and their structures can be protonated in acidic condition, a type of SPE sorbent would be strong-cation exchange (SCX) using for strongly acidic cation exchanger, ion exchanger of organic compounds from aqueous matrix [35].

Due to its effective extraction of the positively charged compounds, the strong-cation exchange solid-phase extraction (SCX-SPE) is suitable for the extraction of phenylalanine, tyrosine and creatinine from urine samples.

CHAPTER 2
REVIEW OF LITERATURE

This chapter was divided to three parts. The first part presents a literature review of the analytical method of phenylalanine and tyrosine in blood (plasma, serum or dried blood spot) for the diagnosis and monitoring of phenylketonuria (PKU) and other inherited metabolic disorders. The second part presents the analytical methods for the determination of phenylalanine, tyrosine and creatinine with the sample preparation in urine samples and the last part presents the analytical methods for the determination of phenylalanine and tyrosine in dietary supplements. The review of literature emphasized on HPLC methods for the chromatographic determination of phenylalanine, tyrosine and creatinine in urine.

2.1 The analytical methods for determination of phenylalanine and tyrosine in blood

Quantitative analysis of phenylalanine and tyrosine in blood is necessary for the diagnosis of PKU and the evaluation of treatment. Phenylalanine and tyrosine were determined and calculated the ratio of Phe/Tyr to decrease the false positive results of PKU diagnosis. Currently, HPLC method contributes significantly in the determination of phenylalanine and tyrosine. Many developed HPLC methods are employed to the simultaneous determination of phenylalanine and tyrosine in blood (plasma, serum or dried blood spot) sample.

The first efficient screening method of PKU was a semi-quantitative bacterial inhibition assay proposed by Robert Guthrie [36]. The test was based on the inhibition of growth of *Bacillus subtilis* ATCC6051 by β-2-thienylalanine in a minimal culture medium, which is prevented by phenylalanine. The Guthrie test is advantageous for screening PKU as the dried blood spot (DBS) obtained in the hospital using a standardized filter paper, called Guthrie card, and sent to the laboratories by mail [9]. However, the Guthrie test is only the screening method and evenly replaced

by advanced techniques such as chromatography which can detect and monitor various inborn diseases.

Many methods for the determination of phenylalanine, tyrosine or several amino acids in blood such as a colorimetry and fluorimetry [37, 38], enzymatic methods [39, 40], gas chromatography [41], capillary electrophoresis [42, 43] and tandem mass spectrometry [44-47] have been proposed; however, those techniques were required derivatization, sophisticated or long-time sample preparation, lack of reproducibility and high-cost analysis.

In this work, we focus on high performance liquid chromatographic (HPLC) or ultra-high performance liquid chromatographic method (UHPLC) because the HPLC and UHPLC method is simple, fast, precise and accurate method which has been utilized for the analysis of phenylalanine and tyrosine and/or creatinine in biological samples such as urine and blood.

2.1.1 The analytical methods for simultaneous determination of phenylalanine and tyrosine in blood samples for the diagnosis and monitoring of PKU

HPLC or UHPLC methods coupled with various detections including ultraviolet detection (UV), photodiode array or diode array detection (PDA or DAD), fluorescence detection (FLD) have been developed for the determination of phenylalanine (Phe) and tyrosine (Tyr) with or without derivatization in blood samples as peripheral capillary blood, dried blood spot (DBS), plasma and serum for the diagnosis and monitoring of PKU as shown in **Table 2.1**.

Table 2.1 Literature review of the analytical methods for simultaneous determination of Phe and Tyr in blood samples for the diagnosis and monitoring of PKU.

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
X. Mo, et al. (2013) [18]	Phe and Tyr	Peripheral capillary blood	-	Hypersil C8 column (6.0 × 300 mm, 10 μm)	HPLC-UV (210 nm)	10	Phe 1.5 Tyr 1.0	Phe 6 – 1512 Tyr 5.5 – 1250	Phe 98.2 Tyr 98.8
F.C. Ladaşiu Ciolacu, et al. (2015) [48]	Phe and Tyr	Plasma	-	Acclaim 120, C18 column, (4.6 x 250 mm, 5 μm)	HPLC-PDA (210 nm)	7	-	Phe 109 – 1834.4 Tyr 42 – 304	-
F. Haghighi, et al. (2015) [49]	Phe and Tyr	Dried blood spot (DBS) and serum	-	Chromolith® RP-18 column (4.6 × 100 mm)	HPLC-PDA (210 nm)	6	Phe 0.04 Tyr 0.04	0.1 – 3200	Phe 92.5 – 103.9 Tyr 88.2 – 104.2

Table 2.1 Literature review of the analytical methods for simultaneous determination of Phe and Tyr in blood samples for the diagnosis and monitoring of PKU (cont.).

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
R. Kand'ár, et al. (2009) [50]	Phe and Tyr	Plasma and dried blood spot (DBS)	-	LiChroCART, RP-18e Purosphere STAR column (4.0 x 125 mm)	HPLC-FLD (Ex: 260 nm, Em: 282 nm)	10	Phe 10 Tyr 5	Phe 10 – 1500 Tyr 5 – 750	Phe 92.0 – 102.8 Tyr 92.6 – 102.9
G. Neurauter, et al. (2013) [51]	Phe and Tyr	Serum and CSF	-	LiChroCART, RP_{18}Purosphere STAR column (4.0 x 55 mm, 3 μm)	HPLC-FLD (Ex: 210 nm, Em: 302 nm)	7	Phe 0.3 Tyr 0.3	Phe 1.25 – 200 Tyr 1.25 – 80	-

Ex, excitation; Em, emission; CSF, cerebrospinal fluid.

Table 2.1 Literature review of the analytical methods for simultaneous determination of Phe and Tyr in blood samples for the diagnosis and monitoring of PKU (cont.).

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
Y. Dale, et al. (2003) [52]	Phe and Tyr	Plasma and dried blood spot (DBS)	Phenylisothio cyanate (PITC)	Reversed-phase C-18 column (4.6 x 150 mm)	HPLC-UV (254 nm)	11	Phe 10 Tyr 10	625 – 10000	Phe 95 and 98 Tyr 93 and 104
P. Allard, et al. (2004) [53]	Phe and Tyr	Dried blood spot (DBS) and plasma	Ninhydrin	Cation-exchange column (4.6 x 60 mm, 3 μm)	HPLC-Vis (440 and 570 nm)	8	-	1 – 3000	Phe 91 ± 4 Tyr 81 ± 5
R. Pecce, et al. (2013) [54]	Phe and Tyr	Dried blood spot (DBS)	OPA	Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm)	HPLC-FLD (Ex: 340 nm, Em 450 nm)	20	Phe 0.1 Tyr 0.5	12 – 1200	Phe 93 – 101 Tyr 92 – 103

Vis, Visible; OPA, *o*-phthalaldehyde.

As shown in **Table 2.1**, HPLC coupled with UV detector or fluorescence detector was applied for the simultaneous analysis of Phe and Tyr in blood for diagnosis and monitoring of PKU. Moreover, the determination of phenylalanine and tyrosine in blood are usually measured by HPLC or UHPLC methods for total amino acids.

2.1.2 The analytical methods for simultaneous determination of phenylalanine and tyrosine with other amino acids in blood samples

Many HPLC or UHPLC methods was developed for the determination of total amino acids (Phe, Tyr and others) in biological sample such as blood and urine. Disturbances in amino acids concentrations are not only related to inborn errors of metabolism, but amino acids can also serve as biomarkers to evaluate intake and nutritional status.

There are many methods for amino acids analysis by HPLC or UHPLC with various detections including ultraviolet detection (UV), photodiode array or diode array detection (PDA or DAD), fluorescence detection (FLD), mass spectrometry (MS) and tandem mass spectrometry (MS/MS) that can be commonly divided into methods with derivatized analysis and methods without derivatization as shown in **Table 2.2.**

Table 2.2 Literature review of the analytical methods for simultaneous determination of Phe and Tyr with other amino acids in blood samples.

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
S. Sedehi, et al. (2018) [55]	Phe and Tyr	Plasma and fruit juices	-	YMC column (250 × 4.6 mm, 5 μm)	HPLC-UV (210 nm)	14	Phe 0.04 Tyr 0.04	0.15 - 6	53.9 – 84.0
M. Zoppa, et al. (2006) [56]	Phe, Tyr (total 40 AAs/isomer), Cre	Dried blood spot (DBS)	-	Discovery C18 column (2.1 × 50 mm, 5 μm)	HPLC–ESI–MS/MS	10	-	Phe 0 – 800 Tyr 0 – 400 Cre 0 – 300	-

AAs, amino acids; ESI, Electrospray ionization.

Table 2.2 Literature review of the analytical methods for simultaneous determination of Phe and Tyr with other amino acids in blood samples (cont.).

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
M. Armstrong, et al. (2007) [57]	Phe, Tyr (total 25 AAs)	Plasma	-	Agilent XDB-C18 column (2.1 x 50 mm, 1.8 μm) with TDFHA as ion-pairing agents	HPLC-TOF-MS	32	-	1.56 – 400	Phe 109.41 Tyr 81.58
M. Olkowicz, et al. (2017) [58]	Phe, Tyr (total 43 AAs)	Plasma	-	Synergi Hydro-RP column (50 × 2.0 mm, 2.5 μm) with NFPA as ion-pairing agents	HPLC-MS/MS	10	Phe 0.01 Tyr 0.05	0.5 – 1000	Phe 99.5 – 104.3 Tyr 97.4 – 98.8

TDFHA, tridecafluoroheptanoic acid; TOF, time-of-flight; NFPA, nonafluoropentanoic acid.

Table 2.2 Literature review of the analytical methods for simultaneous determination of Phe and Tyr with other amino acids in blood samples (cont.).

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
J. Kim, et al. (2017) [59]	Phe, Tyr (total 5 AAs)	Blood	-	CAPCELL CORE ADME column (2.1 x 150 mm, 2.7 μm) with TFA as ion-pairing agents	UPLC–MS/MS	4	Phe 0.002 Tyr 0.011	1 – 50	Phe 99.1 – 102.9 Tyr 100.2 – 101.6
E.L. Schwarz, et al. (2005) [60]	Phe, Tyr (total 23 AAs)	Plasma	OPA for UV and FMOC for FLD	Zorbax Eclipse C18 column AAA (4.6 x 150 mm, 3.5 μm)	HPLC-PDA (338 nm) HPLC-FLD (Ex: 266 nm, Em: 305 nm)	30	-	5 – 2500	-
S.B. Narayan, et al. (2011) [61]	Phe, Tyr (total 31 AAs)	Plasma	AccQTag®	MassTrak AAA Column (2.1 x 150 mm, 1.7 μm)	UPLC-UV (260 nm)	45	Phe 1 Tyr 1	2 – 3500	-

FMOC, 9-fluorenylmethyl chloroformate.

Table 2.2 Literature review of the analytical methods for simultaneous determination of Phe and Tyr with other amino acids in blood samples (cont.).

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
L. Palego, et al. (2012) [62]	Phe, Tyr (total 23 AAs)	Plasma	Phenylisothiocyanate (PITC)	PicoTag C_{18} Column (3.9 x 300 mm), 4 μm	HPLC-PDA (254, 283 nm)	80	-	25 - 400	-
M.P. Frank, et al. (2007) [63]	Phe, Tyr (total 22 AAs)	Plasma	OPA	XTerra RP18 column (4.6 x 100 mm, 5 μm)	HPLC-FLD (Ex: 340 nm, Em 455 nm)	35	-	5 – 1000	Phe 101 Tyr 102
R. Kandar, et al. (2016) [64]	Phe, Tyr (total 21 AAs)	Peripheral venous blood	NDA	Discovery® Bio Wide Pore C18 (4.6 x 150 mm, 5 μm)	HPLC-FLD (Ex: 350 nm, Em 420 nm)	45	Phe 1.2 Tyr 1.1	Phe 3.6 – 148.0 Tyr 3.5 – 139.7	Phe 102.4 Tyr 103.2

NDA, naphthalenedicarboxaldehyde.

Table 2.2 Literature review of the analytical methods for simultaneous determination of Phe and Tyr with other amino acids in blood samples (cont.).

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
J. Ni, et al. (2016) [65]	Phe, Tyr (total 13 AAs)	Serum	N-butyl alcohol–acetyl chloride	Atlantis dC_{18} column (2.1 × 150 mm, 5 μm)	HPLC-MS/MS	14	Phe 0.015 Tyr 0.022	Phe 1 – 256 Tyr 1.25 – 320	Phe 97.1 – 106.2 Tyr 95.7 – 105.2
L. Sun, et al. (2015) [66]	Phe, Tyr and 3 others	Serum	-	Syncronis HILIC column (2.1 x 150 mm, 5 μm)	HILIC-MS/MS (QTRAP)	11	Phe 1.2 Tyr 1.1	Phe 1.2 – 605 Tyr 1.1 – 552	Phe 93.9 – 104.7 Tyr 91.4 – 101.2
H.C.M.T. Prinsen, et al. (2016) [67]	Phe and Tyr (total 36 AAs)	Plasma	-	Acquity BEH Amide column (2.1 × 100 mm, 1.7 μm)	HILIC-ESI-MS/MS (triple quadrupole)	18	-	Phe 0 – 250 Tyr 0.1 – 250	-

As shown in **Table 2.1 and 2.2**, there are many HPLC with any detection methods for the analysis of Phe, Tyr and other amino acids. In order to achieve proper chromatographic separation and sensitivity for a reversed phase HPLC, pre- or post-column derivatization of amino acids was required. Various analytical problems such as an instability of derivatives, interference from a derivatizing reagent, long analysis time, long preparation time and relatively high cost per sample was suffered from all the existing derivatization methods. Therefore, the analytical method without derivatization step eliminates these problems. Since amino acids are high polar and zwitterionic compounds, the retentions on a normal C18 column were almost not chanced. The usage of monolithic column, more polar reversed phase column or the introduction of ion-pairing agents were employed to improve the retention and separation of amino acids. Complex derivatization methods were no longer required and good separation of analytes was achieved.

Recently, the chromatographic method coupled with mass spectrometry for the determination of free amino acids has gained great progress because hydrophilic interaction liquid chromatography (HILIC) method can be used for selective separation of the polar compounds without any derivatization steps [66, 67].

The determination of phenylalanine and tyrosine in urine is preferred than blood because it is non-invasive method and simple sample collection, especially for infants [68]. However, to avoid the 24-hour sampling of the urine sample, creatinine must also be determined for monitoring the concentration of the analytes in the urine. Normalization of urine creatinine is a commonly applied approach to control for deviations in flow rate of urine, assuming the creatinine in urine excretion is constant across and within individuals [69]. The analytical methods for the determination of phenylalanine, tyrosine and creatinine in urine was presented in the following section.

2.2 The analytical methods for determination of phenylalanine, tyrosine and creatinine in urine

This research focused on the determination of phenylalanine, tyrosine in urine collected by spot urine collection, therefore the determination of creatinine was also determined for the correction of the analyte concentration with the urinary creatinine concentration. Generally, HPLC methods coupled with various detections have been applied for the determination of phenylalanine, tyrosine and other amino acids in urine with the usage of Jaffe method for the determination of creatinine.

2.2.1 The analysis of phenylalanine and tyrosine by chromatographic method and creatinine by colorimetric method in urine

The general method for the determination of creatinine in biological fluids is the Jaffe method (colorimetry). This reaction was firstly proposed by Max Jaffe in 1886 [70]. Jaffe observed that a red color formed when creatinine reacted with picric acid in an alkaline medium. This method may have few problems; some compounds in blood and urine, for example, sugars, urea, uric acid, pyruvate, dopamine, acetone, acetoacetic acid, fructose, glucose, ascorbate, cefoxitin, cephalotin, cefatril and cefazolin interfered with this technique [71]. As shown in **Table 2.3**, some researchers determined phenylalanine, tyrosine and other amino acids by the chromatographic method and used the Jaffe method or other colorimetric methods for the determination of creatinine.

Table 2.3 Literature review of the analytical methods for the determination of Phe and Tyr by chromatographic method and Cre by colorimetric method in urine samples.

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
M. Piraud, et al. (2005) [72]	Phe, Tyr (total 76 AAs)	Urine and plasma	-	Modulo-cart QS UptiSphere C18 (2 × 50 mm, 3 μm) with perfluorinated carboxylic acid as ion-pairing agents	HPLC-ESI-MS/MS	20	Phe 0.1 Tyr 0.6	0 - 500	Phe 104 – 110 Tyr 93 – 103
	Cre	Urine			Jaffe method				

Table 2.3 Literature review of the analytical methods for the determination of Phe and Tyr by chromatographic method and Cre by colorimetric method in urine samples (cont.).

Author (Year)	Analytes	Sample	Derivatizing reagent	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
R. Joyce, et al. (2016) [73]	Phe, Tyr (total 18 AAs)	Urine	-	HILIC ACQUITY UPLC BEH amide column (2.1 × 100 mm, 1.7 μm)	HILIC-UPLC-qTOF-MS	18	Phe 0.0121 Tyr 0.0213	Phe 0.1 – 12 Tyr 0.25 – 30	-
	Cre	Urine	Jaffe method						
G. Giordano, et al. (2012) [74]	Phe, Tyr (total 40 AAs/isomer)	Urine, blood and DBS	-	Discovery C 18 (2.1 × 50 mm, 5 μm)	HPLC-ESI-MS/MS	10	-	Phe 0 – 600 Tyr 0 – 500 Cre 0 – 300	-
	Cre	Urine	Colorimetric method (using 3,5-dinitrobenzoic acid)						

It would be convenient to simultaneous determination of phenylalanine, tyrosine and creatinine in urine for the high throughput analysis resulting in fast and accurate monitoring of phenylalanine and tyrosine level. The chromatographic method for the simultaneous determination of phenylalanine, tyrosine and creatinine was presented in the next section.

2.2.2 The analytical methods for simultaneous determination of phenylalanine, tyrosine and creatinine in urine

In 1991, Y. Yokoyama and research group [28] proposed the analytical method for the simultaneous analysis of amino acids (phenylalanine, tyrosine and others) and creatinine in urine by cation exchange chromatography with UV detection. The preparative low capacity cation exchange resin column was used for the separation of amino acids and creatinine in urine. The analytes were simultaneously monitored at 210 nm. The method was applied to the analysis in the urine samples from patients with an inborn error metabolism of amino acid, such as PKU. The ratios of phenylalanine to creatinine concentration in the urine of patients were determined correctly and were differed from the healthy people's urine. In addition, Y. Yokoyama, et al. have developed the analytical methods using cation-exchange column for the simultaneous determination of amino acids and creatinine for many years as shown in **Table 2.4**.

Table 2.4 Literature review of the analytical methods for the simultaneous determination of Phe, Tyr and Cre in urine samples.

Author (Year)	Analytes	Sample	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
Y. Yokoyama, et al. (1991) [28]	Phe, Tyr, Cre and Trp	Urine	Column (4.6 x 100 mm) packed with a cation-exchange resin (sulphonated PS-DVB, 4% DVB)	HPLC-UV (210 nm)	25	Cre 1	5 – 200	Phe 96.2 – 98.7 Cre 98.3 – 101.1
Y. Yokoyama, et al. (1992) [75]	Phe, Tyr, Cre and other 8 compounds (AAs and organic acids)	Urine	Inertsil ODS-2 (4.6 x 250 mm, 5 μm)	HPLC-UV (210 nm)	70	-	2 – 500	Phe 98.3 Tyr 99.4

Trp, tryptophan; PS-DVB, polystyrene-divinylbenzene; DVB, divinylbenzene.

Table 2.4 Literature review of the analytical methods for the simultaneous determination of Phe, Tyr and Cre in urine samples (cont.).

Author (Year)	Analytes	Sample	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
Y. Yokoyama, et al. (1996) [27]	Phe, Tyr, Cre, Crn, His and Trp	Urine	Inertsil C4 (4.6 x 150 mm, 5 μm) with sodium dodecyl sulfate (SDS) as ion-pairing agents	HPLC-UV (210 nm) and HPLC-FLD post-column derivatized by OPA (Ex: 340 nm, Em: 430 nm)	80	-	UV: 1 – 500 FLD: 0.05 - 15	-
Y. Yokoyama, et al. (2000) [76]	Phe, Tyr, Cre and other 7 compounds (AAs and organic acids)	Urine	CAPCELL PAK C_{18} UG80 (4.6 x 150 mm, 5 μm) coated withhexadecylsulfonate	HPLC-UV (254 nm)	60	Phe 0.1 Tyr 0.1 Cre 0.02	0.5 – 2000	97 – 104

AAs, amino acids; Crn, creatine; His, histidine.

Table 2.4 Literature review of the analytical methods for the simultaneous determination of Phe, Tyr and Cre in urine samples (cont.).

Author (Year)	Analytes	Sample	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
Y. Yokoyama, et al. (2004) [77]	Phe and Tyr and other 14 AAs	Urine	Self-packed column (4.6 × 150 mm, low-capacity sulfoacylated macroreticular PS-DVB)	HPLC-FLD post-column derivatized by OPA (Ex: 340 nm, Em: 430 nm)	70	-	0.1 – 10	-
Y. Yokoyama, et al. (2005) [25]	Phe, Tyr, Cre, Crn, His and Trp	Urine	Self-packed column (4.6 × 150 mm x 5 μm, low-capacity cation exchange resin, sulfoacylated macro-porous PS-DVB)	HPLC-UV (210 nm)	25	-	2 – 200	-

Table 2.4 Literature review of the analytical methods for the simultaneous determination of Phe, Tyr and Cre in urine samples (cont.).

Author (Year)	Analytes	Sample	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
Y. Yokoyama, et al. (2005) [26]	Phe, Tyr, Cre, Crn, Met, His and Trp	Urine	Self-packed column (4.6 × 150 mm x 5 μm, low-capacity cation exchange resin, sulfoacylated macro-porous PS-DVB)	HPLC-UV (210 nm)	27	Phe 0.02 Tyr 0.02 Cre 0.02	1 – 1000	Almost 100
Y. Yokoyama, et al. (2013) [78]	Phe and Tyr and other 18 AAs	Urine	Column (4.6 x 70 mm) packed with a sulfoacylating a highly crosslinked macroreticular PS-DVB copolymer (3 μm)	HPLC-UV (210 nm) and HPLC-FLD post-column derivatized by OPA (Ex: 340 nm, Em: 430 nm)	25	Phe 0.005 Tyr 0.006	FLD: 0.05 – 20	-

Met, methionine.

Table 2.4 Literature review of the analytical methods for the simultaneous determination of Phe, Tyr and Cre in urine samples (cont.).

Author (Year)	Analytes	Sample	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
Y. Yokoyama, et al. (2014) [24]	Phe, Tyr, Cre, Crn and other 7 AAs	Urine	MCI® GEL CHK40/C04 cation-exchange column (4.6 x 150 mm) packed with sulfo-functionalized highly cross-linked macroreticular PS-DVB (3.67 μm)	HPLC-UV (210 nm)	30	Phe 0.02 Tyr 0.01 Cre 0.02	1 – 500	-
Y. Yokoyama, et al. (2015) [23]	Phe, Tyr, Cre, Crn and other 10 AAs	Urine	MCI®GEL CHK45/C04 column (4.6 x 150 mm) packed with carboxy-functionalized polymethacrylate cation-exchange resin (functional group: dicarboxylic acid, 4 μm)	HPLC-UV (210 nm)	15	Phe 0.27 Tyr 0.29 Cre 0.34	5 – 500	Phe 80 Tyr 101 Cre 95

Table 2.4 Literature review of the analytical methods for the simultaneous determination of Phe, Tyr and Cre in urine samples (cont.).

Author (Year)	Analytes	Sample	Column	Method/ Detector	Analysis time (min)	Detection limit (μM)	Linear range (μM)	%Recovery (%)
C. Seo, et al. (2019) [79]	Phe, Tyr, Cre and other 32 compounds	Urine, plasma and tissue	Synergi Hydro-reverse phase (4.6 × 150 mm x 4 μm) with HFBA as ion-pairing agents	HPLC-MS/MS	22	Phe 0.002 Tyr 0.006 Cre 0.008	0.03 - 6	Phe 101.4 Tyr 101.6 Cre 98.5

HFBA, heptafluorobutyric acid.

As shown in **Table 2.4**, Y. Yokoyama, et al. had been developed the new synthesized cation exchange packing materials in the use of the chromatographic determination of amino acids and also creatinine in urine samples. Currently, C. Seo and research group [79] developed LC–MS/MS method with the introduction of ion-pairing agents for the simultaneous determination of 35 non-derivatized metabolites including amino acids and creatinine in biological samples such as urine plasma and tissue.

As mentioned above, hydrophilic interaction liquid chromatography (HILIC) method coupled with MS/MS detector can be used for selective separation and good retention of the polar compounds without any derivatization steps [66, 67]. On the other hand, if sensitivity is not the majority point, advanced and high-cost detection techniques are not necessary. The HILIC method shows the advantage of using mobile phase with the low UV cutoff (acetonitrile rich-mobile phase) supporting for the spectrophotometric detection (UV or PDA) of amino acids at low wavelength [80].

In this work, we interested in HILIC method because it is suitable for analyzing and separation of small polar compounds in complex samples which could be developed for the rapid chromatographic analysis. The UV detection was used for the determination without derivatization of phenylalanine, tyrosine and creatinine due to its inherent ultraviolet absorption. However, the matrices in urine are a complication, sample pretreatment and preconcentration method should be applied in this work.

2.2.3 Sample preparation for the determination of phenylalanine, tyrosine and creatinine in urine sample

Urine is composed of more than 95% water. Other constituents include ammonia, phosphate, sodium, sulfate, creatinine, proteins, urea and other inorganic and organic compounds processed by the liver and kidney, including metabolites and drugs [81]. As a sample collection for analysis, the advantages of urine compared with blood, urine can be collected in a large amount of samples by a non-invasive method. The sampling repetition of urine is feasible. Moreover, because of the glomerular filtration, urine contains lower protein concentration (60 – 80 g L^{-1} in serum, 0.5 – 1 g L^{-1} in urine), lipids and other macromolecules [82].

A step for sample pretreatment and preconcentration is usually important for biological samples. Selection of the technique for this task related to the properties of the analytes and their concentrations, the matrix in the sample, amount of the sample and determination method [81]. Because of the complex matrices in urine, the sample preparation as a pretreatment (clean-up) and preconcentration is necessary before the chromatographic analysis of phenylalanine, tyrosine and creatinine.

2.2.3.1 Sample pretreatment

When utilizing HPLC coupled with MS for the detection of amino acids, derivatization is not necessary, and the sample preparation can be simplified as dilution [79, 83] or deproteinization [73]. Dilution is supposed as one of the sample pretreatment methods prior to chromatographic determination. The capacity of dilution relies on the selected diluents, for example, the mobile phase is selected as the diluent for chromatographic analysis [82].

Nevertheless, the removal of matrices still not achieved, some sample extractions may be applied to reduce the complex sample matrices as UV detection is not a specific technique. It must be taken into account that low wavelengths applied for amino acids will decrease the selectivity and sensitivity of detection. The lower the detection wavelength used the more interferences will occur in the analysis [84].

Normally, the dilution of urine samples with water or buffer is carried out in order to reduce the matrix effect and the pH adjusted appropriately prior to sample addition of the SPE procedure. SPE has been proved to be effective in decresing the matrix effect [85].

2.2.3.2 Solid-phase extraction (SPE)

Solid phase extraction (SPE) is a general extraction method for the analysis of biological samples. SPE is used the amount of organic solvent less than liquid-liquid extraction (LLE) and the formation of emulsion does not occur. Preconcentration of the analytes can be achieved by evaporation of the eluent such as nitrogen blowing. The strong cation exchange cartridges were used for extraction of amino acid from the samples and eliminated matrices prior to chromatographic

analysis because the structure of amino acids can be protonated in acidic condition [57, 83, 85, 86]. Due to its effective extraction of the positively charged compounds, the strong-cation exchange solid-phase extraction (SCX-SPE) is suitable for the extraction of phenylalanine, tyrosine and creatinine from urine samples.

From the review of literature, only Y. Yokoyama research group proposed various synthesized cation exchange packing materials in HPLC column for the determination of amino acids and also creatinine in urine samples [23-28, 75-78]. All methods were based on the use of strong cation exchange resin (sulfopropyl phase) for sample pretreatment and preconcentration of Phe, Tyr, Cre and other compounds in urine samples. A 100 µL of filtrated urine was applied to the conditioned cation-exchange resin (SP-Toyopearl 650 M, sulfopropyl phase) column (6 x 65 mm, glass made) and permeated the preparative column with water. The column was washed to eliminate acidic and/or neutral compounds from the column and eluted the cationic compounds such as amino acids and creatinine by 1 – 2 mL of 0.1 M ammonia. This step was taken about 30 minutes. Then, the fraction was freeze-dried in order to remove ammonia and redissolved the residue or acidified by adding phosphoric acid in order to injection to the chromatographic instrument.

Moreover, the application note of Macherey-Nagel application database [83], Guide to Solid Phase Extraction [86] and X. Tang [85] were also proposed the use of strong cation exchange solid-phase extraction for the analysis of amino acids in human urine [83] and rat urine sample [85] including other biological fluid, human serum [57] as shown in **Table 2.5**.

Table 2.5 Literature review of the solid phase extraction (SPE) for amino acid analysis in urine samples.

Author (Year)	Analytes	Sample	SPE	Eluting solvent	Method/ Detector	LOD (μM)	LOQ (μM)	Note
Y. Yokoyama, et al. (2015) [23]	Phe, Tyr, Cre, Crn and other 10 AAs	Human urine	Cation-exchange resin (sulfopropyl phase)	0.1 M ammonia	HPLC-UV (210 nm)	Phe 0.27 Tyr 0.29 Cre 0.34	-	Freeze dried to remove ammonia
Macherey-Nagel application database (2018) [83]	Amino acids	Human urine	Cation-exchange CHROMABOND® SA (= SCX) (sulfophenyl phase)	1 M ammonia solution	-	-	-	Freeze dried to remove ammonia
M. Armstrong, et al. (2007) [57]	Phe, Tyr (total 25 AAs) with labelled AAs (I.S.)	Human plasma	Cation-exchange (Strata X-C) (sulfophenyl phase)	5% ammonium hydroxide in methanol	HPLC-TOF-MS	-	Phe 1.56 Tyr 1.56	Vacuum evaporation of the ammonium hydroxide

Table 2.5 Literature review of the solid phase extraction (SPE) for amino acid analysis in urine samples (cont.).

Author (Year)	Analytes	Sample	SPE	Eluting solvent	Method/ Detector	LOD (μM)	LOQ (μM)	Note
X. Tang, et al. (2014) [85]	Phe, Tyr (total 26 AAs) with labelled AAs (I.S.)	Rat urine	Strong cation-exchange SCX (Chrom-Matrix) (sulfophenyl phase)	5% ammonium hydroxide in methanol	HPLC-MS/MS	Phe 0.08 Tyr 0.90	Phe 0.10 Tyr 1.10	Evaporated to dryness under nitrogen at 35 °C

I.S., internal standard.

As we mentioned previously about advantages of the sample preparation methods, we have interested on the dilution and the extraction method based on SCX-SPE for pretreatment urine samples prior to chromatographic analysis.

2.3 The analytical methods for determination of phenylalanine and tyrosine in dietary supplement

The control of phenylalanine in blood to a normal level for brain development is the goal of PKU treatment. PKU is treated by a low phenylalanine concentration diet such as vegetables and fruits, and the main source of amino acids (such as tyrosine) and protein equivalent is supplied by a Phe-free or low Phe protein and amino acids substitute. Therefore, the analytical methods for the determination of phenylalanine and tyrosine in food, infant formula, pharmaceutical product and dietary supplement is necessary.

In this work, the proposed HILIC-UV method were focused on the simultaneous determination of phenylalanine, tyrosine and creatinine in urine for the diagnosis and screening of PKU. However, it is an opportunity that the proposed method can be applied to other samples such as dietary supplement.

The reversed-phase HPLC method with pre-column derivatization was widely used for the simultaneous determination of phenylalanine and tyrosine or other amino acids in food. Derivatizing reagent were commonly used as same as blood samples such as o-phenylisothiocyanate (PITC) [87], phthaldialdehyde (OPA) [88, 89], 9-fluorenylmethyl-chloroformate (FMOC) [89, 90] and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [91].

As mentioned above, various analytical problems such as an instability of derivatives, interference from derivatizing reagent and long preparation times were suffered from the derivatization methods. Therefore, the analytical method without derivatization step could be eliminated these problems. The HILIC method can be used for selective separation of the polar compounds without any derivatization steps. Complex derivatization methods were no longer required and good separation of analytes was achieved.

CHAPTER 3
RESEARCH METHODOLOGY

The HILIC technique for the determination of phenylalanine, tyrosine and creatinine was developed. The research methodology of this work was described in this section consisting of instrumentations, apparatus, chemicals, reagent and sample preparations and procedures of the method.

3.1 Instrumentations and apparatus

The details of the instruments and laboratory equipment of this research were listed in this part.

3.1.1 High performance liquid chromatography with photodiode array detection (HPLC-DAD)

The details of a high-performance liquid chromatography coupled with photodiode array detector (HPLC-DAD) was summarized in **Table 3.1**.

Table 3.1 HPLC-DAD system specifications.

Instrument	Model	Company
High performance liquid chromatography (HPLC)	Nexera LC–30A	Shimadzu (Japan)
- Autosampler	SIL–30AC	Shimadzu (Japan)
- Column oven	CTO–20AC	Shimadzu (Japan)
- Degasser	DGU-20A5	Shimadzu (Japan)
- Diode array detector (DAD)	SPD–20A	Shimadzu (Japan)
- Pump	LC–30AD	Shimadzu (Japan)
- System software	Lab Solution (version 5.81 SP1)	Shimadzu (Japan)

Table 3.1 HPLC-DAD system specifications (cont.).

Instrument	Model	Company
Column	Inertsil HILIC column (3.0 mm x 150 mm x 3 µm, dihydroxypropyl) with HILIC guard column (3.0 mm x 10 mm x 3 µm)	GL Sciences (Japan)

3.1.2 Apparatus

The laboratory equipment used in this work for the determination of phenylalanine, tyrosine and creatinine was showed in **Table 3.2**.

Table 3.2 Equipments.

Equipment	Model	Company
Analytical balance	AB304-S	Mettler Toledo (Switzerland)
Auto-micropipette	Research model Volume: 2 – 20, 20 – 200, 100 – 1,000 and 500 – 5,000 µL	Eppendorf (Germany)
Diaphragm Vacuum pump	GM–0.5	Biobase (China)
pH meter	C860	Consort (Belgium)
pH electrode	SP10B pH standard electrode	Consort (Belgium)
Nitrogen evaporator	MD200	C.E. Instruments (Thailand)
Nylon membrane filter	CHROMAFIL O-20/15 MS (0.20 µm, 47 mm)	Xiboshi (China)
Shaker machine	Shaker 3006	Gesellschaft für Labortechnik (Germany)

Table 3.2 Equipments (cont.).

Equipment	Model	Company
Type I water purification system	ELGASTAT UHQ PS.	Elga (England)
Vortex mixer	Vortex-2 Genie G-560E	Scientific Industries (USA)
Ultrasonicator	RK156	BANDELIN electronic GmbH & Co. KG (Germany)

3.2 Chemicals

The chemicals of this work were analytical reagent and HPLC grade as listed in **Table 3.3**.

Table 3.3 Chemicals and reagents.

Chemicals and reagents	Formula	Company
Acetic acid	$C_2H_4O_2$	Carlo erba (France)
Acetonitrile	C_2H_3N	Lab Scan (Thailand)
Ammonia solution 30%	NH_3	Carlo erba (France)
Ammonium acetate	$C_2H_7NO_2$	Merck (Germany)
Ammonium formate	CH_5NO_2	Sigma (USA)
Creatinine (anhydrous)	$C_4H_7N_3O$	Sigma (USA)
Ethanol	C_2H_6O	Lab Scan (Thailand)
Formic acid	CH_2O_2	Fisher (UK)
Hydrochloric acid 37%	HCl	Lab Scan (Thailand)
L-phenylalanine	$C_9H_{11}NO_2$	Sigma (USA)
L-tyrosine	$C_9H_{11}NO_3$	Sigma (USA)
Methanol	CH_4O	Lab Scan (Thailand)
Type I water	H_2O (18.2 MΩcm^{-1})	Elga (England)
α-methyl-L-phenylalanine	$C_{10}H_{13}NO_2$	Sigma (USA)

3.3 Urine sample

The urine samples were collected from 40 volunteers. Inclusion criteria of the volunteer were a healthy male or female aged 18 – 45 years. For the analysis, two urine samples from each male and female aged range between 18 – 20, 21 – 30, 31 – 40 and 41 – 45 years old were sampled from the collected urine samples.

The volunteers were given a urine container to take home and instructed to collect about 50 mL of urine sample in the morning. All urine samples were stored at 4 °C until analysis. The analysis was carried out within 24 hours after sample collection.

3.4 Dietary supplement sample

Single-nutrient and multi-nutrients (Phe, Tyr with other nutrients such as vitamins and plant extracts) dietary supplements were purchased from stores in Thailand. The samples were kept in dark and dry place at room temperature.

Twenty capsules of the dietary supplement were accurately weighed to acquire an average weight of powder. The powder from twenty capsules was unwrapped and homogeneously mixed prior to analysis. A multi-nutrients dietary supplement sample was chosen as a representative sample for the study of an optimal condition for the extraction, separation and determination of phenylalanine and tyrosine in dietary supplements.

3.5 Preparation of standard solutions and chemical reagents

The standard solutions and chemical reagents of the research were prepared as follows:

3.5.1 Standard solutions

3.5.1.1 Phenylalanine standard solutions

(1) primary stock standard solution, 5,000 mg L^{-1}

A primary stock standard solution of phenylalanine at a concentration of 5,000 mg L^{-1} was prepared by accurately weighing of 0.2500 g phenylalanine and dissolving into 50.00 mL of 0.1 M hydrochloric acid in a volumetric flask. The stock standard solution was stored in a refrigerator at 4 °C until use.

(2) secondary stock standard solution, 1,000 mg L^{-1}

A secondary stock standard solution of phenylalanine was prepared at a concentration of 1,000 mg L^{-1}. This stock solution was prepared from primary stock standard solution by pipetting 2,000 µL and adjusting the volume with 0.1 M hydrochloric acid in a 10-mL volumetric flask. The stock standard solution was stored in a refrigerator at 4 °C until use.

3.5.1.2 Tyrosine standard solutions

(1) primary stock standard solution, 5,000 mg L^{-1}

A primary stock standard solution of tyrosine was prepared at a concentration of 5,000 mg L^{-1} as same as the primary stock standard solution of phenylalanine (see **Section 3.5.1.1(1)**).

(2) secondary stock standard solution, 1,000 mg L^{-1}

A secondary stock standard solution of tyrosine was prepared at a concentration of 1,000 mg L^{-1} as same as the secondary stock standard solution of phenylalanine (see **Section 3.5.1.1(2)**).

3.5.1.3 Creatinine standard solutions

(1) primary stock standard solution, 25,000 mg L^{-1}

A primary stock standard solution of creatine was prepared at a concentration of 5,000 mg L^{-1} by accurately weighing of 1.2500 g creatinine and dissolving into 50.00 mL of 0.1 M hydrochloric acid in a volumetric flask. The stock standard solution was stored in a refrigerator at 4 °C until use.

(2) secondary stock standard solution, 10,000 mg L^{-1}

A secondary stock standard solution of creatinine was prepared at a concentration of 1,000 mg L^{-1} by pipetting 4,000 µL of primary stock

standard solution and adjusting the volume with 0.1 M hydrochloric acid in a 10-mL volumetric flask. The stock standard solution was stored in a refrigerator at 4 ºC until use.

3.5.1.4 α-methyl phenylalanine internal standard solutions

(1) primary stock internal standard solution, 5,000 mg L^{-1}

A primary stock internal standard solution of α-methyl phenylalanine at a concentration of 5,000 mg L^{-1} was prepared by accurately weighing of 0.0500 g and dissolving into 10.00 mL of 0.1 M hydrochloric acid in a volumetric flask. The stock standard solution was stored in a refrigerator at 4 ºC until use.

(2) secondary stock internal standard solution, 1,000 mg L^{-1}

A secondary stock internal standard solution of α-methyl phenylalanine was prepared at a concentration of 1,000 mg L^{-1} as same as the secondary stock standard solution of phenylalanine (see **Section 3.5.1.1(2)**).

3.5.1.5 Mixture of standard and internal standard solution for the optimization of HILIC conditions

A mixture of standard and internal standard solution consisted of 20 mg L^{-1} of phenylalanine, 20 mg L^{-1} of tyrosine, 500 mg L^{-1} of creatinine and 50 mg L^{-1} of α-methyl phenylalanine. The mixture solutions were prepared as follows:

- pipetted 200 µL of a secondary stock standard solution of phenylalanine (1,000 mg L^{-1}) into a 10-mL volumetric flask.
- pipetted 200 µL of a secondary stock standard solution of tyrosine (1,000 mg L^{-1}) into a 10-mL volumetric flask.
- pipetted 500 µL of a secondary stock standard solution of creatinine (10,000 mg L^{-1}) into a 10-mL volumetric flask.
- pipetted 500 µL of a secondary stock internal standard solution of α-methyl phenylalanine (1,000 mg L^{-1}) into a 10-mL volumetric flask.

and then adjusted the volume to the mark with acetonitrile. These solutions were used for the optimization of HILIC conditions in **Section 3.6.1**.

3.5.1.6 Mixture of standard and internal standard solution for the analysis of urine samples

A mixture of standard and internal standard solution consisted of 10 mg L^{-1} of phenylalanine, 10 mg L^{-1} of tyrosine, 500 mg L^{-1} of creatinine and 30 mg L^{-1} of α-methyl phenylalanine (these concentrations meant to be the concentration of the analytes after extraction through SCX-SPE). The mixture solutions were prepared as follows:

- pipetted 10 µL of a secondary stock standard solution of phenylalanine (1,000 mg L^{-1}) into a 10-mL volumetric flask.

- pipetted 10 µL of a secondary stock standard solution of tyrosine (1,000 mg L^{-1}) into a 10-mL volumetric flask.

- pipetted 50 µL of a secondary stock standard solution of creatinine (10,000 mg L^{-1}) into a 10-mL volumetric flask.

- pipetted 30 µL of a secondary stock internal standard solution of α-methyl phenylalanine (1,000 mg L^{-1}) into a 10-mL volumetric flask.

and then adjusted the volume to the mark with 0.1 M hydrochloric acid. These solutions were used for the optimization of solid-phase extraction (SPE) and validation of the developed method in **Section 3.6.2.2** and **Section 3.6.2.3**.

3.5.1.7 Mixture of standard and internal standard solution for the analysis of the dietary supplement samples

A mixture of standard and internal standard solution consisted of 10 mg L^{-1} of phenylalanine, 10 mg L^{-1} of tyrosine and 30 mg L^{-1} of α-methyl phenylalanine. The mixture solutions were prepared as follows:

- pipetted 100 µL of a secondary stock standard solution of phenylalanine (1,000 mg L^{-1}) into a 10-mL volumetric flask.

- pipetted 100 µL of a secondary stock standard solution of tyrosine (1,000 mg L^{-1}) into a 10-mL volumetric flask.

- pipetted 300 µL of a secondary stock internal standard solution of α-methyl phenylalanine (1,000 mg L^{-1}) into a 10-mL volumetric flask.

and then adjusted the volume to the mark with acetonitrile. A mixture of standard and internal standard solution consisted of 50 mg L^{-1} of phenylalanine, 50 mg L^{-1} of tyrosine and 30 mg L^{-1} of α-methyl phenylalanine were also prepared. These solutions were used for the validation of the developed method for dietary supplement determination in **Section 3.6.3.2**.

3.5.2 Hydrochloric acid, 0.1 M

Hydrochloric acid at the concentration of 0.1 M was prepared by pouring 8.3 mL of concentrated hydrochloric acid (37% w/w) using a measuring cylinder into a beaker containing type I water. The total volume of the solution was adjusted to 1,000 mL with type I water.

3.5.3 Ammonium formate buffer, 50 mM

To prepare 50 mM of ammonium formate buffer pH 3.0, 1.5765 g of ammonium formate crystalline solid was dissolved with about 400 mL of type I water. Then, the solution was adjusted pH to pH 3.0 with 6 M formic acid and adjusted the volume end up to 500.00 mL in a volumetric flask. This buffer was employed as an aqueous of the mobile phase.

3.5.4 Methanol: hydrochloric acid, 0.1 M (1:1, v/v)

Methanol : 0.1 M of hydrochloric acid (1:1, v/v) was prepared for the conditioning of SPE in **Section 3.6.2.2(2)**. This solution was freshly prepared by mixing 100 mL of methanol and 100 mL of 0.1 M hydrochloric acid into a 250-mL beaker.

3.5.5 Ammonia in ethanol, 0.5 M

An eluting reagent, 0.5 M of ammonia in ethanol, was prepared by pouring 14.5 mL of concentrated ammonia (30%) into a beaker and adjusting the volume to 250 mL with ethanol. This reagent was used as the eluting reagent of SPE in **Section 3.6.2.2(5)**.

3.6 Experimental

The proposed method was based on hydrophilic interaction liquid chromatography (HILIC) technique for the separation of phenylalanine, tyrosine and creatinine in urine and additionally applied to separate phenylalanine and tyrosine in

the dietary supplement. The HILIC conditions were therefore optimized. Furthermore, the sample preparation of urine and the dietary supplement was optimized prior to introduction of samples into the chromatographic system. The validation of the method and sample analysis of urine and dietary supplement samples were also described in this section.

3.6.1 Optimization of HILIC conditions

The condition of HILIC technique was optimized for simultaneous separation of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (internal standard). The stationary phase of HILIC column is dihydroxypropyl (diol) group bonded silica gel which retains highly polar compounds. The optimal condition of each parameter was considered based on separation (resolution), peak shape, sensitivity and analysis time of the analytes.

3.6.1.1 Detection wavelength

In this research, the diode array detector (DAD) was employed in order to simultaneously detect absorbances of the analytes at different wavelengths and additionally can be used for confirmation of compound identity. The detection wavelength was scanned from 190 to 400 nm and chosen based on the maximum absorbance to obtain the highest sensitivity and selectivity of each analyte.

3.6.1.2 Mobile phase

The condition of the mobile phase was investigated for simultaneous separation and determination of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine. The optimization of the mobile phase was focused on pH of buffer, composition and flow rate.

(1) pH of buffer and mobile phase composition

A common mobile phase for HILIC technique which is water-miscible polar organic solvents such as acetonitrile and methanol with a slight amount of water or buffer was studied. According to the pH durability of the column which is in the range of pH 2 – 7.5, a buffer solution as an aqueous phase of the mobile phase

was investigated at the pH of 3 – 7 with ionic additives namely ammonium acetate and ammonium formate.

The composition of mobile phase (acetonitrile:buffer at any pH) was studied at the ratios of 80:20, 81:19, 82:18, 83:17, 84:16, 85:15 and 90:10 v/v at the flow rate of 0.8 mL min^{-1} and 4 µL of injection volume. The selection of buffer and mobile phase composition was based on the separation of analytes, peak shape and analysis time.

(2) Flow rate

The flow rate of mobile phase was optimized to achieve fast analysis and the good separation. The suitable mobile phase, acetonitrile mixed with 50 mM of ammonium formate pH 3.0 at the ratio of 84:16 v/v, was used to optimize the flow rates from 0.6 to 0.8 mL min^{-1}.

3.6.1.3 Injection volume

The optimization of an injection volume was examined at the volume of 1, 2, 3, 4 and 5 µL with the optimal condition of the mobile phase. The highest sensitivity with the correlation of good precision and the good peak shape was applied to decide the optimal volume of injection.

Table 3.4 The optimal chromatographic conditions of HILIC for the determination of phenylalanine, tyrosine and creatinine.

Parameter	Optimal condition
Column (stationary phase)	Analytical column: Inertsil HILIC column (3.0 mm x 150 mm x 3 µm) (dihydroxypropyl groups bonded to silica gel) Guard column: Inertsil HILIC column (3.0 mm x 10 mm x 3 µm) (dihydroxypropyl groups bonded to silica gel)
Mobile phase	Acetonitrile:50 mM of ammonium formate pH 3.0 at the ratio of 84:16 v/v
Flow rate	0.8 mL min^{-1}
Detection wavelength	210 nm for phenylalanine and α-methyl phenylalanine (I.S.), 225 nm for tyrosine and 234 nm for creatinine
Injection volume	4 µL

The optimal condition of HILIC (**Table 3.4**) was applied to analyze of phenylalanine, tyrosine and creatinine in urine (**Section 3.6.2**) and phenylalanine and tyrosine in the dietary supplement (**Section 3.6.3**) along with α-methyl phenylalanine as an internal standard.

3.6.2 Analysis of urine

The optimal condition of SPE was studied to clean-up and preconcentration of the analytes in urine. The mixed standard solution (**Section 3.5.1.6**) and diluted urine sample (**Section 3.6.2.1**) were employed for optimizing the SPE conditions.

3.6.2.1 Sample dilution

One milliliter of urine sample was spiked with 30 µL of the secondary stock internal standard solution of α-methyl phenylalanine (1,000 mg L^{-1}) and then diluted to 10.00 mL with 0.1 M hydrochloric acid in a volumetric flask. This diluted urine was used to optimize the SPE conditions.

3.6.2.2 Optimization of solid-phase extraction (SPE)

Sample preparation of urine sample was carried out using strong cation exchange solid-phase extraction (SCX-SPE) for the simultaneous extraction of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.). Various parameters were studied to achieve the best extracted condition as detailed below.

(1) Type of sorbent

The equal amount of two different types of absorbent, benzenesulfonic acid modified silica and aliphatic sulfonic acid bonded silica with Na^+ counter-ion were investigated and compared the extraction efficiency. The mixed standard solution and the reagent blank were used for studying this parameter with the following SPE condition:

- conditioned SPE with 25 mL of each conditioning reagent consisting of methanol, 1:1 (v/v) of methanol:0.1 M hydrochloric acid and 0.1 M hydrochloric acid.

- loaded 1 mL of mixed standard solution or reagent blank at pH 1 into SPE.

- washed the SPE with 5 mL of type I water.

- eluted the analytes with 5 mL of 0.5 M ammonia solution.

The eluate was evaporated to dryness under nitrogen gas at 60°C and then reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) prior to injection into the HILIC system.

(2) Conditioning

SPE conditioning is an important step in order to provide repeatable interaction between the sorbent and analytes. The various volumes of the conditioning reagent was studied in order to produce an environment which is appropriate for extraction of the analytes.

The volume of each conditioning reagent, methanol, 1:1 (v/v) of methanol:0.1 M hydrochloric acid and 0.1 M hydrochloric acid, was optimized by 3 mL (one tube volume), 6 mL (two tube volume), 9 mL (three tube volume) and 25 mL (5 times volume proportional to sorbent weight). The mixed standard solution was used for studying this parameter with the following SPE condition:

- conditioned SPE with 3, 6, 9 or 25 mL of each conditioning reagent.

- loaded 1 mL of mixed standard solution at pH 1 into SPE.

- washed the SPE with 5 mL of type I water.

- eluted the analytes with 5 mL of 0.5 M ammonia in ethanol.

The eluate was evaporated to dryness under nitrogen at 60°C and then reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) prior to injection into the HILIC system.

(3) Loading

pH of the sample solution plays an important role in the extraction of the analytes due to their structure. The pH of the loading sample was studied in the range of pH 1 – 4 in order to obtain the protonated structure of the analytes. The mixed standard solution was adjusted the pH to 1, 2, 3 and 4 by 0.1 M

hydrochloric acid and 1 M sodium hydroxide solution to study this parameter with following SPE condition:

- conditioned SPE with 9 mL of each conditioning reagent.
- loaded 1 mL of mixed standard solution at pH 1 – 4 to SPE.
- washed the SPE with 5 mL of type I water.
- eluted the analytes with 5 mL of 0.5 M ammonia in methanol.

The eluate was evaporated to dryness under nitrogen gas at 60°C and then reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) prior to injection into the HILIC system.

(4) Washing

Washing of the sorbent was achieved with a washing reagent to eliminate matrices which can be retained on SCX-SPE and then eluted with the analytes. The mixed standard solution and the diluted urine sample were used for investigating types and volumes of the washing reagent.

(4.1) Type of washing reagent

Various types of washing reagent at fixed volume were investigated to wash the matrices of the urine sample as follows: 4 mL of type I water, 4 mL of methanol, 2 mL of type I water and then 2 mL of methanol and 4 mL of type I water:methanol at a ratio of 1:1 (v/v).

The diluted urine sample was used for studying this parameter with the following SPE condition:

- conditioned SPE with 9 mL of each conditioning reagent.
- loaded 1 mL of mixed standard solution at pH 1 to SPE.
- washed the SPE with 4 mL (total volume) of various washing reagents.
- eluted the analytes with 5 mL of 0.5 M ammonia in methanol.

The eluate was evaporated to dryness under nitrogen gas at 60°C and then reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) prior to injection into the HILIC system.

(4.2) Washing volume

The washing volumes of methanol was optimized in the range of 1 – 5 mL to wash the matrices in urine which can be retained on the SCX sorbent. The diluted urine sample was used for studying this parameter with the following SPE condition:

- conditioned SPE with 9 mL of each conditioning reagent.
- loaded 1 mL of mixed standard solution at pH 1 into SPE.
- washed the SPE with 1 – 5 mL of methanol.
- eluted the analytes with 5 mL of 0.5 M ammonia in methanol.

The eluate was evaporated to dryness under nitrogen gas at 60°C and then reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) prior to injection into the HILIC system.

(5) Eluting

Various types of eluting reagent (solvent) and volume were studied to elute the analytes but leaves behind any matrices which not expelled in the washing step from SCX-SPE.

(5.1) Type of eluting reagent

Various types of eluting reagent consisting of 0.5 M ammonia in type I water, 0.5 M ammonia in methanol, 0.5 M ammonia in ethanol and 0.5 M ammonia in acetonitrile at fixed volume (4 mL) were studied to elute the analytes from SPE sorbent.

The mixed standard solution along with diluted urine sample was used for studying this parameter with the following SPE condition:

- conditioned SPE with 9 mL of each conditioning reagent.
- loaded 1 mL of mixed standard solution at pH 1 into SPE.
- washed the SPE with 4 mL of methanol.
- eluted the analytes with 5 mL of 0.5 M ammonia in various types of solvents.

The eluate was evaporated to dryness under nitrogen gas at 60°C and then reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) prior to injection into the HILIC system.

(5.2) Eluting volume

Different volumes of the optimal eluting reagent were studied to elute the analytes from SPE sorbent. The suitable eluting reagent for SCX-SPE was 0.5 M ammonia in ethanol. The volume of eluting reagent was studied in the range of 1 – 5 mL.

The mixed standard solution and diluted urine sample were used for studying this parameter with the following SPE condition:

- conditioned SPE with 9 mL of each conditioning reagent.
- loaded 1 mL of mixed standard solution at pH 1 into SPE.
- washed the SPE with 4 mL of methanol.
- eluted the analytes with 1 – 5 mL of 0.5 M ammonia in ethanol.

The eluate was evaporated to dryness under nitrogen gas at 60°C and then reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) prior to injection into the HILIC system.

3.6.2.3 Method validation

The developed HILIC with SPE for urinary analysis was validated using the Appendix F: Guidelines for Standard Method Performance Requirements by Association of Official Analytical Communities (AOAC) [92] as a guideline. Several analytical performances including internal calibration curve, the limit of detection (LOD), the limit of quantitation (LOQ), precision and accuracy were validated from sample preparation to HILIC analysis.

(1) Internal calibration curve

The internal calibration curves were plotted between peak area of the analytes divided by peak area of an internal standard (α-methyl phenylalanine) (the y-axis) and the series of each analyte concentrations (the x-axis). The coefficient of determination was calculated to indicate linearity.

Mixed standard solutions with seven different concentrations of phenylalanine (1 – 400 mg L^{-1}), tyrosine (0.5 – 200 mg L^{-1}), creatinine (3 – 3,000 mg L^{-1}) and 30 mg L^{-1} of α-methyl phenylalanine were prepared to construct the internal calibration curves. The mixed standard solutions were passed through SCX-SPE as the extraction of the analytes from the urine sample which described below in **Section 3.6.2.4**. The extraction and HILIC analysis of the mixed standard solutions were performed with three replications. The linear equations were used for calculation of phenylalanine, tyrosine and creatinine concentrations in the urine samples.

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

Limit of detection (LOD) is the lowest concentration of extracted phenylalanine, tyrosine and creatinine through SCX-SPE which can be determined by three times of signal to noise ratio (3S/N). A concentration that gives detectable signal related to the concentration of phenylalanine, tyrosine and creatinine at ten times signal to noise ratio (10S/N) was a limit of quantification (LOQ). This analytical performance was accomplished by the triplicate injection of the lower concentration of each analyte.

(3) Precision

The precision of the proposed method was studied by replicated determination of the urine sample. According to the AOAC requirements, the precisions in term of intra-day and inter-day precision were evaluated by the HorRat method.

(3.1) Intra-day precision

The intra-day precision was obtained by injection of ten extracted urine samples (from the same pool) in three replications within a day. The intra-day precision was calculated using the relative standard deviation (%RSD(r)) of ten extracted urine sample in three replicated injections.

(3.2) Inter-day precision

The inter-day precision was obtained by injection of ten extracted urine sample (from the same pool) in three replications within three days. The analysis of three extracted urine each day was performed except the four extracted urine on the first day. The inter-day precision of each analyte was calculated

using the relative standard deviation (%RSD(r)) of ten extracted urine sample in three replicated injections within three days.

(4) Accuracy

The accuracy of the proposed method was evaluated in term of recovery. The recoveries of SCX-SPE and HILIC determination were assessed by determining the concentrations of the analytes in the urine samples compared with the analytes in the fortified urine samples (fortifying with 10 mg L^{-1} of phenylalanine, 10 mg L^{-1} of tyrosine, 500 mg L^{-1} of creatinine and 30 mg L^{-1} of **α**-methyl phenylalanine). The percentage of the recovery was calculated as

$$\% \text{ Recovery} = \frac{C_f - C_u}{C_A} \times 100$$

where C_f = concentration of the analytes in fortified urine samples,

C_u = concentration of the analytes in unfortified urine samples,

C_A = concentration of the analytes added to urine samples.

3.6.2.4 Application to urine

Urine samples were prepared prior to load on SPE as described in **Section 3.6.2.1**. One milliliter of urine sample was fortified with 30 µL of 1,000 mg L^{-1} **α**-methyl phenylalanine and then diluted to 10.00 mL with 0.1 M hydrochloric acid. These diluted urines were extracted through SCX-SPE.

The strong cation exchange solid-phase extraction (SCX-SPE) with 500 mg weight of SCX sorbent and 3 mL of cartridge volume was employed for extraction. The conditioning step of SCX-SPE was carried out as follows: 9 mL of methanol, 9 mL of 1:1 (v/v) of methanol:0.1 M hydrochloric acid and then 9 mL of 0.1 M hydrochloric acid.

A 1.00 mL of the diluted urine was applied to condition the SCX-SPE and then the SCX-SPE was washed by 4.00 mL of methanol in order to elimination the matrices. The analytes were eluted with 4.00 mL of 0.5 M ammonia in ethanol. All the eluate was collected in a 10-mL glass test tube with screw cap and evaporated to dryness under nitrogen gas at 60°C (about 30 minutes). Then, the solid

(solute) was reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) by vortexing for 30 seconds. The reconstituted solution was filtrated through a 0.2 µm nylon syringe filter prior to injection into the HILIC system.

3.6.3 Analysis of dietary supplement

Sample preparation for the determination of phenylalanine and tyrosine in the dietary supplement samples were studied. A parameter was varied while keeping other parameters fixed in order to achieve the highest extraction efficiency.

3.6.3.1 Sample extraction

The optimization of sample extraction was performed using multi-nutrients dietary supplement sample (comprised of phenylalanine and tyrosine with other nutrients such as vitamins and plant extracts) with three aliquots and three replications.

(1) Extraction solvent

Twenty capsules of the dietary supplement were unwrapped and mixed the sample powder together. The homogeneous powders of dietary supplements were extraction or dissolution with various types of solvent as detailed below:

- 0.1 M of hydrochloric acid (HCl): the solvent of standard solutions.
- Type I water: the common polar solvent to extract polar compounds.
- 50 mM of ammonium formate buffer pH 3: aqueous of mobile phase
- Mobile phase, acetonitrile:50 mM of ammonium formate pH 3.0 at the ratio of 84:16 (v/v).

Types of extraction solvent were studied with an equal volume of solvents. The homogeneous powders of multi-nutrients dietary supplement (Sample D) were accurately weighed of 0.0250 g in a plastic tube with a screw cap. 750 µL of the secondary stock internal standard solution of α-methyl phenylalanine (1,000

mg L^{-1}) and 24.25 mL of extraction solvent were added to the tube. A solution was agitated using ultrasonication for 30 minutes. One milliliter of the extracted solution was diluted to 10.00 mL with acetonitrile in a volumetric flask and then filtrated through a 0.2 µm nylon syringe filter prior to injection into the chromatographic system.

(2) Agitation method and time

The highest extraction efficiency and the shortest extraction time were studied to obtain the highest performance of the extraction method. Three different agitation methods along with the extraction time were studied as follows:

- Ultrasonicator: 5, 15, 30 and 45 min.
- Horizontal shaker: 1, 5, 10, 15 and 30 min.
- Vortex: 0.5, 1, 2, 3, 4 and 5 min.

The multi-nutrients dietary supplement (Sample D) powder was accurately weighed of 0.0250 g in a plastic tube with a screw cap. 750 µL of the secondary stock internal standard solution of α-methyl phenylalanine (1,000 mg L^{-1}) and 24.25 mL of 0.1 M hydrochloric acid as extraction solvent was added to the tube. A solution was agitated using various agitation methods and extraction times as mentioned above. One milliliter of the extracted solution was diluted to 10.00 mL with acetonitrile in a volumetric flask and then filtrated through a 0.2 µm nylon syringe filter prior to injection into the chromatographic system.

3.6.3.2 Method validation

The proposed HILIC with extraction method was validated using the Appendix K: Guidelines for Dietary Supplements and Botanicals, Part I: AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals by Association of Official Analytical Communities (AOAC) [93] as a guideline. Several analytical performances including internal calibration curve, the limit of detection (LOD), the limit of quantitation (LOQ), precision and accuracy were validated from sample preparation to HILIC analysis.

(1) Internal calibration curve

The internal calibration curves were plotted between peak area of the analytes divided by peak area of an internal standard (α-methyl

phenylalanine) (the y-axis) and the series of each analyte concentrations (the x-axis). The coefficient of determination was calculated to indicate linearity.

Mixed standard solutions with seven different concentrations of phenylalanine (1 – 500 mg L^{-1}), tyrosine (1 – 500 mg L^{-1}) and 30 mg L^{-1} of α-methyl phenylalanine were prepared to construct the internal calibration curve. The HILIC analysis of the mixed standard solutions was performed with three replications. The linear equations were used for calculation of phenylalanine and tyrosine concentration in dietary supplement samples.

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

Limit of detection (LOD) is the lowest concentration of the standard solution of phenylalanine and tyrosine which can be determined by three times of signal to noise ratio (3S/N). A concentration that gives detectable signal related to the concentration of phenylalanine and tyrosine at ten times signal to noise ratio (10S/N) was a limit of quantification (LOQ). This analytical performance was accomplished by the triplicate injection of the lower concentration of each analyte.

(3) Precision

The precision of the proposed method was studied by replicated determination of a multi-nutrients dietary supplement sample. According to the AOAC requirements, the precisions in term of intra-day and inter-day precision were evaluated by the HorRat method.

(3.1) Intra-day precision

The intra-day precision was obtained by injection of ten extracted multi-nutrients dietary supplement samples in three replications within a day. The intra-day precision was calculated using the relative standard deviation (%RSD(r)) of ten extracted dietary supplements with three replicated injections.

(3.2) Inter-day precision

The inter-day precision was obtained by injection of ten extracted multi-nutrients dietary supplement in three replications within ten days. The inter-day precision of each analyte was calculated using the relative standard deviation (%RSD(r)) of ten extracted dietary supplement sample in three replicated injections.

(4) Accuracy

The accuracy of the proposed method was evaluated in term of recovery. The recoveries of extraction and HILIC determination were assessed by determining the concentrations of the analytes in all dietary supplement samples compared with the analytes in the fortified dietary supplement samples (fortifying with 10 and 50 mg L^{-1} of phenylalanine, tyrosine and 30 mg L^{-1} of α-methyl phenylalanine). The percentage of the recovery was calculated as

$$\% \text{ Recovery} = \frac{C_f - C_u}{C_A} \times 100$$

where C_f = concentration of the analytes in fortified dietary supplement samples,

C_u = concentration of the analytes in unfortified dietary supplement samples,

C_A = concentration of the analytes added to dietary supplement samples.

3.6.3.3 Application to dietary supplement

The homogeneous powders of the multi-nutrients dietary supplement were accurately weighed of 0.0250 g in a plastic tube with a screw cap. 750 µL of the secondary stock internal standard solution of α-methyl phenylalanine (1,000 mg L^{-1}) and 24.25 mL of 0.1 M hydrochloric acid as extraction solvent were added to the tube. The solution was vortexed for 3 minutes. One milliliter of the extracted solution was diluted to 10.00 mL with acetonitrile in a volumetric flask and then filtrated through a 0.2 µm nylon syringe filter prior to injection into the HILIC system.

CHAPTER 4
RESULTS AND DISCUSSION

This research presented the simple and fast HILIC method for the simultaneous determination of phenylalanine, tyrosine and creatinine. The proposed method not only can be applied to urine but can also be applied to analyze phenylalanine and tyrosine in dietary supplements.

This chapter was divided into three sections consisting of the development of HILIC with diode array detection (DAD) method for the simultaneous determination of phenylalanine, tyrosine and creatinine with α-methyl phenylalanine as an internal standard (I.S.). The second section was the optimization of the solid-phase extraction (SPE) as the sample preparation for urinary analysis and the last section was presented the optimization of the extraction or dissolution of phenylalanine and tyrosine in dietary supplement.

4.1 Optimization of HILIC conditions

Due to several advantages of HILIC mentioned in **Section 1.2**, the HILIC technique was selected for simultaneous separation of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (internal standard). The stationary phase of HILIC column is dihydroxypropyl (diol) group bonded silica gel which retains highly polar compounds.

The optimal condition of each parameter was considered based on separation (resolution), peak shape, sensitivity and analysis time of the analytes.

4.1.1 Detection wavelength

According to phenylalanine, tyrosine, creatinine and α-methyl phenylalanine, their structures have chromophores which can absorb UV light with the different maximum absorption at any wavelengths. The diode array detector (DAD) was used in this research in order to concurrent detect the absorbances at different

wavelengths. The detection wavelength was chosen based on the maximum absorbance to obtain the highest sensitivity and selectivity of each analyte as shown in **Figure 4.1**.

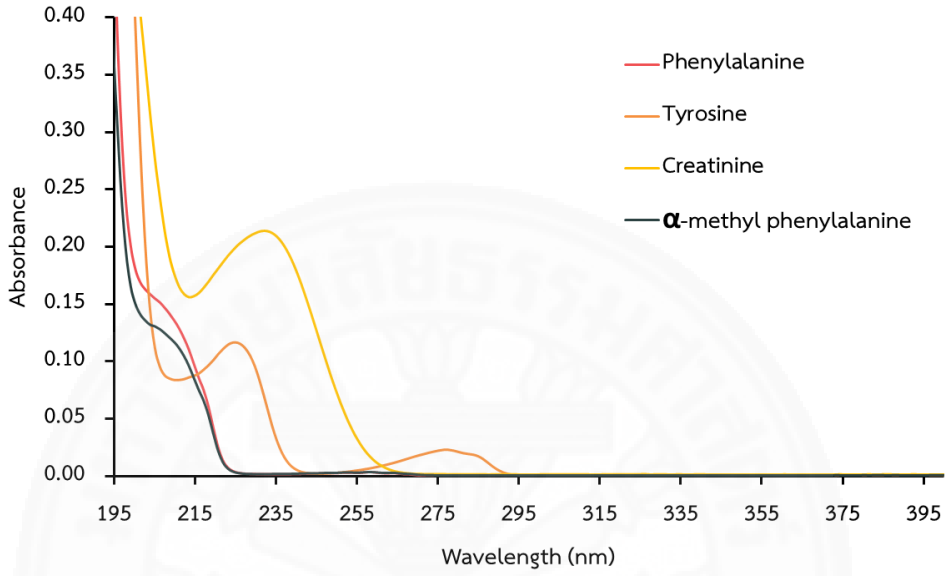

Figure 4.1 UV absorption spectra of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine at a concentration of 200, 200, 100 and 150 mg L^{-1}, respectively.

The maximum wavelength of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine in 0.1 M hydrochloric acid and mobile phase medium was obtained at 210, 225, 234 and 210 nm, respectively. These wavelengths were used as detection wavelength in order to obtain the highest sensitivity of the method.

4.1.2 Mobile phase

The conditions of the mobile phase comprising of pH of buffer, composition and flow rate were studied for simultaneous separation of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine.

4.1.2.1 pH of buffer and mobile phase composition

A common mobile phase for HILIC technique which is water-miscible polar organic solvents such as acetonitrile and methanol with a slight amount of water or buffer was studied [2]. Since the detection wavelength of phenylalanine

and α-methyl phenylalanine (I.S.) is 210 nm, the appropriated organic solvent of the mobile phase must have UV cut-off less than 210 nm as acetonitrile (190 nm).

According to the pH durability of the column which is in the range of pH 2 – 7.5, a buffer solution as an aqueous phase of the mobile phase was investigated at the pH of 3 – 7. Ionic additives, such as ammonium acetate and ammonium formate, are typically used to control the pH of the mobile phase and ion strength. In HILIC separation, they can also conduce to the analyte polarity, leading to differently induce in retention [2].

In this work, the buffer of the mobile phase such as 50 mM of ammonium formate pH 3.0, 50 mM of ammonium formate pH 4.0, 50 mM of ammonium acetate pH 4.0, 50 mM of ammonium acetate pH 5.0, 50 mM of ammonium acetate pH 6.0 and 50 mM of ammonium acetate pH 7.0 were studied to achieve the chromatographic separation of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine in the mixed standard solution. The mobile phase consisting of acetonitrile and buffer was varying its ratio at 80:20, 81:19, 82:18, 83:17, 84:16, 85:15 and 90:10 (v/v). The preliminary results of buffer pH 6.0 and 7.0 were shown in **Figure 4.2**. The bad peak shape of the analytes was obtained; consequently, the mobile phase consisting of buffer at pH 6.0 and 7.0 was not considered to employ in this research.

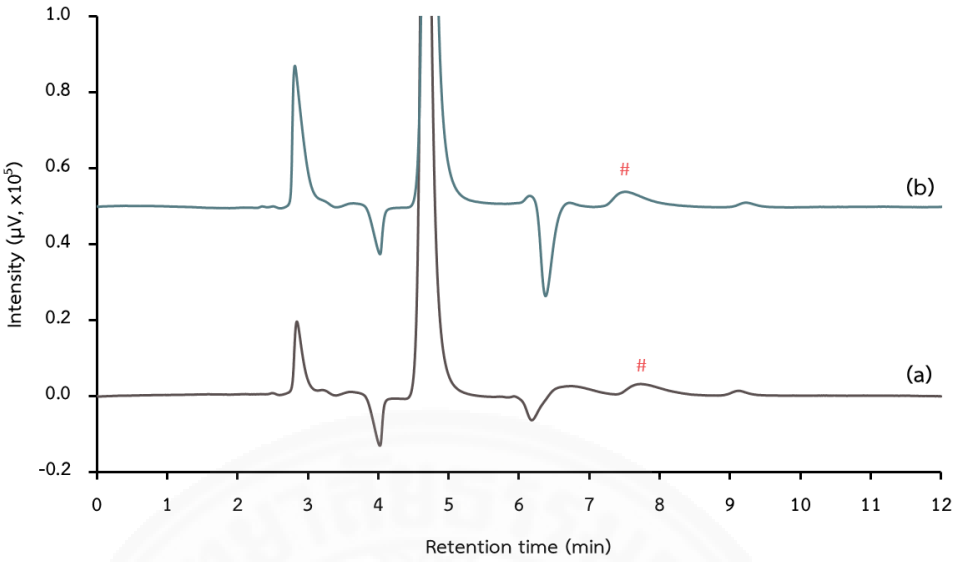

Figure 4.2 HILIC chromatograms of mixed standard solution at wavelength 210 nm for the determination of phenylalanine (#). The conditions of mobile phase were acetonitrile mixed with (a) 50 mM of ammonium formate pH 6.0 and (b) 50 mM of ammonium formate pH 7.0 at the ratio of 80:20 (v/v); flow rate 0.3 mL min^{-1}.

The optimal condition of the pH and the mobile phase ratio was considered based on the separation between analytes and matrix, resolution (R_s) > 1.5, tailing factor < 1.5 and the minimum analysis time. HILIC chromatograms of any pH and ratio of the mobile phase were shown in **Figure 4.3 – 4.6** at the wavelength of 210 nm for phenylalanine and α-methyl phenylalanine (IS). Additional HILIC chromatograms at the wavelength of 225 nm for tyrosine and 234 nm for creatinine were presented in **Appendix A (Figure A1 – A8)**.

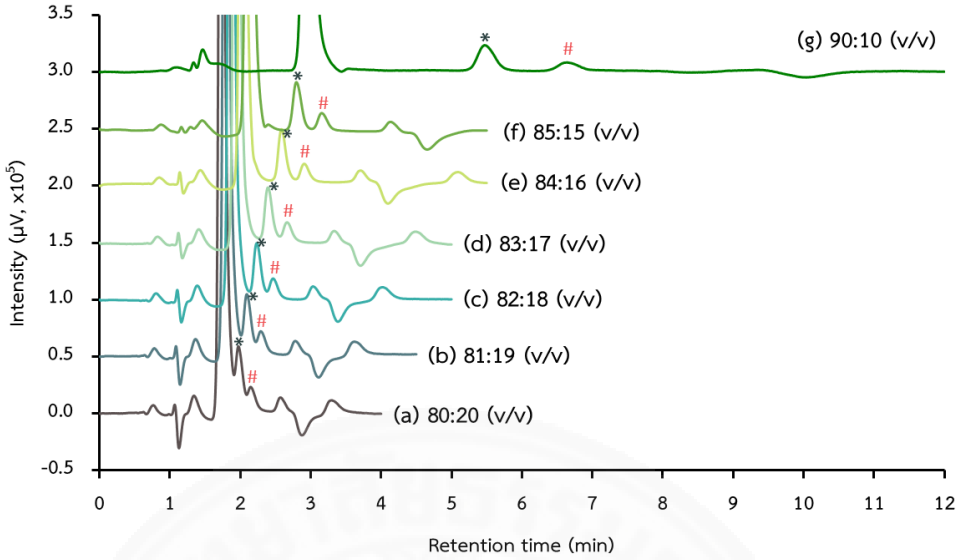

Figure 4.3 HILIC chromatograms of mixed standard solution at wavelength 210 nm for the determination of phenylalanine (#) and α-methyl phenylalanine (*). The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium formate pH 3.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

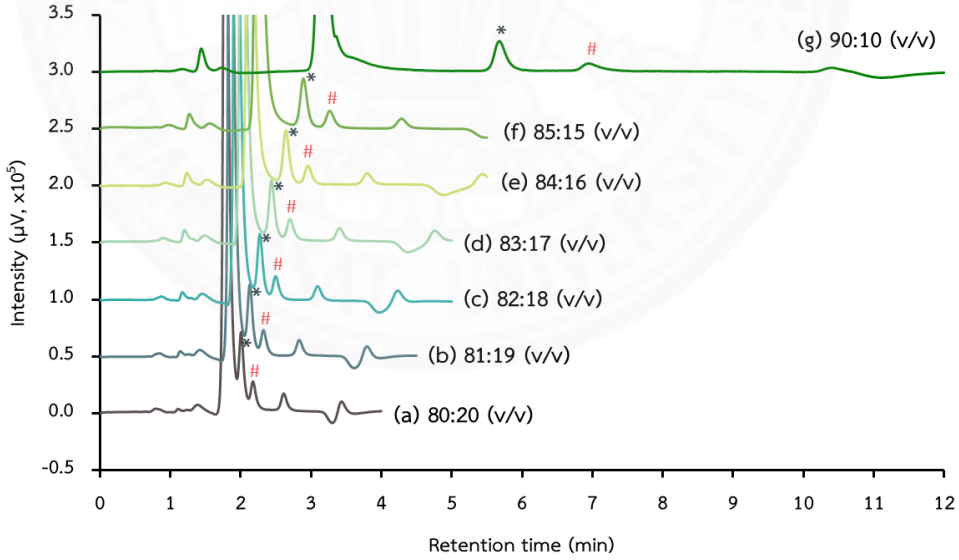

Figure 4.4 HILIC chromatograms of mixed standard solution at wavelength 210 nm for the determination of phenylalanine (#) and α-methyl phenylalanine (*). The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium formate pH 4.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

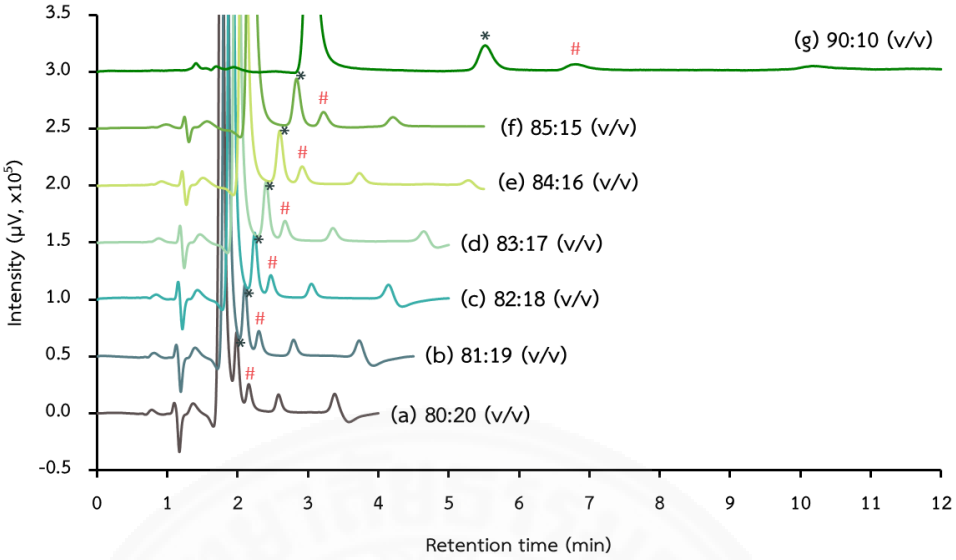

Figure 4.5 HILIC chromatograms of mixed standard solution at wavelength 210 nm for the determination of phenylalanine (#) and α-methyl phenylalanine (*). The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium acetate pH 4.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

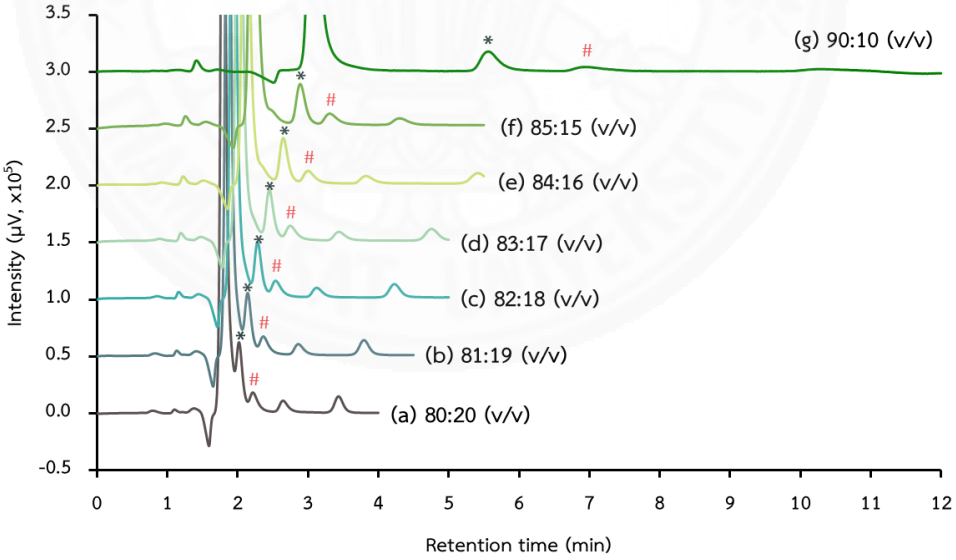

Figure 4.6 HILIC chromatogram of mixed standard solution at wavelength 210 nm for the determination of phenylalanine (#) and α-methyl phenylalanine (*). The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium acetate pH 5.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

The best peak shape of all analytes was achieved when the buffer at pH 3.0 was used as the aqueous phase of the mobile phase. If the ratio of the acetonitrile (organic phase) is increased, the retention time is then longer because of the weaker strength of the mobile phase. The HILIC chromatogram at a wavelength of 210 nm was necessarily examined because it can be used for detection of phenylalanine, α-methyl phenylalanine and almost interferences which can absorb UV light at this wavelength.

The mobile phase comprising of acetonitrile:50 mM of ammonium formate pH 3.0 at the ratio of 84:16 (v/v) led to the best separation, good peak shape and the shortest analysis time (more informations of resolution (R_s), tailing factor and analysis time were presented in **Appendix B)**. Therefore, acetonitrile:50 mM of ammonium formate pH 3.0 at the ratio of 84:16 (v/v) was selected as the mobile phase of this work.

4.1.2.2 Flow rate

Flow rate affects the retention time of the analytes as well as the analysis time. Then, the appropriate flow rate is necessary to be studied. Flow rate of 0.6, 0.7 and 0.8 mL min^{-1} was studied with acetonitrile mixed with 50 mM ammonium formate pH 3.0 (84:16, v/v) as the mobile phase. The fastest analysis time with the maintenance of the separation of all analytes was considered to choose the flow rate. Moreover, the limiting factors in the choosing of flow rate are column and instrument pressure limitations. The column pressure limit was 2900 psi.

The results of varying flow rate at 0.6, 0.7 and 0.8 mL min^{-1} were showed in **Figure 4.7**. The higher flow rate was operated, the faster analysis time was obtained. The analysis time was 7, 6 and 5 minutes when the flow rate of 0.6, 0.7 and 0.8 mL min^{-1} was operated, respectively. The HILIC chromatograms of each flow rate were presented in **Appendix C (Figure C1 – C3)**. While the flow rate of 0.8 mL min^{-1} provided the fastest analysis time with the good separation of the analytes and matrices, the pressure of the column was near the limitation. As a result, a flow rate of 0.8 mL min^{-1} was chosen for this research.

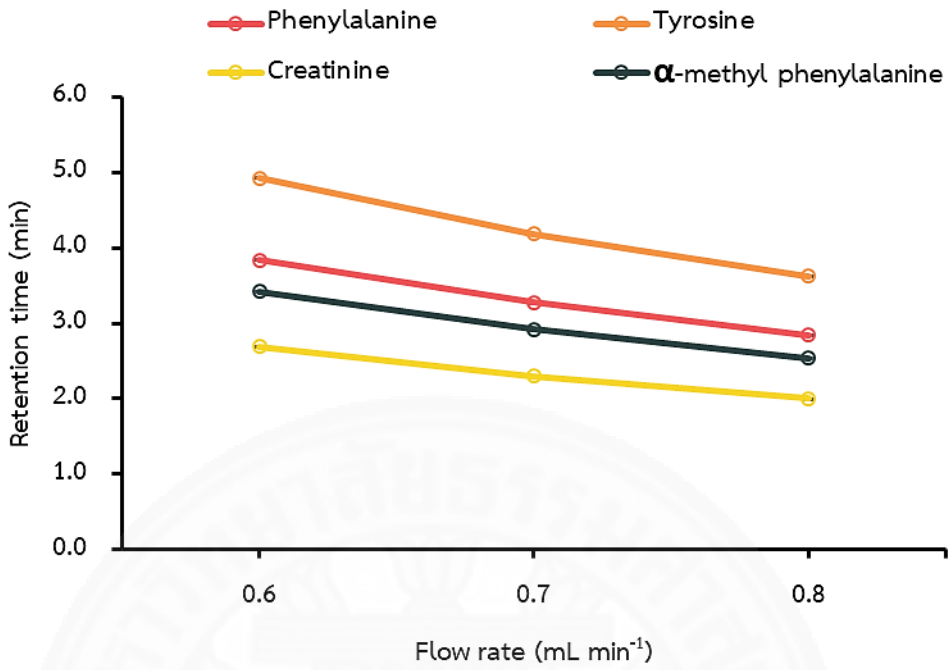

Figure 4.7 The retention time of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (IS). The conditions of the mobile phase were acetonitrile mixed with 50 mM of ammonium formate pH 3.0 at the ratio of 84:16 (v/v) with flow rates of 0.6, 0.7 and 0.8 mL min^{-1}.

4.1.3 Injection volume

To enhance sensitivity especially for the urine analysis of phenylketonurics which have a low amount of tyrosine, investigation of injection volume is important. The increase of sample volume can enhance sensitivity until it overloads the column. Volume overload leads to altered peak shapes. Thus, the appropriate injection volume was studied.

Injection volume was studied at the volume of 1, 2, 3, 4 and 5 µL with the optimal condition of the mobile phase (acetonitrile mixed with 50 mM of ammonium formate pH 3.0 at the ratio of 84:16 v/v and flow rate of 0.8 mL min^{-1}). The selection criteria were based on the obtaining of the highest sensitivity of the method with the correlation of good precision and the good peak shape. **Figure 4.8** showed HILIC chromatograms of mixed standard solution at the wavelength of 210 nm for the determination of phenylalanine and α-methyl phenylalanine, additional HILIC

chromatograms at the wavelength of 225 nm for tyrosine and 234 nm for creatinine were presented in **Appendix D** and **Table 4.1** showed the precisions and the tailing factor of the analytes when the injection volumes were 1, 2, 3, 4 and 5 µL.

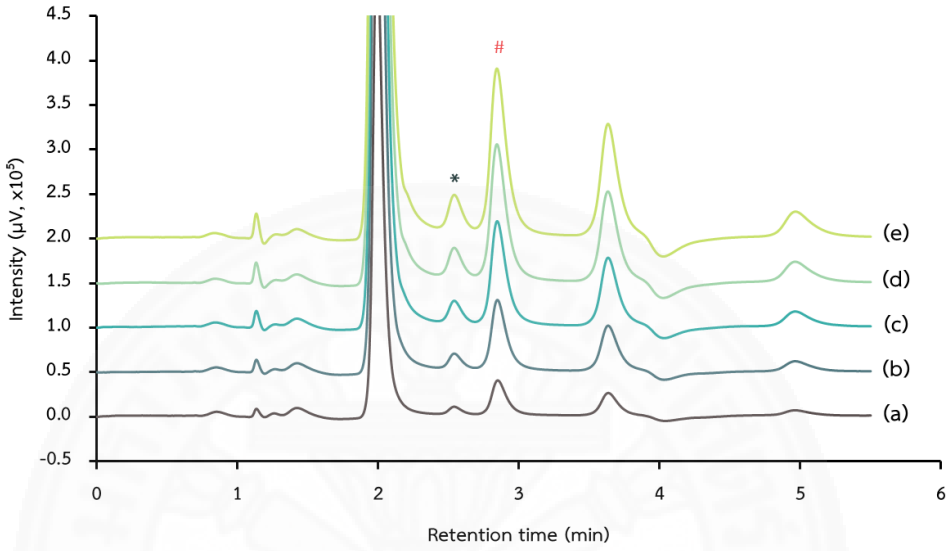

Figure 4.8 HILIC chromatograms of mixed standard solution at wavelength 210 nm for the determination of phenylalanine (#) and α-methyl phenylalanine (*). The conditions of mobile phase were 84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0 at flow rate of 0.8 mL min^{-1} with the injection volumes (a) 1 µL, (b) 2 µL, (c) 3 µL, (d) 4 µL and (e) 5 µL.

Table 4.1 The precision and the tailing factor of each analyte at the injection volume of 1, 2, 3, 4 and 5 µL

Injection	Precision (Repeatability; %RSD)[a]				Tailing Factor[b]			
volume (µL)	Phe	Tyr	Cre	I.S.	Phe	Tyr	Cre	I.S.
1	1.47	2.91	1.63	2.91	1.32	1.20	1.46	1.15
2	1.19	1.98	0.62	2.02	1.32	1.22	1.46	1.16
3	0.45	0.82	0.52	0.71	1.38	1.22	1.48	1.17
4	0.32	0.85	0.41	0.71	1.38	1.23	1.48	1.21
5	0.23	0.46	0.34	0.28	1.39	1.24	1.53	1.21

[a] n = 5; [b] The calculation method of USP (see **Appendix E**).

The increase of injection volume enhanced sensitivity and the better precision was reached. However, the peak shape was broadening due to the increased tailing factor. The injection volume of 4 µL was chosen as the optimal injection volume for this work because it gave high sensitivity with the good peak shape (tailing factor < 1.5).

4.2 Analysis of urine

4.2.1 Sample preparation

Due to the complicated matrices in urine, sample preparation for determination of phenylalanine, tyrosine and creatinine in urine was studied consisting of sample clean-up and preconcentration step.

4.2.1.1 Sample dilution

Normally, the dilution of urine with water or buffer is carried out in order to reduce the matrix effect and the pH adjusted appropriately prior to sample addition of the SPE procedure. In this research, urine samples were diluted 10 times with 0.1 M of hydrochloric acid according to the sample pretreatment procedure [85] and the recommendation sample pretreatment (pH adjustment) from Macherey-Nagel application database [83], and this dilution step was conformed to an experimental result in Section 4.2.1.2 (3).

4.2.1.2 Optimization of solid phase extraction (SPE)

A sample preparation of urine sample was carried out using solid-phase extraction (SPE). An appropriate type of sorbent was considered for the simultaneous extraction of phenylalanine, tyrosine, creatinine and **α**-methyl phenylalanine (I.S.). The structures of phenylalanine, tyrosine and **α**-methyl phenylalanine (I.S.) contain an amino group and the structure of creatinine which is imidazole compound contains amine groups. **Figure 4.9 – 4.12** showed the structures of analytes that are protonated form in the pH range of 1 – 4. This research used strong cation exchange (SCX) solid-phase extraction as the sample preparation due to its effective extraction of the positively charged compounds.

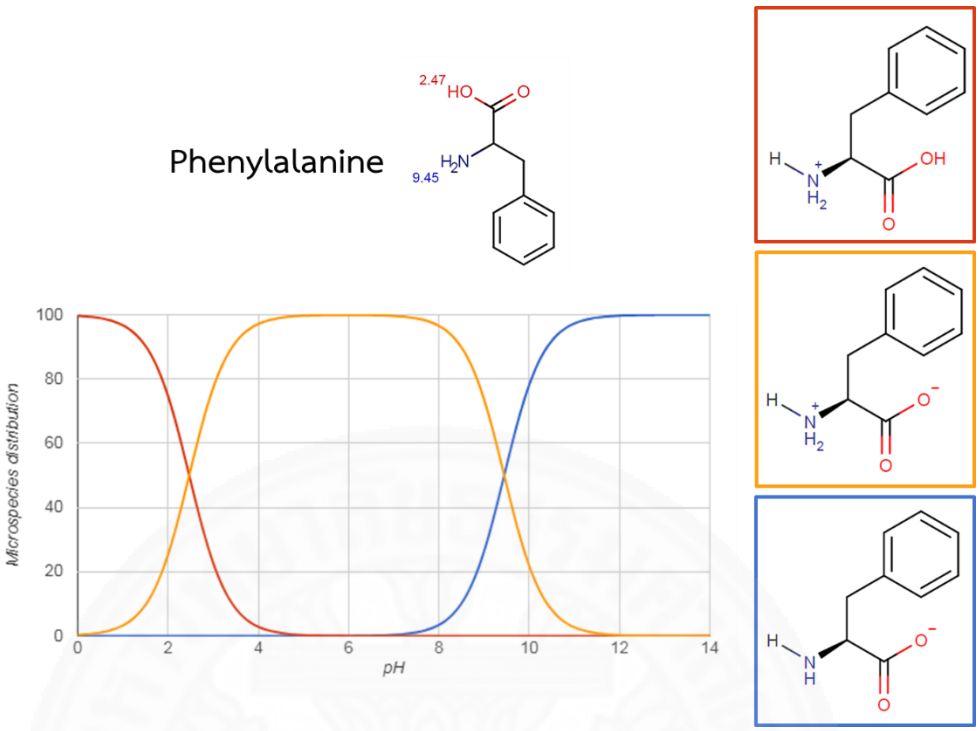

Figure 4.9 The dissociation of phenylalanine at any pH [4]

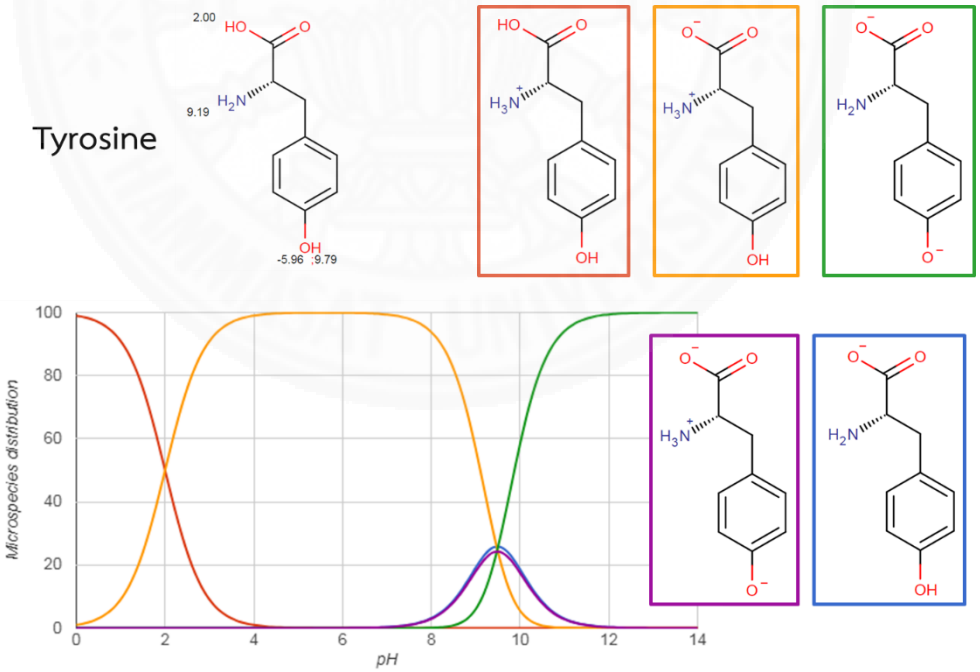

Figure 4.10 The dissociation of tyrosine at any pH [94]

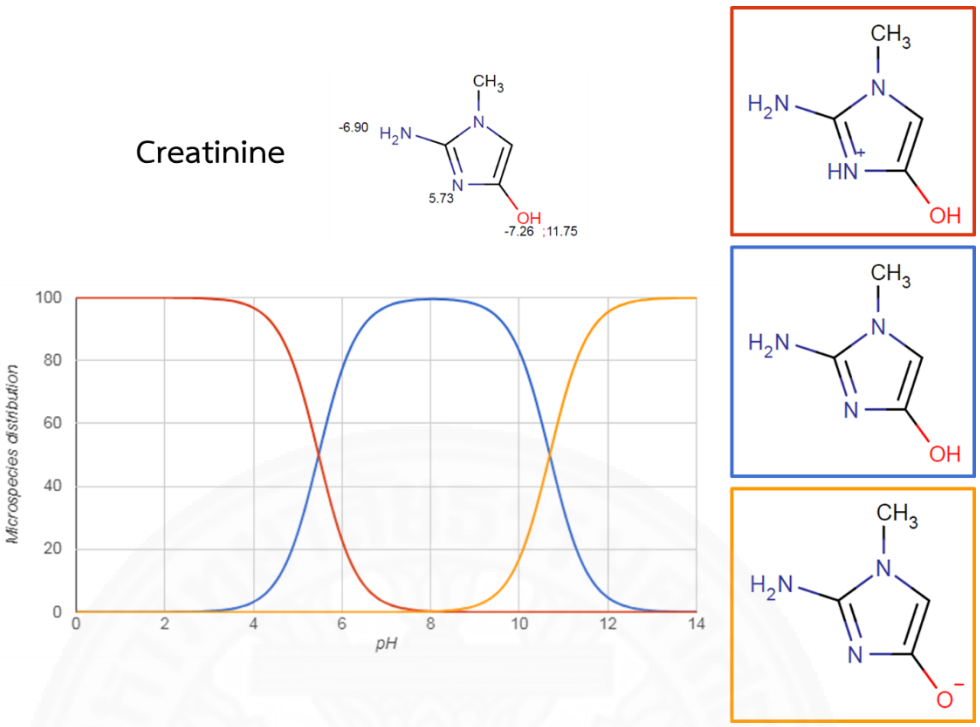

Figure 4.11 The dissociation of creatinine at any pH [94]

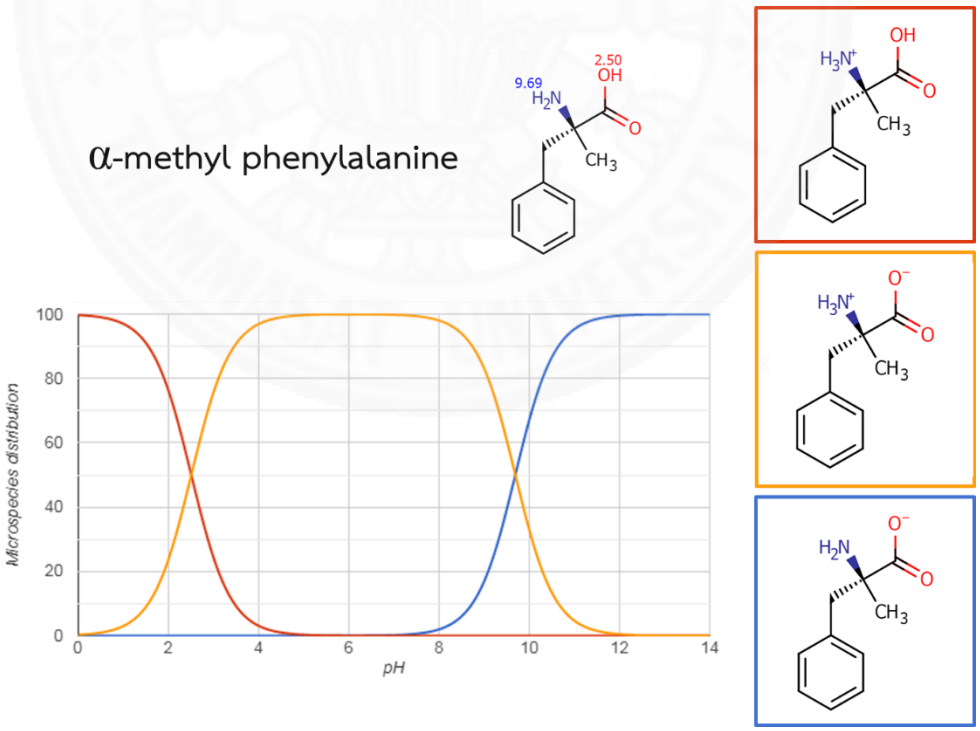

Figure 4.12 The dissociation of α-methyl phenylalanine (I.S.) at any pH [94]

Various parameters of solid-phase extraction such as a functional group of strong cation exchange, type and volume of conditioning reagent, pH of loading sample, type and volume of washing and eluting reagent were studied in this work in order to obtain the highest extraction efficiency.

(1) Type of sorbent

Two types of sorbent namely benzenesulfonic acid modified silica [83] and aliphatic sulfonic acid bonded silica with Na^+ counter-ion [86] with the equal weight of adsorbent were investigated and compared the extraction efficiency. The preliminary results demonstrated that HILIC chromatograms of the reagent blank and the mixed standard solution using benzenesulfonic acid modified silica as SPE were obtained and interfered by matrix as shown in **Figure 4.13 – 4.14**.

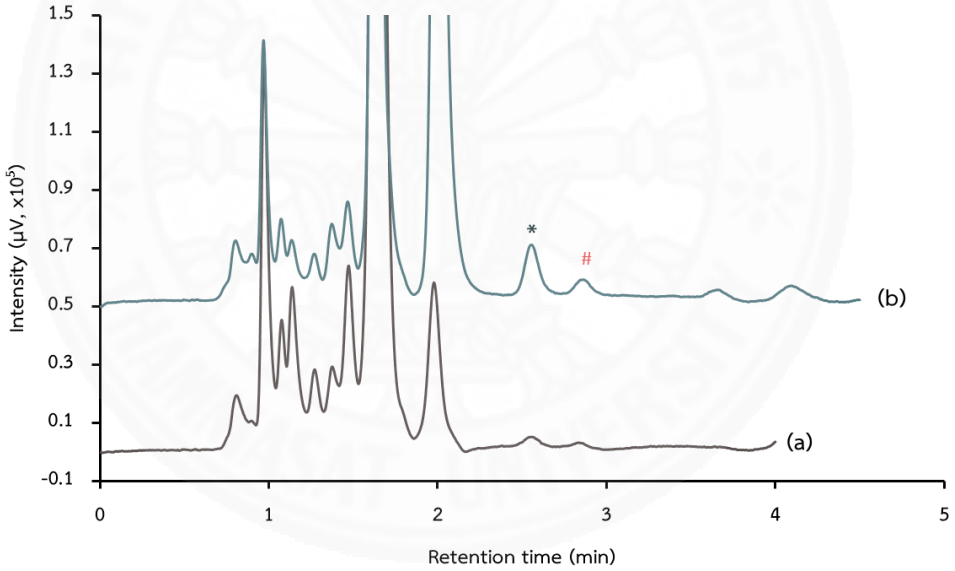

Figure 4.13 HILIC chromatograms at wavelength 210 nm of
(a) Reagent blank extracted through benzenesulfonic acid modified silica as SCX-SPE.
(b) Mixed standard solution of phenylalanine (#) and α-methyl phenylalanine (*) extracted through benzenesulfonic acid modified silica as SCX-SPE.

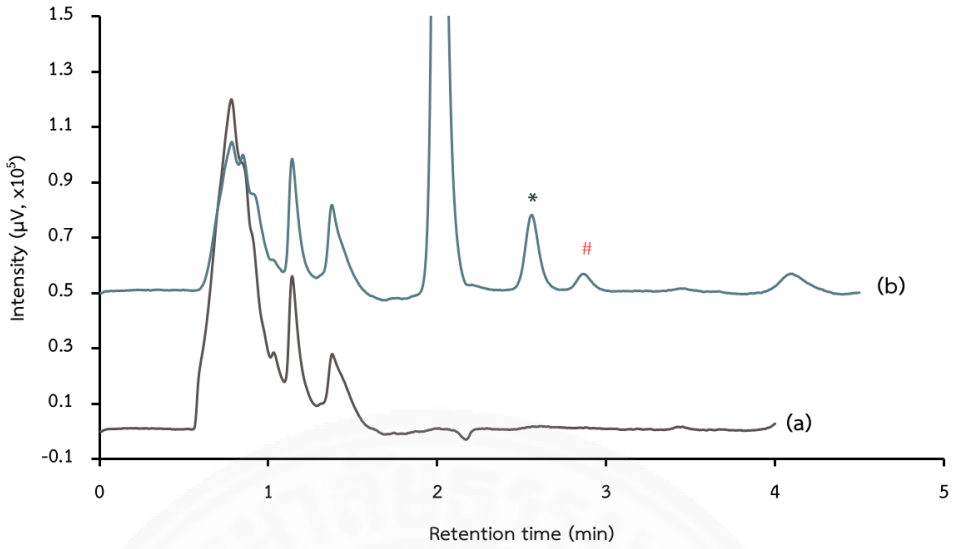

Figure 4.14 HILIC chromatograms at wavelength 210 nm of
(a) Reagent blank extracted through aliphatic sulfonic acid bonded silica as SCX-SPE.
(b) Mixed standard solution of phenylalanine (#) and α-methyl phenylalanine (*) extracted through aliphatic sulfonic acid bonded silica as SCX-SPE.

In this work, the aliphatic sulfonic acid bonded silica as a strong cation exchange solid-phase extraction was therefore selected for the simultaneous extraction of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine in standard solution and urine sample.

(2) Conditioning

SPE conditioning is an important step in order to provide repeatable interaction between the sorbent and analytes. This step prepares the sorbent prior to adsorption of the analytes [83]. Strong cation exchange sorbent which used for samples in aqueous solution or polar solvent should be conditioned with the water-miscible solvent, then an appropriate pH of an aqueous solution. Normally, one tube-full volume of conditioning reagent was introduced to the SPE sorbent before extracting the sample [86]. Moreover, Macherey-Nagel application database [83] recommends the conditioning of SCX sorbent (100 mg weight and 1 mL of cartridge volume) utilizing 5 mL of methanol followed by 5 mL of 1:1 (v/v) of methanol:0.1 M hydrochloric acid and then 5 mL of 0.1 M hydrochloric acid.

In this research, 500 mg weight of SCX sorbent and 3 mL of cartridge volume was employed for extraction. The various volumes of the conditioning reagent were studied in order to produce an environment which is appropriate for extraction of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine. The volume of each conditioning reagent was optimized by 3 mL (one tube volume) in accordance with the manufacturer protocol [86], 6 mL (two tube volume), 9 mL (three tube volume) and 25 mL (5 times volume proportional to sorbent weight) as the Macherey-Nagel application database protocol [83].

The results were shown in **Figure 4.15**. The y-axis in the left-hand side represents the peak area of phenylalanine, tyrosine and α-methyl phenylalanine and the y-axis in the right-hand side represents the peak area of only creatinine. The higher volume of conditioning reagent was operated, the higher extraction efficiency was obtained and reached the constant when 9 and 25 mL of conditioning regent was performed. Nevertheless, the usage of a higher volume of the conditioning reagent affected the operation time. The volume of 9 mL of each conditioning reagent was therefore selected as the optimal condition in order to achieve the highest extraction efficiency with the fastest operation time. The conditioning step of SCX-SPE was carried out as follows: 9 mL of methanol, 9 mL of 1:1 (v/v) of methanol:0.1 M hydrochloric acid and then 9 mL of 0.1 M hydrochloric acid.

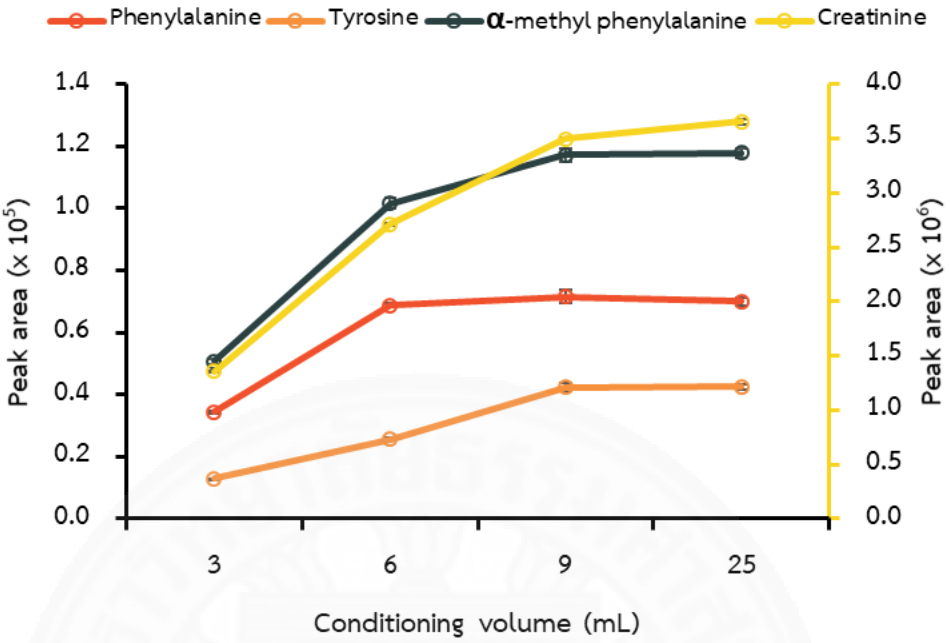

Figure 4.15 The extraction efficiency of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) obtained when the SPE was conditioned with the volume of 3, 6, 9 and 25 mL of each conditioning reagent.

(3) Loading

Sample loading or sample application was performed by accurate transfer of the sample to an SPE cartridge. The analytes in the sample must be in a form which is appropriate with the conditioning SCX-SPE. The retention of the analytes on the SCX-SPE depended on the pH of the sample, because of the cation exchange interaction mechanism. At lower pH conditions, the cation exchange interaction between analytes and SCX-SPE were strong and the analytes should be effectively retained on the SCX-SPE cartridge. Therefore, the samples must be adjusted to lower pH.

As shown in **Figure 4.9 – 4.12**, the analytes were protonated to cation or zwitterion in acidic conditions (pH 1 – 4). In this work, the pH of the loading sample was studied in order to obtain the highest extraction efficiency. The mixed standard solution was adjusted the pH to 1, 2, 3 and 4 by 0.1 M hydrochloric acid and 1 M sodium hydroxide solution.

The result showed that the highest extraction efficiency was reached at pH 1 and lost the extraction efficiency when the loading pH was up. As shown in **Figure 4.16,** phenylalanine and tyrosine was non-detectable when the sample pH was adjusted to pH 3 and 4. At higher pH value, the cation exchange interaction became weak and the analytes may lose in the pre-mature elution. According to pH 1, the highest fraction of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) in the protonated form was conduced as shown in **Figure 4.9 – 4.12**.

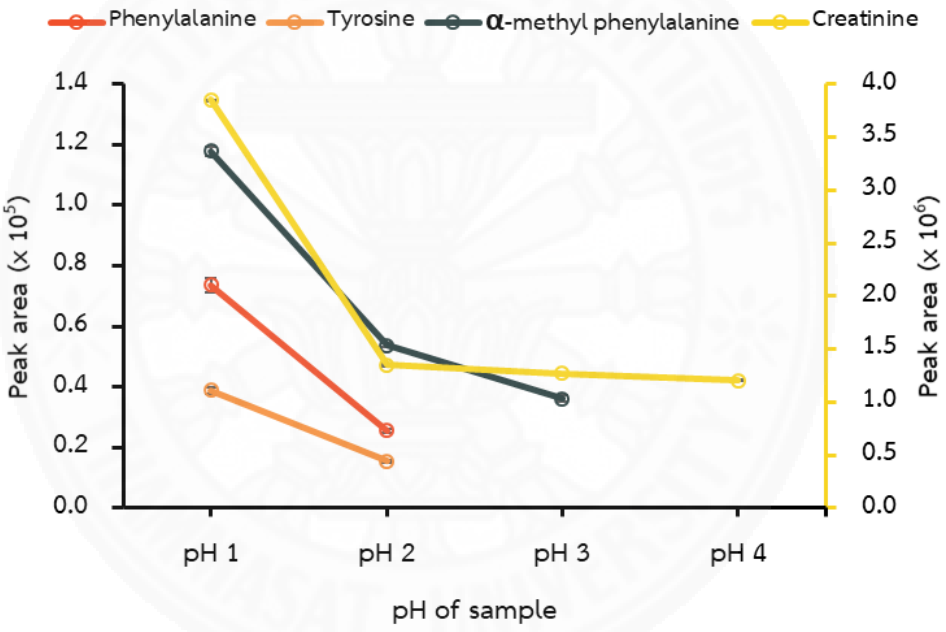

Figure 4.16 The effect of loading pH on the extraction efficiency of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) from the mixed standard solution.

Accordingly, the sample pH adjustment to pH 1 with 1 mL of loading sample was chosen as the optimal condition of the loading step in order to achieve the highest extraction efficiency and the fast sample preparation time.

(4) Washing

Washing of the sorbent is usually achieved with washing reagent to eliminate matrices which can be retained on SCX-SPE and then eluted with the analytes. Some matrices may interfere the separation of the chromatographic system; consequently, these matrices should be washed with the suitable washing reagent. Typically, the matrices were washed off using the same solvent of the sample, or another solution that was not eluted the analytes from the SCX sorbent in this step. Usually, one tube-full volume of washing solution is required [86].

(4.1) Type of washing reagent

The same solution of the sample which is 0.1 M hydrochloric acid and type I water [86] was preliminarily examined as washing reagents with washing volume ranged of 1 – 5 mL. The preliminary test was performed with the mixed standard solution. When the SCX-SPE was washed off using 0.1 M of hydrochloric acid, the SCX sorbent was dissolved as the white precipitate in the eluate. The functional group of SCX sorbent may be degradation leading to non-retained of the analytes. When the SCX-SPE was washed off using 1 – 3 mL of type I water, the SCX sorbent was slightly dissolved because the volume of type I water was not enough to wash off all of 0.1 M hydrochloric acid from the conditioning and loading step. The content of phenylalanine, creatinine and α-methyl phenylalanine was extracted and kept constant since 4 mL of type I water was applied. However, the higher volume of type I water (4 and 5 mL) was employed, the content of tyrosine was decreased. Because of its high polarity property, tyrosine can be washed off with type I water during the washing step (**Figure 4.17**).

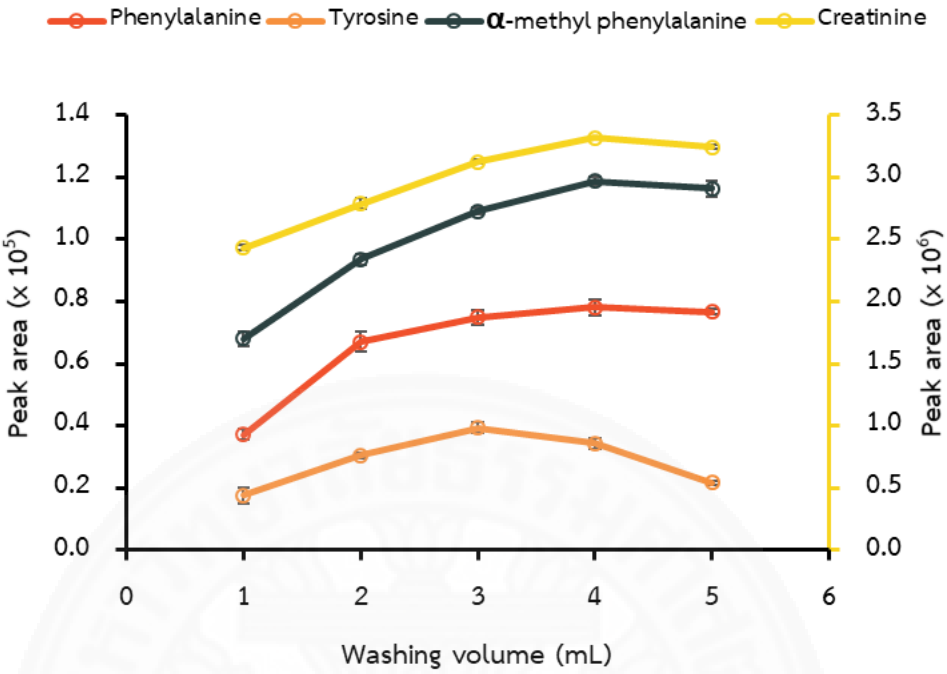

Figure 4.17 The effect of various volume of type I water as washing reagent on the extraction efficiency of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) from the mixed standard solution.

In order to avoid the removed of tyrosine during the washing step, the lower polarity solvent such as methanol were also considered. Consequently, types of washing reagent at fixed volume were investigated to wash the matrices of the urine sample as follows:

- 4 mL of type I water [83, 95]
- 4 mL of methanol [57, 85]
- 2 mL of type I water followed by 2 mL of methanol
- 4 mL of type I water:methanol at a ratio of 1:1 (v/v)

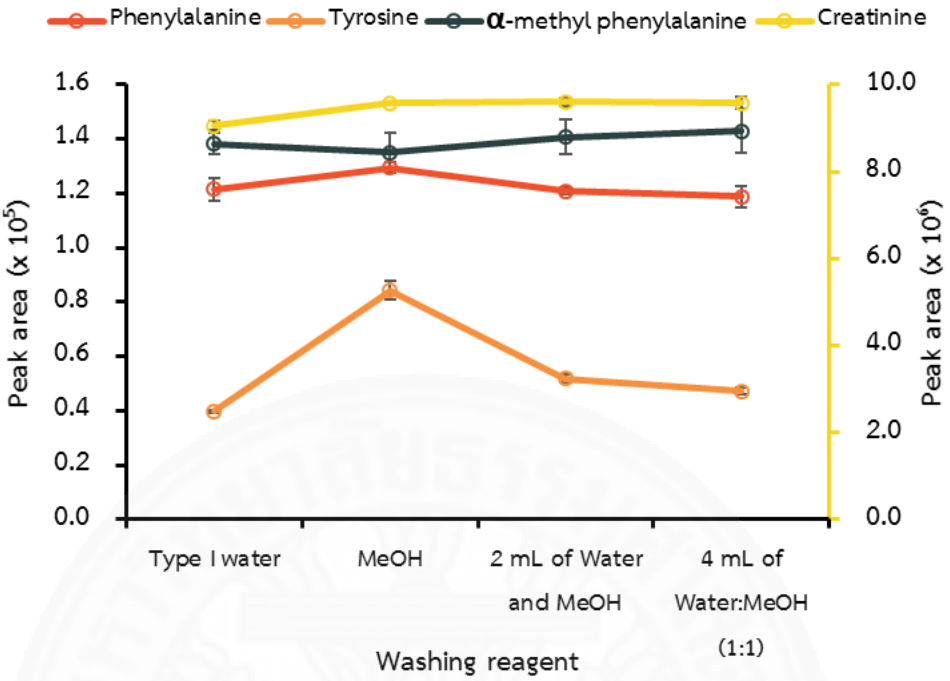

Figure 4.18 The effect of various washing reagent on the extraction efficiency of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) from the urine sample.

As can be seen from **Figure 4.18**, when the different types of washing reagent were applied, the extracted content of phenylalanine, creatinine and α-methyl phenylalanine was not significantly different except the tyrosine. The highest content of tyrosine was obtained when methanol was using as the washing reagent. Because the polarity of this reagent was lower than the others which consist of type I water, tyrosine was not eluted in this step. Consequently, methanol was selected as a washing reagent.

(4.2) Washing volume

The washing volume of methanol was optimized to wash the matrices in urine which can be retained on the SCX sorbent. The washing volume was decided based on the elimination of matrices which observed from the UV spectrum of extracted analytes from urine sample comparing mixed standard solution and still provided the highest extraction efficiency.

The results indicate that the higher volume of methanol was implemented, the more elimination of matrices was carried out. As shown in **Figure 4.19,** the UV spectrum of the extracted analytes from the urine sample was nearly similar to the mixed standard solution especially phenylalanine. Phenylalanine was detected at the wavelength of 210 nm that almost matrices can be absorbed light at this wavelength. Additional UV spectra of the extracted phenylalanine from the urine sample with the usage of 3 mL methanol comparing with 4 mL methanol as washing reagent were presented in **Appendix F**.

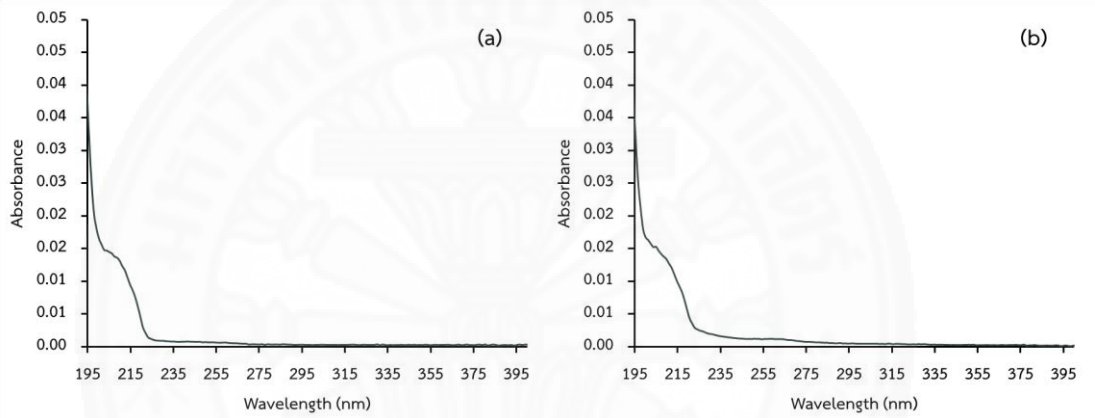

Figure 4.19 UV absorption spectra of (a) the extracted phenylalanine from the mixed standard solution comparing with (b) the extracted phenylalanine from the urine sample with the usage of 4 mL methanol as washing reagent.

As shown in **Figure 4.20**, when 1 and 2 mL of methanol were employed, the peak area of phenylalanine and tyrosine was higher and then decreased until kept constant at 3 mL. Because methanol can be eliminated the co-eluted matrix of the analytes peak especially phenylalanine which can be interfered from many matrices.

In order to achieve the efficient elimination of matrices, the highest sensitivity of all analytes and the shortest operation time of the washing step, 4 mL of methanol was therefore chosen as the washing reagent.

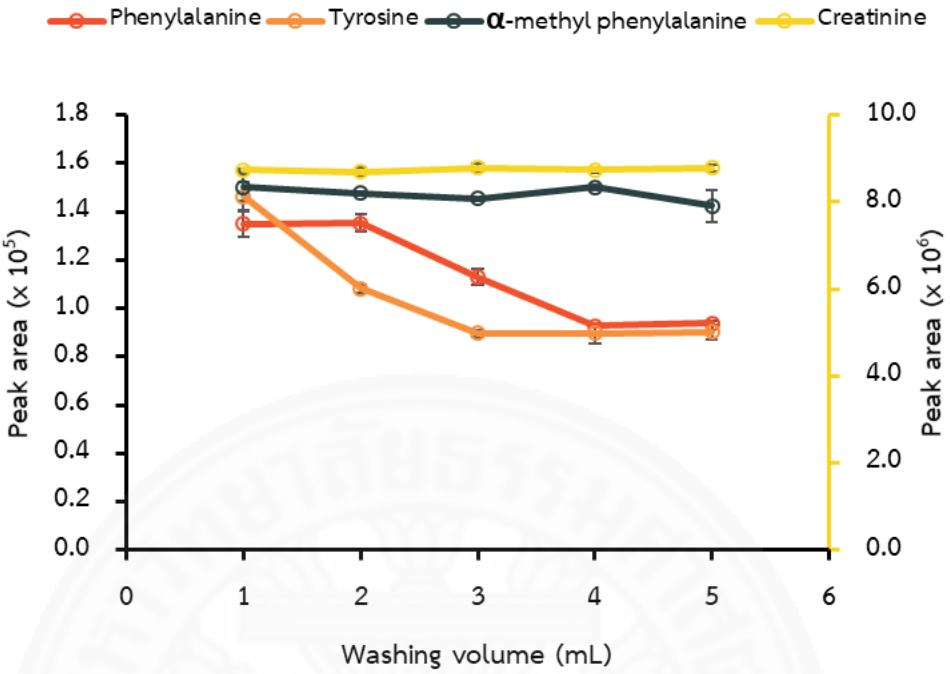

Figure 4.20 The effect of varied volume of methanol as washing reagent on the extracted content through SCX-SPE of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) from the urine sample.

(5) Eluting

Eluting step of SCX-SPE is an important step. The eluting reagent should remove the analytes but leaves behind any matrices which not expelled in the washing step. The eluting step was normally done with a reagent or solvent at 2 pH units above the analytes' pKa for neutralizing the analytes, or through a different cation which can displace the retained analytes on the SCX sorbent [86]. Therefore, 0.5 M of ammonia solution was considered as the eluting reagent because it has the properties as follows: the pH of 0.5 M ammonia solution is about pH 9 and it can easily be evaporated to preconcentration the analytes. The extracted samples were reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) before injected to HILIC system so that the pH of injection solution was in the range of column pH durability and the effect of refractive index was reduced when the detector was DAD or UV detector. This research studied the type of solvent and the volume of eluting reagent.

(5.1) Type of eluting reagent (solvent)

Various solvents of 0.5 M ammonia solution consisting of type I water, methanol, ethanol and acetonitrile were studied in both mixed standard solution and urine sample.

As shown in **Figure 4.21 – 4.22**, the extracted amount of phenylalanine, creatinine and α-methyl phenylalanine from mixed standard solution and urine sample tended to be increased when the eluting reagents were 0.5 M ammonia in acetonitrile, 0.5 M ammonia in type I water, 0.5 M ammonia in methanol which gave the same extraction efficiency with 0.5 M ammonia in ethanol, respectively. On the other hand, the highest extraction efficiency of tyrosine was achieved while 0.5 M ammonia in ethanol was utilizing as an eluting reagent. The polarity of 0.5 M ammonia in ethanol was matched with the polarity of all analytes.

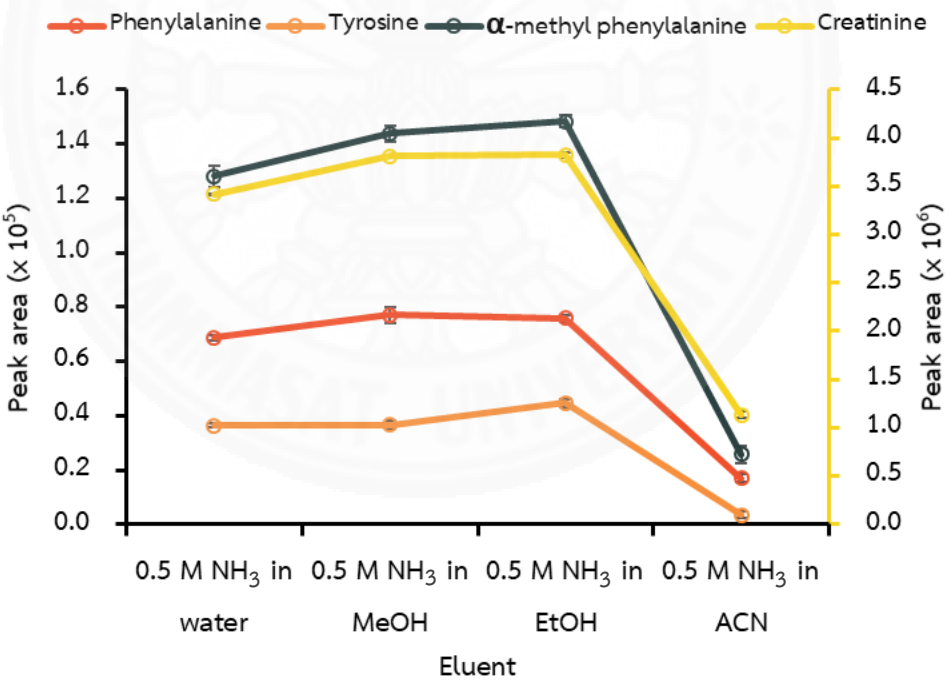

Figure 4.21 The effect of various eluting reagent on the extraction efficiency of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) from the mixed standard solution.

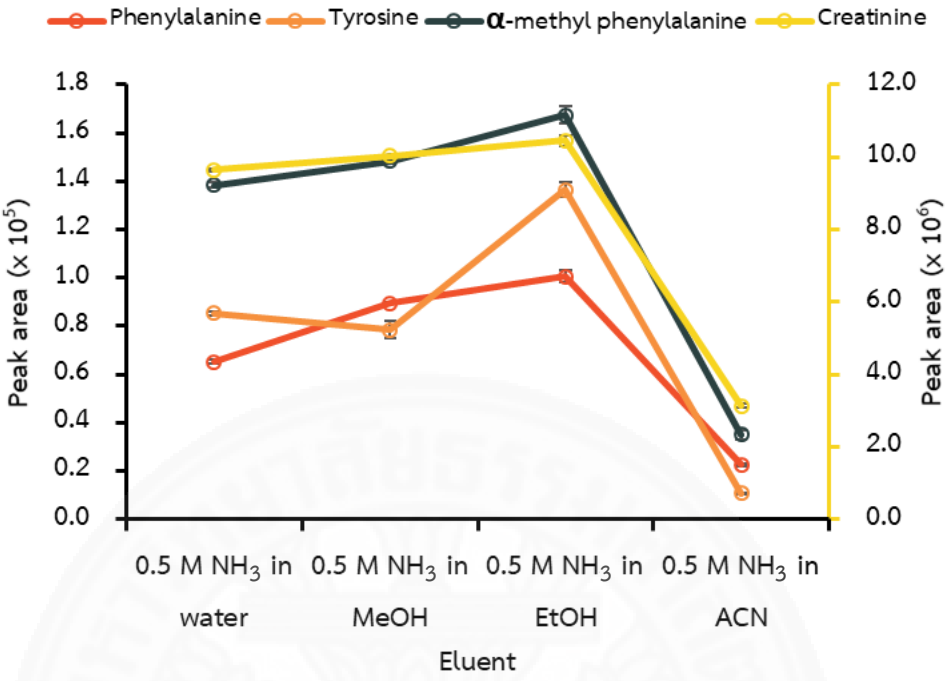

Figure 4.22 The effect of various eluting reagent on the extraction efficiency of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) from the urine sample.

(5.2) Eluting volume

The optimal volume of 0.5 M ammonia in ethanol was examined in both mixed standard solution and urine sample so as to obtain the highest extraction efficiency of all analytes (**Figure 4.23 – 4.24**).

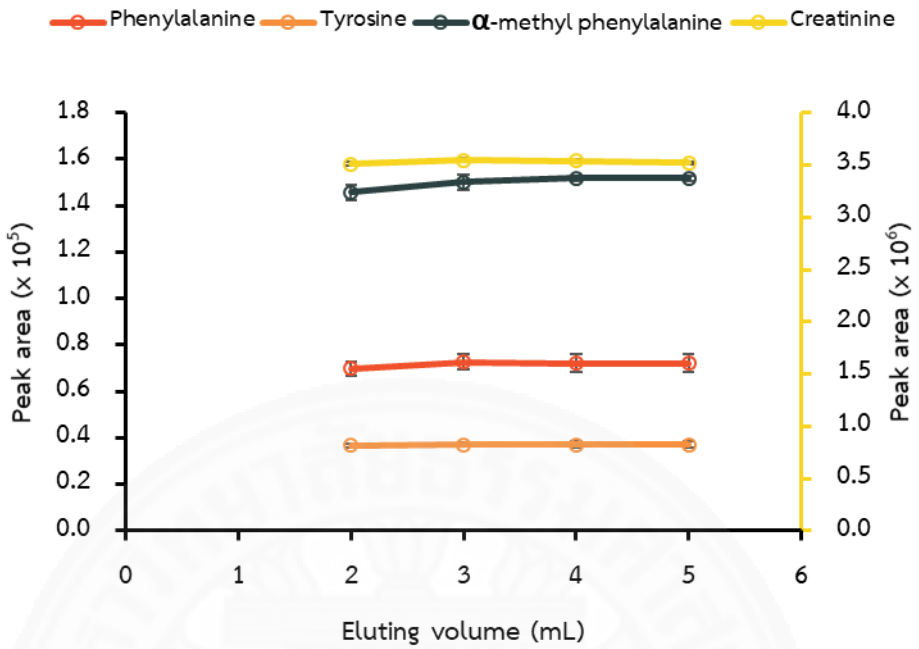

Figure 4.23 The effect of eluting volume of 0.5 M ammonia in ethanol as an eluting reagent on the extraction efficiency of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) from the mixed standard solution.

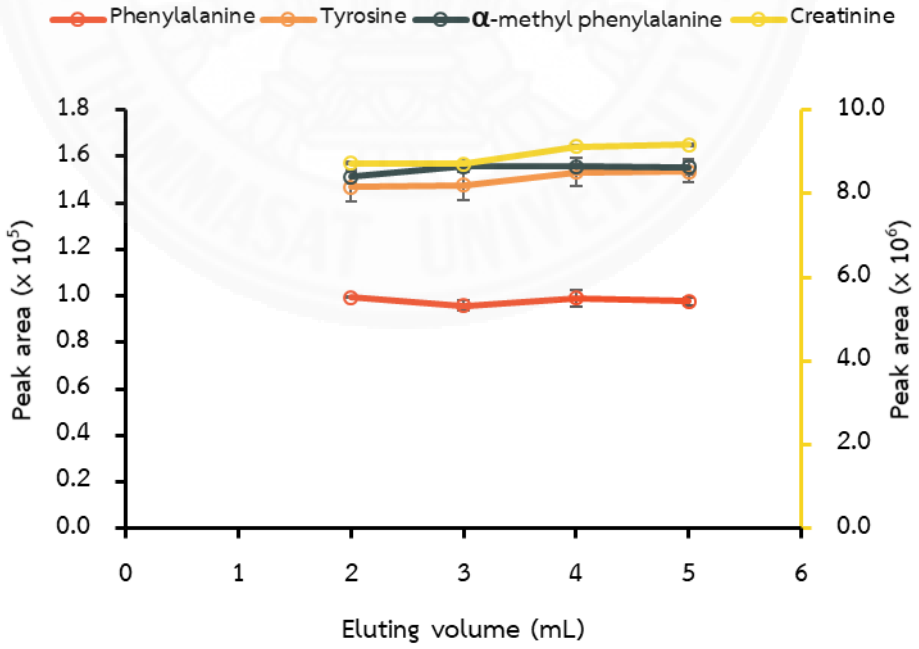

Figure 4.24 The effect of eluting volume of 0.5 M ammonia in ethanol as an eluting reagent on the extraction efficiency of phenylalanine, tyrosine, creatinine and α-methyl phenylalanine (I.S.) from the urine sample.

From the results, all of the extracted analytes from the mixed standard solution and the urine sample were demonstrated a similar trend. When 1 mL of 0.5 M ammonia in ethanol was implemented as eluent, the analytes were non-detectable because the volume of the eluent was not sufficient for eluting in comparing with 500 mg weight of SCX sorbent. The low extracted amount of all analytes was not enough to detect by the HILIC technique.

The higher volume of eluent was applied, the higher amount of the extracted analytes was obtained until kept constant at 3 mL of the eluent. Due to the higher concentration of the analytes in the urine sample, the volume of the eluent was selected at 4 mL of 0.5 M ammonia in ethanol as the eluting reagent to ensure that the analytes were completely eluted from the SCX sorbent. The optimal condition of SCX-SPE was summarized in the **Table 4.2**.

All the eluates of mixed standard solution and urine sample were evaporated to dryness under nitrogen gas at 60°C for 30 minutes and then reconstituted with 100 µL of mobile phase (84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0) prior to injection into the HILIC system.

Table 4.2 The optimal condition of SCX-SPE for the determination of phenylalanine, tyrosine and creatinine in urine.

Parameter	Optimal condition
Type of sorbent	Aliphatic sulfonic acid bonded silica
Conditioning reagent and volume	Step 1: 9 mL of methanol Step 2: 9 mL of 1:1 (v/v) of methanol:0.1 M hydrochloric acid Step 3: 9 mL of 0.1 M hydrochloric acid
Loading volume and pH	1 mL of sample (adjusted to pH 1)
Washing reagent and volume	4 mL of methanol
Eluting reagent and volume	4 mL of 0.5 M ammonia in ethanol

4.2.2 Method validation

The developed HILIC with SPE for urinary analysis was validated using the Appendix F: Guidelines for Standard Method Performance Requirements by Association of Official Analytical Communities (AOAC) as a guideline [92].

4.2.2.1 Internal calibration curve

Internal calibration curves were carried out using seven standard concentrations with three replicate injections of mixed standard solution extracted through SCX-SPE. The internal calibration curves were plotted between peak area of the analytes divided by peak area of an internal standard (α-methyl phenylalanine) (the y-axis) and the series of each analyte concentrations (the x-axis). A high coefficient of determination (e.g., >0.99) is often recommended as evidence of the goodness of fit [93]. The results showed that the working ranges of the method were linear in the range of 1 – 400 mg L^{-1} with the acceptable coefficient of determination (r^2) of 0.9998 for phenylalanine, 0.5 – 200 mg L^{-1} with r^2 = 0.9997 for tyrosine and 3 – 3000 mg L^{-1} with r^2 = 0.9990 for creatinine (**Figure 4.25**).

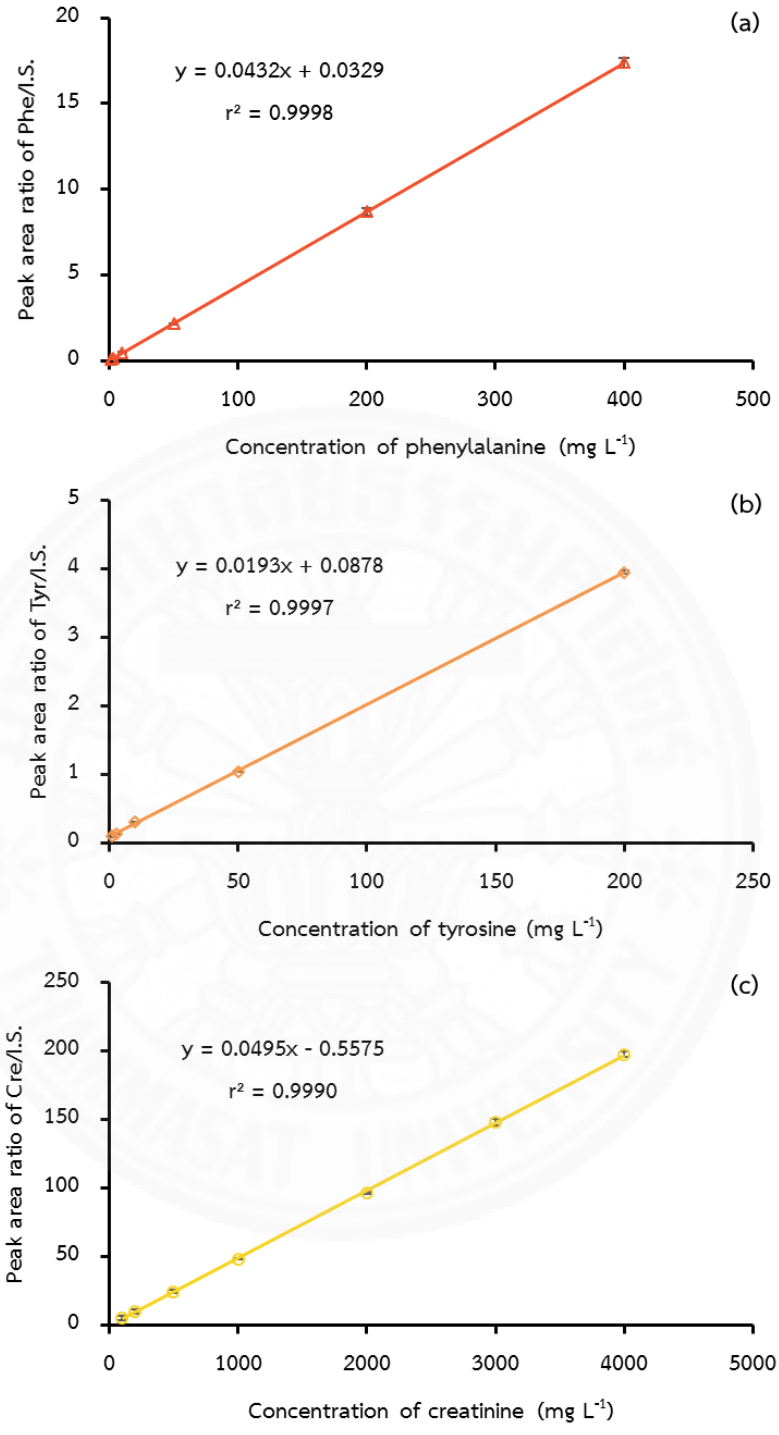

Figure 4.25 The internal calibration curve and coefficient of determination of (a) phenylalanine, (b) tyrosine and (c) creatinine.

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

Limit of detection (LOD) is the lowest concentration of extracted phenylalanine, tyrosine and creatinine through SCX-SPE which can be determined by three times of signal to noise ratio (3S/N). The LODs of the proposed method were 0.5, 0.1 and 1 mg L^{-1} of phenylalanine, tyrosine and creatinine, respectively. A concentration that gives detectable signal related to the concentration of phenylalanine, tyrosine and creatinine at ten times signal to noise ratio (10S/N) was a limit of quantification (LOQ). The LOQs of this method were 1, 0.5 and 3 mg L^{-1} of phenylalanine, tyrosine and creatinine, respectively.

4.2.2.3 Precision

According to the AOAC requirements, the precisions in term of intra-day and inter-day precision were evaluated by the HorRat method. The predicted relative standard deviation (PRSD(R)) calculated from the Horwitz formula and the ratio of the repeatability relative standard deviation (RSD(r)) calculated from the data to the PRSD(R) value to achieve $HorRat_r$ value. The corresponding within-laboratory $HorRat_r$ value was assigned with limits of acceptability of 0.3 to 1.3. The Horwitz formula and $HorRat_r$ equation of both precisions (Appendix F: Guidelines for Standard Method Performance Requirements by AOAC) were presented as follows:

Horwitz formula: $PRSD(R)(\%)^* = 2C^{-0.15}$

where C is expressed as a mass fraction.

$HorRat_r$ equation: $HorRat_r = RSD(r)(\%)/PRSD(R)(\%)$

*Within-laboratory acceptable predicted target values for repeatability are given at 1/2 of PRSD(R).

(1) Intra-day precision

The intra-day precision was obtained by injection of ten extracted urine samples (from the same pool) in three replications within a day. The results illustrated that the %RSD(r) of phenylalanine, tyrosine and creatinine were 2.87% ($HorRat_r$ = 0.26), 3.72% ($HorRat_r$ = 0.33) and 2.76% ($HorRat_r$ = 0.54), respectively.

(2) Inter-day precision

The inter-day precision was obtained by injection of ten extracted urine samples (from the same pool) in three replications within three days. The analysis of three extracted urine each day was performed except the four extracted urine on the first day. The results illustrated that the %RSD(r) of phenylalanine, tyrosine and creatinine were 4.77% ($HorRat_r$ = 0.42), 5.58% ($HorRat_r$ = 0.50) and 5.15% ($HorRat_r$ = 0.78), respectively.

Due to the usage of an internal standard for improving the precisions of the method, some $HorRat_r$ values were not within the limits of acceptability. In conclusion, the developed method has a good intra-day and inter-day precision within the acceptability of AOAC requirements.

4.2.2.4 Accuracy

The accuracy of the proposed method was evaluated in term of recovery. The recoveries were determined in 16 urine samples by fortifying with 10 mg L^{-1} of phenylalanine, 10 mg L^{-1} of tyrosine and 500 mg L^{-1} of creatinine. As shown in **Table 4.4**, the recoveries ranged between 88 – 105% and 88 – 108% of phenylalanine and tyrosine, respectively, with the range of the acceptable mean recovery (AOAC) between 80 – 110%. For creatinine, the recoveries ranged within 96 – 104% with the range of the acceptable mean recovery (AOAC) between 95 – 105%.

The method validation of the developed HILIC technique coupled with SPE for urinary analysis and the criteria was summarized in **Table 4.3**.

Table 4.3 Method validation of the HILIC method for the analysis of phenylalanine, tyrosine and creatinine in urine.

Parameter	Results			Criteria[a]
	Phenylalanine	Tyrosine	Creatinine	
Internal calibration curve[b]	1 – 400 (r^2 = 0.9998)	0.5 – 200 (r^2 = 0.9997)	3 – 3,000 (r^2 = 0.9990)	
Limit of detection (LOD)[b]	0.5	0.1	1	3S/N
Limit of quantification (LOQ)[b]	1	0.5	3	10S/N
Precision (%RSD)				
- intra day[c]	2.87 % ($HorRat_r$ = 0.26)	3.72 % ($HorRat_r$ = 0.33)	2.76 % ($HorRat_r$ = 0.54)	$HorRat_r$ = 0.3 – 1.3
- inter day[c]	4.77 % ($HorRat_r$ = 0.42)	5.58 % ($HorRat_r$ = 0.50)	5.15 % ($HorRat_r$ = 0.78)	$HorRat_r$ = 0.3 – 1.3

[a] Criteria of Appendix F: Guidelines for Standard Method Performance Requirements [92]

[b] mg L^{-1}

[c] Ten replications (n = 10)

4.2.3 Application to urine

The developed method was applied to simultaneously determine phenylalanine, tyrosine and creatinine in 16 urine samples collected from the male and female healthy volunteers aged between 20 – 44 years old. The representative HILIC chromatogram of male (aged 34 years old) and female (aged 39 years old) urine and fortified urine sample was demonstrated as **Figure 4.26 – Figure 4.29**, respectively. The results of the urine analysis were summarized in **Table 4.4**.

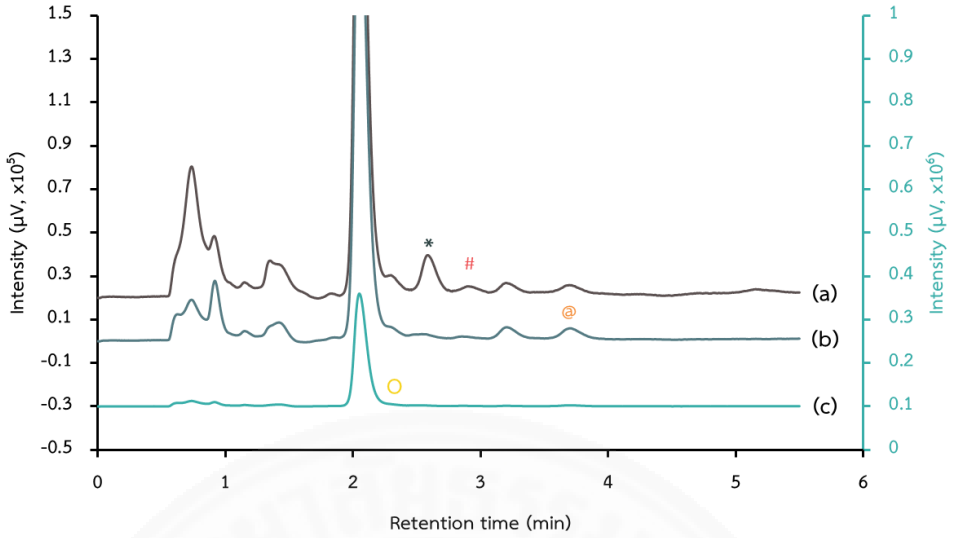

Figure 4.26 HILIC chromatograms of male urine sample at wavelength of (a) 210 nm for phenylalanine (#) and α-methyl phenylalanine (*), (b) 225 nm for tyrosine (@) and (c) 234 nm for creatinine (O).

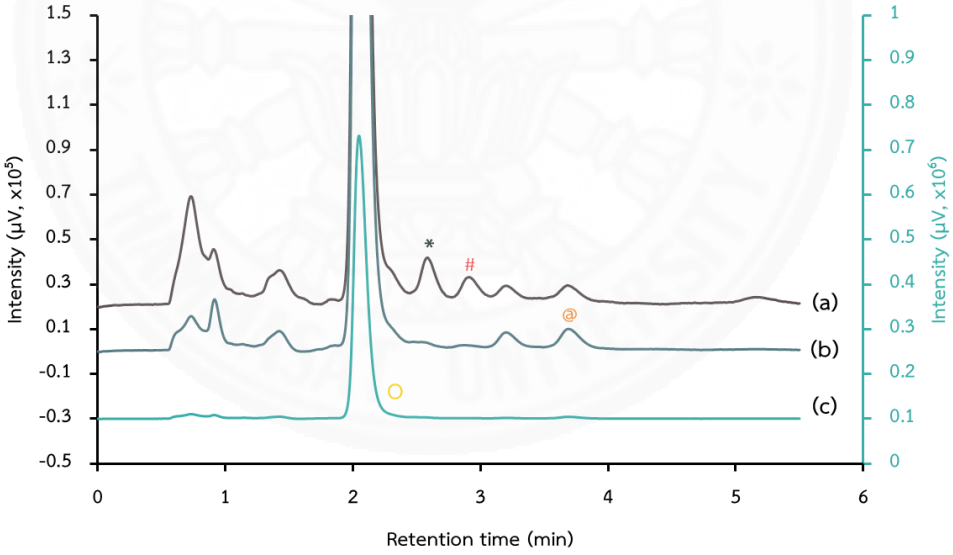

Figure 4.27 HILIC chromatograms of male urine sample fortifying with 10 mg L^{-1} of phenylalanine, 10 mg L^{-1} of tyrosine and 500 mg L^{-1} of creatinine at wavelength of (a) 210 nm for phenylalanine (#) and α-methyl phenylalanine (*), (b) 225 nm for tyrosine (@) and (c) 234 nm for creatinine (O).

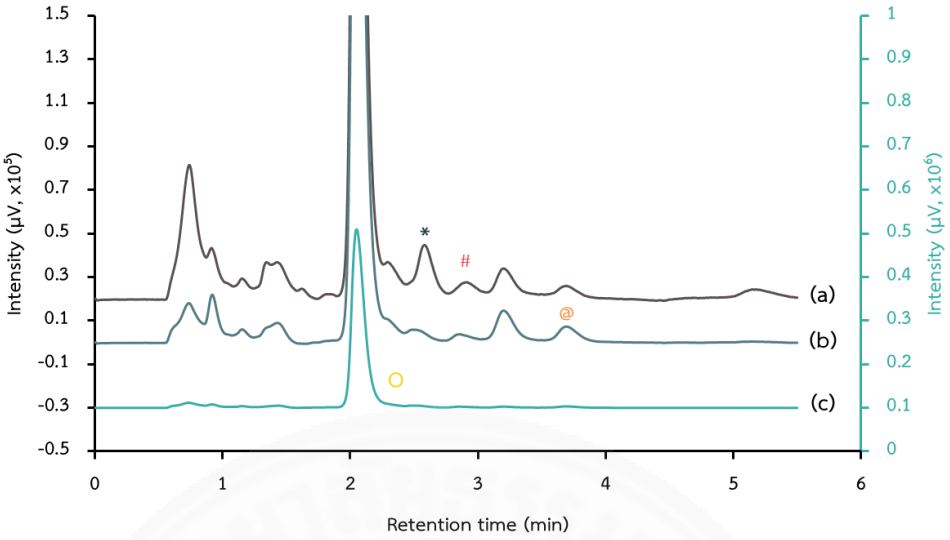

Figure 4.28 HILIC chromatograms of female urine sample at wavelength of (a) 210 nm for phenylalanine (#) and α-methyl phenylalanine (*), (b) 225 nm for tyrosine (@) and (c) 234 nm for creatinine (O).

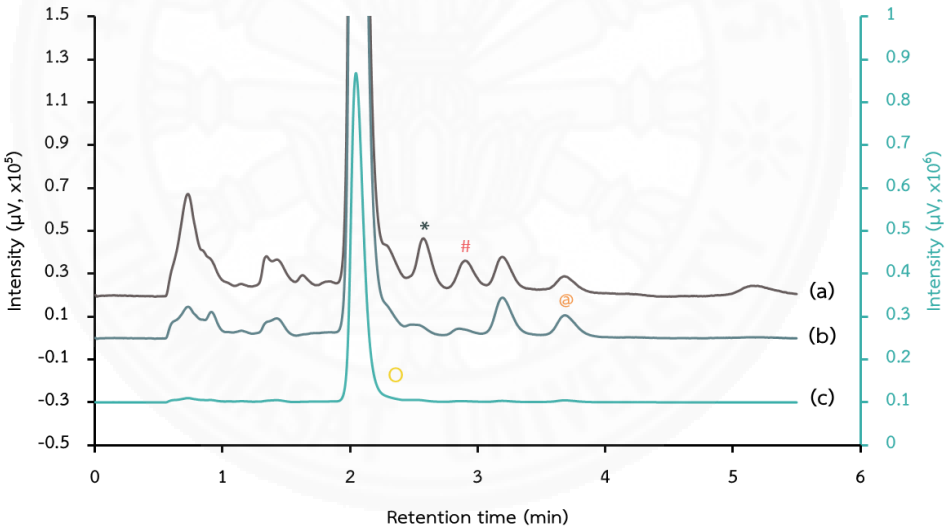

Figure 4.29 HILIC chromatograms of female urine sample fortifying with 10 mg L^{-1} of phenylalanine, 10 mg L^{-1} of tyrosine and 500 mg L^{-1} of creatinine at wavelength of (a) 210 nm for phenylalanine (#) and α-methyl phenylalanine (*), (b) 225 nm for tyrosine (@) and (c) 234 nm for creatinine (O).

The results showed that concentration of phenylalanine, tyrosine and creatinine in urine was in the range of 1.81 – 26.51, 2.68 – 34.07 and 227.54 – 1,394 mg L^{-1}, respectively. In this research, the collections of urine samples were

collected as a spot urine collection. The endogenous creatinine was frequently employed as an internal standard to collect changeable urine concentration, the creatinine ratio is important to evaluate the significant levels of metabolites in urine [26]. The creatinine ratio was commonly calculated as mg L^{-1} Phe divided by mg L^{-1} Cre (Phe/Cre ratio) and mg L^{-1} Tyr divided by mg L^{-1} Cre (Tyr/Cre ratio) which ranged between 0.005 – 0.0222 and 0.007 – 0.047, respectively. The results of this research were compared with the others as shown in **Table 4.5**. Phe/Cre ratio of urine samples determined by the proposed method (0.005 – 0.022) was in the range of Phe/Cre ratio determined by Y. Yokoyama et al. which the lowest value was 0.004 and the highest value was 0.050. Tyr/Cre ratio (0.007 – 0.0047) was also in the range of Tyr/Cre ratio determined by the others (0.001 – 0.0069). It can be concluded that the Phe/Cre and Tyr/Cre ratio analyzed in this research were agreed with others. The determined Phe/Tyr ratio was slightly less than the others; however, it could still be utilized for finding the correlation of this ratio between urine and blood. In comparison with the creatinine ratio of phenylketonuric urines from other researches, Tyr/Cre ratio was not different from normal urine, however; Phe/Cre ratio of phenylketonurics was hundreds of times higher than the normal levels. The proposed method can provide the Phe/Cre ratio for the diagnostic markers of PKU.

In order to diagnose phenylketonuria, the determination of phenylalanine and tyrosine to calculate the phenylalanine to tyrosine ratio and use of this Phe/Tyr ratio as a cut off value was normally carried out in blood. The Phe/Tyr ratio of phenylketonurics' blood was higher than normal blood which in agreement with urine sample (**Table 4.5**). Nevertheless, the correlation of Phe/Tyr ratio in blood and urine of normal and phenylketonuric should be further studied in order to provide data which can support to optimize the clinical sensitivity and specificity of the PKU diagnosis test in urine sample instead of blood analysis. The developed method can provide Phe/Tyr ratio in urine for the diagnostic markers of PKU.

Table 4.4 The content of phenylalanine, tyrosine and creatinine in the urine sample, recovery percentage and its ratio.

Aged Range (Year)	Gender (Age)	Phenylalanine (Phe)		Tyrosine (Tyr)		Creatinine (Cre)		Phe/Cre ratio	Tyr/Cre ratio	Phe/Tyr ratio
		Content ($mg\ L^{-1}$) (Mean ± SD)	%Recovery (Mean ± SD)	Content ($mg\ L^{-1}$) (Mean ± SD)	%Recovery (Mean ± SD)	Content ($mg\ L^{-1}$) (Mean ± SD)	%Recovery (Mean ± SD)			
18 – 20	Male (20)	12.05±0.08	101.66±0.04	30.83±0.06	100.24±0.04	1073.05±8.58	95.61±0.23	0.011	0.029	0.391
	Male (20)	5.16±0.13	93.47±0.21	2.68±0.10	97.38±0.21	374.34±1.27	101.77±5.04	0.014	0.007	1.927
	Female (20)	26.51±1.22	105.30±0.20	20.99±0.69	91.24±0.07	1276.80±9.41	96.74±0.17	0.021	0.016	1.263
	Female (20)	22.71±0.78	97.46±0.07	34.07±0.40	106.87±0.07	1393.58±26.83	99.87±0.05	0.016	0.024	0.667
21 – 30	Male (21)	3.80±0.07	93.78±0.11	3.95±0.14	89.27±0.04	505.42±9.38	96.42±3.76	0.008	0.008	0.962
	Male (26)	10.34±0.27	96.49±0.08	5.86±0.18	102.53±0.20	476.34±1.49	95.79±0.08	0.022	0.012	1.766
	Female (26)	11.92±0.56	101.68±0.06	8.34±0.40	93.71±0.47	680.76±2.28	103.41±0.04	0.018	0.012	1.430
	Female (26)	12.65±0.57	99.65±0.47	9.01±0.22	107.75±0.86	940.57±37.40	98.32±0.10	0.013	0.010	1.404
31 – 40	Male (34)	1.81±0.07	99.67±0.01	9.90±0.45	100.64 0.07	227.54±2.14	99.54±0.01	0.008	0.044	0.183
	Male (34)	1.95±0.07	87.80±0.37	8.61±0.18	104.27±0.09	386.35±5.57	98.74±0.06	0.005	0.022	0.226
	Female (39)	9.53±0.22	102.54±0.40	17.64±0.33	103.00±0.11	512.62±2.69	103.15±3.86	0.019	0.034	0.540
	Female (40)	5.34±0.18	95.45±0.03	9.16±0.14	95.15±0.04	398.59±2.01	97.30±0.01	0.013	0.023	0.583

Table 4.4 The content of phenylalanine, tyrosine and creatinine in the urine sample, recovery percentage and its ratio (cont.).

Aged Range (Year)	Gender (Age)	Phenylalanine (Phe)		Tyrosine (Tyr)		Creatinine (Cre)		Phe/Cre ratio	Tyr/Cre ratio	Phe/Tyr ratio
		Content ($mg\ L^{-1}$) (Mean ± SD)	%Recovery (Mean ± SD)	Content ($mg\ L^{-1}$) (Mean ± SD)	%Recovery (Mean ± SD)	Content ($mg\ L^{-1}$) (Mean ± SD)	%Recovery (Mean ± SD)			
41 – 50	Male (42)	15.05±0.24	99.39±0.14	19.15±0.48	88.33±2.56	1145.59±3.89	98.90±5.24	0.013	0.017	0.786
	Male (43)	11.59±0.26	101.95±0.73	19.13±0.92	106.96±0.12	885.35±17.03	103.65±0.09	0.013	0.022	0.606
	Female (42)	9.84±0.35	94.95±0.43	28.64±0.88	92.01±0.09	817.51±7.55	103.51±1.56	0.012	0.035	0.344
	Female (44)	3.75±0.11	97.72±0.31	18.98±0.56	104.81±0.05	403.11±6.56	102.12±0.02	0.009	0.047	0.197

Table 4.5 The ratio of phenylalanine/creatinine, tyrosine/creatinine and phenylalanine/tyrosine in urine from this work comparing to others.

Author (Year, Country)	Normal			Phenylketonuric		
	Phe/Cre ratio	Tyr/Cre ratio	Phe/Tyr ratio	Phe/Cre ratio	Tyr/Cre ratio	Phe/Tyr ratio[a]
This work (2019, Thailand)	0.005 – 0.022	0.007 – 0.047	0.183 – 1.927	-[b]	-	-
Y. Yokoyama et al. (2015, Japan) [23]	0.025 – 0.032	-	-	0.50 – 0.82	-	-
Y. Yokoyama et al. (2014, Japan) [24]	0.004 – 0.039	0.001 – 0.044	0.795 – 4.000	0.200 – 0.820	0.006 – 0.072	6.67 – 41.67
Y. Yokoyama et al. (2005, Japan) [25]	0.007 – 0.044	n.d.[c]	-	0.165 – 0.680	n.d.	-
Y. Yokoyama et al. (2005, Japan) [26]	0.008 – 0.050	0.013 – 0.069	0.217 – 2.083	0.162 – 0.521	0.040 – 0.133	3.301 – 6.650
Y. Yokoyama et al. (1996, Japan) [27]	n.d.	n.d.	n.d.	0.173 – 0.750	0.008 – 0.016	20.731 – 37.759
Y. Yokoyama et al. (1991, Japan) [28]	0.005 – 0.015	0.003 – 0.015	0.541 – 1.727	0.3025 – 0.7613	0.0050 – 0.0541	14.083 – 63.000

[a] calculated by this work; [b] - is not reported; [c] n.d. is non-detectable

4.3 Analysis of dietary supplement

Sample preparation in dietary supplement samples for determination of phenylalanine and tyrosine including extraction solvent, agitation method and extraction time were studied. A parameter was varied while keeping the others fixed in order to obtain the highest extraction efficiency.

4.3.1 Sample extraction

A multi-nutrients dietary supplement sample was the representative sample for the study of an optimal condition for the extraction (or dissolution) of dietary supplement because this sample comprised phenylalanine, tyrosine and other nutrients such as vitamins and plant extracted which can be interfered the chromatographic separation.

4.3.1.1 Extraction solvent

All the dietary supplements came in the form of powder in a capsule. In order to determine phenylalanine and tyrosine, the homogeneous powders of dietary supplements were extraction or dissolution with an appropriate solvent. Various types of solvent were investigated as detailed below:

(1) 0.1 M of hydrochloric acid (HCl): the solvent of standard solutions.

(2) Type I water: the common polar solvent to extract polar compounds.

(3) 50 mM of ammonium formate buffer pH 3: aqueous of mobile phase

(4) Mobile phase, acetonitrile:50 mM of ammonium formate pH 3.0 at the ratio of 84:16 (v/v).

Types of extraction solvent were studied with an equal volume of solvents and using ultrasonication for 30 minutes as an agitation method. **Figure 4.30** shows the invariable content of phenylalanine extracted from 4 types of solvent. However, the highest content of tyrosine was extracted by 0.1 M of hydrochloric acid and the content of extracted tyrosine was decreased when the

extraction solvent was type I water, 50 mM of ammonium formate buffer pH 3.0 and the mobile phase (acetonitrile:50 mM of ammonium formate pH 3.0 at the ratio of 84:16 v/v), respectively. According to the polarity and pH of 0.1 M hydrochloric acid, tyrosine can be easily extracted or dissolved. This research selected 0.1 M of hydrochloric acid as the extraction solvent which provided the highest extraction efficiency.

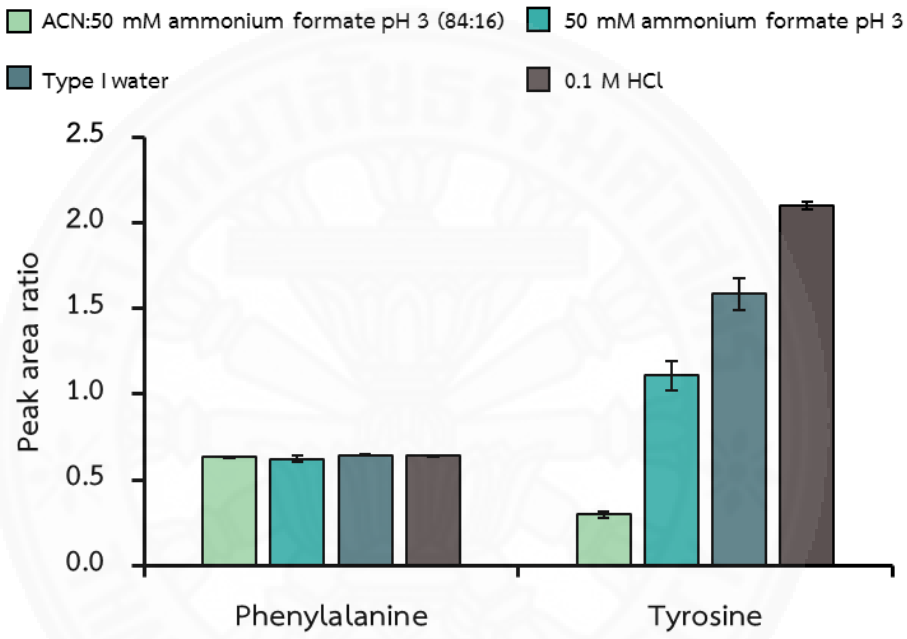

Figure 4.30 The effect of various extraction solvents on phenylalanine and tyrosine extraction efficiency from a multi-nutrient dietary supplement sample (three aliquots and three replicates of injection for each solvent).

4.3.1.2 Agitation method and time

To achieve the highest performance of the extraction method, the highest extraction efficiency and the shortest extraction time were an objective of this study. The optimal extraction solvent, 0.1 M of hydrochloric acid, were employed to study this parameter. In this work, different agitation methods and their extraction time were examined as follows:

(1) Ultrasonicator: 5, 15, 30 and 45 min (**Figure 4.31(a)**).

(2) Horizontal shaker: 1, 5, 10, 15 and 30 min (**Figure 4.31(b)**).

(3) Vortex: 0.5, 1, 2, 3, 4 and 5 min (**Figure 4.31(c)**).

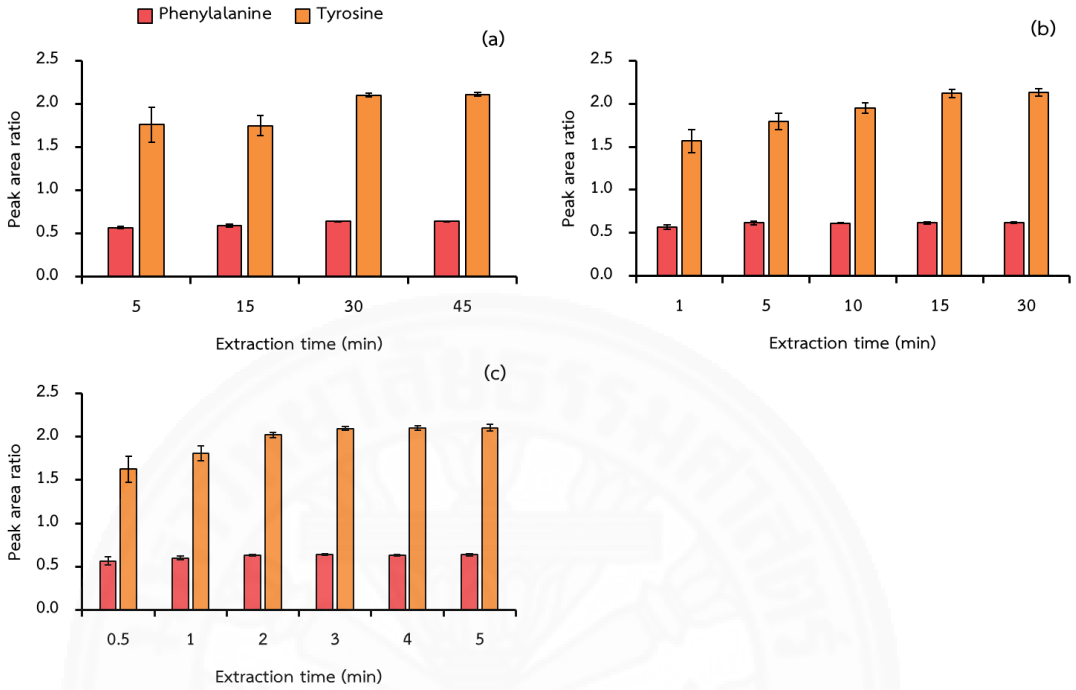

Figure 4.31 The effect of various agitation methods with varying of extraction time on phenylalanine and tyrosine extraction efficiency from multi-nutrient dietary supplement sample (three aliquots and three replicates of injection for each solvent) (a) ultrasonicator, (b) horizontal shaker and (c) vortex.

The results showed that three different agitation methods gave a similar trend. When the long extraction time was performed, the extracted content of especial tyrosine was increased and then kept constant. The best extraction condition of different agitation methods was 30 minutes of ultrasonicator, 15 minutes of horizontal shaker and 3 minutes of vortex which gave the same extraction efficiency of phenylalanine and tyrosine. In conclusion, the vortex extraction for 3 minutes was chosen as the optimal extraction method which provided good extraction efficiency and the shortest extraction time.

4.3.2 Method validation

The proposed HILIC and extraction method was validated using the Appendix K: Guidelines for Dietary Supplements and Botanicals, Part I: AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals by Association of Official Analytical Communities (AOAC) as a guideline [93].

4.3.2.1 Internal calibration curve

Internal calibration curves were carried out using seven standard concentrations with three replicate injections of the mixed standard solution. The internal calibration curves were plotted between peak area of the analytes divided by peak area of an internal standard (α-methyl phenylalanine) (the y-axis) and the series of each analyte concentrations (the x-axis). A high coefficient of determination (e.g., >0.99) is often recommended as evidence of the goodness of fit [93]. The results showed that the working ranges of the method were linear in the range of 1 – 500 mg L^{-1} with the acceptable coefficient of determination (r^2) of 0.9999 for phenylalanine, and 1 – 500 mg L^{-1} with r^2 = 0.9999 for tyrosine (**Figure 4.32**).

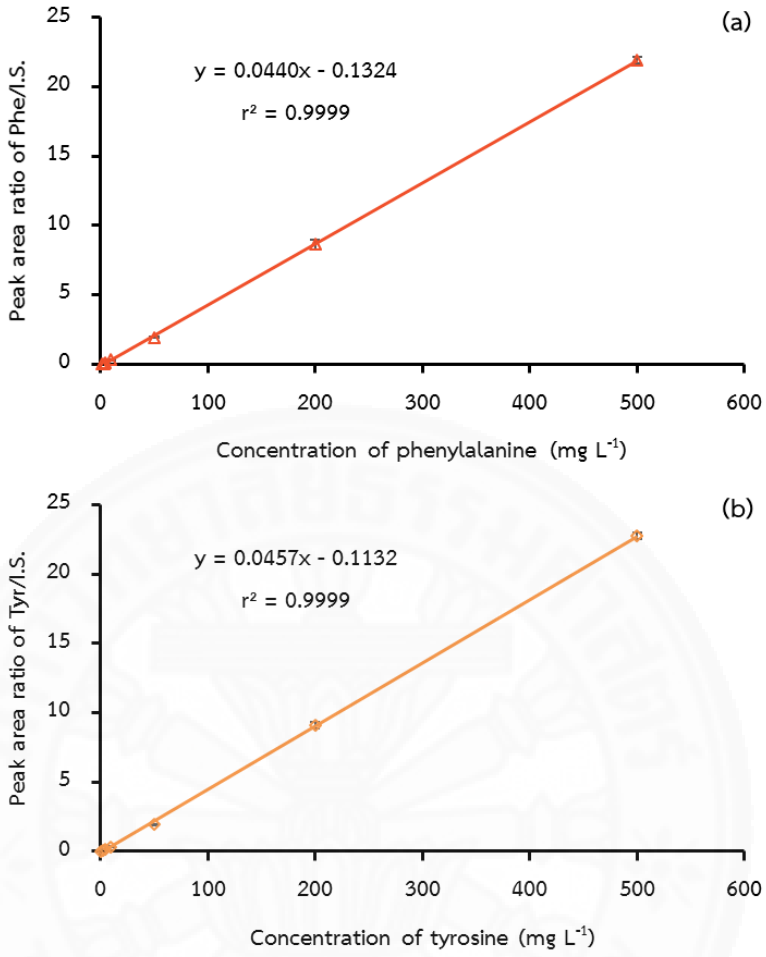

Figure 4.32 The internal calibration curve and coefficient of determination of (a) phenylalanine and (b) tyrosine.

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

Limit of detection (LOD) is the lowest concentration of extracted phenylalanine and tyrosine which can be determined by three times of signal to noise ratio. The LODs of the proposed method were 0.7 and 0.3 mg L^{-1} of phenylalanine and tyrosine, respectively. A concentration that gives detectable signal related to the concentration of phenylalanine and tyrosine at ten times signal to noise (10S/N) was a limit of quantification (LOQ). The LOQs of this method were 1 and 0.8 mg L^{-1} of phenylalanine and tyrosine, respectively.

4.3.2.3 Precision

According to the AOAC requirements, the precisions in term of intra-day and inter-day precision were evaluated by the HorRat method. The predicted relative standard deviation (PRSD(R)) calculated from the Horwitz formula and the ratio of the repeatability relative standard deviation (RSD(r)) calculated from the data to the PRSD(R) value to achieve $HorRat_r$ value. The corresponding within-laboratory $HorRat_r$ value was assigned with limits of acceptability of 0.5 to 2.0. The Horwitz formula and $HorRat_r$ equation of both precisions (Appendix K: Guidelines for Dietary Supplements and Botanicals, Part I: AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals by AOAC) were presented as follows:

Horwitz formula: $PRSD(R)(\%) = C^{-0.15}$

where C is expressed as a mass fraction.

$HorRat_r$ equation: $HorRat_r = RSD(r)(\%)/PRSD(R)(\%)$

(1) Intra-day precision

The intra-day precision was obtained by injection of ten extracted multi-nutrients dietary supplements in three replications within a day. The results illustrated that the %RSD(r) of phenylalanine and tyrosine were 0.73% ($HorRat_r$ = 0.18) and 0.93% ($HorRat_r$ = 0.23), respectively.

(2) Inter-day precision

The inter-day precision was obtained by injection of ten extracted multi-nutrients dietary supplement in three replications within ten days. The results illustrated that the %RSD(r) of phenylalanine and tyrosine were 1.75% ($HorRat_r$ = 0.29) and 1.76% ($HorRat_r$ = 0.29), respectively.

Due to the usage of an internal standard for improving the precisions of the method, some $HorRat_r$ values were not within the limits of acceptability. In conclusion, the developed method has a good intra-day and inter-day precision within the acceptability of AOAC requirements.

4.3.2.4 Accuracy

The accuracy of the proposed method was evaluated in term of recovery. The recoveries were determined in all dietary supplement samples by fortifying with 10 and 50 mg L^{-1} of phenylalanine and tyrosine. As shown in **Table 4.7**, the recoveries ranged between 95.48 – 101.84% and 96.55 – 101.97% of phenylalanine and tyrosine, respectively, with the range of the acceptable mean recovery (AOAC) between 95 – 102%.

The method validation of the HILIC method for the determination of phenylalanine and tyrosine in dietary supplement and the criteria was summarized in **Table 4.6**.

Table 4.6 Method validation of the HILIC method for the analysis of phenylalanine and tyrosine in dietary supplement.

Parameter	Results		Criteria[a]
	Phenylalanine	Tyrosine	
Internal calibration curve[b]	1 – 500 (r^2 = 0.9999)	1 – 500 (r^2 = 0.9999)	$r^2 > 0.99$
Limit of detection (LOD)[b]	0.7	0.3	3S/N
Limit of quantification (LOQ)[b]	1	0.8	10S/N
Precision (%RSD)			
- intra day[c]	0.73 % ($HorRat_r$ = 0.18)	0.93 % ($HorRat_r$ = 0.23)	$HorRat_r$ = 0.5 – 2
- inter day[c]	1.75 % ($HorRat_r$ = 0.29)	1.76 % ($HorRat_r$ = 0.29)	$HorRat_r$ = 0.5 – 2

[a] Criteria of Appendix K: Guidelines for Dietary Supplements and Botanicals, Part I: AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals [93]

[b] mg L^{-1}

[c] Ten replicates (n = 10)

4.3.3 Application to dietary supplement

The developed method was applied to simultaneously determine phenylalanine and tyrosine in eight dietary supplement samples including the single and the multi-nutrients dietary supplement. As presented in **Table 4.7**, the content of phenylalanine and tyrosine was in the range of 149 – 577 and 396 – 499 mg/capsule, respectively. There was no significant difference at 95% confidence interval for comparing the contents of phenylalanine and tyrosine between the amount obtained from the proposed method and the nutrition facts labeled amount by paired *t*-Test ($t_{stat} > t_{critical}$). The representative HILIC chromatogram of multi-nutrients dietary supplement sample (Sample D, Lot No. I) was demonstrated as **Figure 4.33** which indicated that the peaks of phenylalanine, tyrosine and internal standard were completely separated from matrices.

Table 4.7 The contents of phenylalanine and tyrosine in dietary supplement sample and its recovery percentage.

Sample	Phenylalanine				Tyrosine			
	Label (mg/capsule)	Contents (mg/capsule)[a]	Recovery (%)[a]		Label (mg/capsule)	Contents (mg/capsule)[a]	Recovery (%)[a]	
			10 mg L^{-1} [b]	50 mg L^{-1} [b]			10 mg L^{-1} [b]	50 mg L^{-1} [b]
A (Lot No. I) [*]	500	500.24 ± 1.00	99.04 ± 0.73	98.71 ± 0.23	-[c]	n.d.[d]	101.10 ± 0.01	96.78 ± 0.23
A (Lot No. II) [*]	500	501.50 ± 1.38	97.64 ± 0.39	96.38 ± 0.46	-	n.d.	101.83 ± 0.32	99.08 ± 0.74
B (Lot No. I) [*]	-	n.d.	101.22 ± 0.28	96.56 ± 0.08	500	497.36 ± 2.76	99.68 ± 0.91	97.19 ± 0.44
B (Lot No. II) [*]	-	n.d.	97.75 ± 0.07	98.20 ± 0.27	500	499.20 ± 1.17	101.45 ± 0.56	100.89 ± 0.42
C (Lot No. I) [**]	576.45	573.79 ± 1.01	99.58 ± 0.27	96.20 ± 0.76	-	n.d.	101.53 ± 0.08	96.55 ± 0.86
C (Lot No. II) [**]	576.45	577.40 ± 3.88	95.48 ± 0.52	101.84 ± 0.92	-	n.d.	101.97 ± 0.10	101.16 ± 0.14
D (Lot No. I) [**]	150	150.71 ± 2.09	101.31 ± 0.73	101.06 ± 0.40	400	400.72 ± 3.55	101.85 ± 0.50	98.95 ± 0.99
D (Lot No. II) [**]	150	148.70 ± 0.41	98.34 ± 0.04	99.69 ± 1.43	400	395.76 ± 2.75	99.28 ± 0.94	101.89 ± 0.37

[a] Three replicate injections (n=3) (Mean±SD);

[b] Fortified concentration;

[c] - is not labeled;

[d] n.d. is non-detectable;

[*] Single nutrient formulation;

[**] Multi-nutrients formulation.

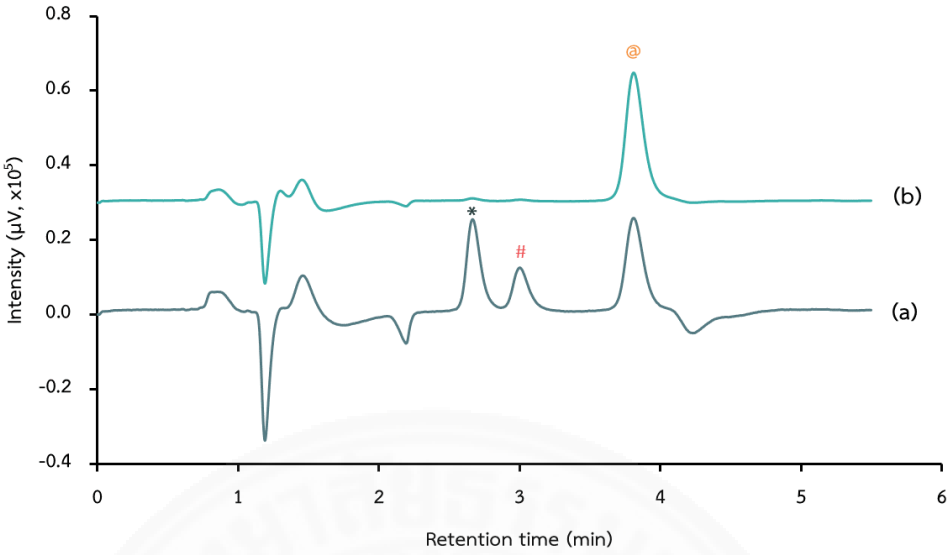

Figure 4.33 HILIC chromatograms of multi-nutrients dietary supplement sample D (Lot No. I) at wavelength of (a) 210 nm for phenylalanine (#) and α-methyl phenylalanine (*) and (b) 225 nm for tyrosine (@).

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

This research developed a new method based on hydrophilic interaction liquid chromatography (HILIC) for the simultaneous determination of phenylalanine (Phe), tyrosine (Tyr) and creatinine (Cre) in urine collected by the spot urine collection and the simultaneous determination of Phe and Tyr in dietary supplement.

The developed method was divided into three parts which consisted of the optimization of HILIC conditions for the simultaneous determination of Phe, Tyr and Cre, the application of the proposed method for the determination of Phe, Tyr and Cre in urine samples with the optimization of SCX-SPE as sample preparation and the application of this proposed method to determine phenylalanine and tyrosine along with an optimization of the extraction method for the analysis of dietary supplement.

The HILIC technique coupled with diode array detector (DAD) was optimized to achieve the best condition for the separation and determination of Phe, Tyr, Cre and α-methyl phenylalanine (I.S.). The stationary phase of the HILIC column (3.0 mm x 150 mm x 3 µm) is dihydroxypropyl (diol) group bonded silica gel which retains highly polar compounds as the analytes of this research. The use of DAD was not only confirmed the presence of the analytes for qualification analysis, but the detection with three detection wavelengths (210 nm for Phe and I.S., 225 nm for Tyr and 234 nm for Cre) was also achieved a simultaneous detection for quantification analysis. The condition of the mobile phase comprising of pH of buffer, composition and flow rate was studied to achieve the best separation (resolution), peak shape, sensitivity and analysis time of the analytes. The optimal condition of the mobile phase was 84:16 v/v of acetonitrile and 50 mM ammonium formate pH 3.0 with isocratic elution at a flow rate of 0.8 mL min^{-1}. The injection volume was 4 µL. The optimal condition of HILIC was applied to two types of sample, urine and dietary supplement, with the optimization of sample preparation.

Due to the complicated matrices in urine, sample preparation for determination of Phe, Tyr and Cre in urine was studied. The SCX-SPE was employed due to its effective extraction of the positively charged compounds as the analytes in acidic condition. Various parameters of SCX-SPE such as a functional group of strong cation exchange, type and volume of conditioning reagent, pH of loading sample, type and volume of washing and eluting reagent were studied in this work in order to obtain the highest extraction efficiency. The optimal condition of SCX-SPE was summarized as follows: The SCX-SPE with 500 mg weight of SCX sorbent (aliphatic sulfonic acid bonded silica phase) was employed for extractions. The conditioning step of SCX-SPE was carried out using 9 mL of methanol, 9 mL of 1:1 (v/v) of methanol:0.1 M hydrochloric acid and then 9 mL of 0.1 M hydrochloric acid. The urine sample was diluted 10 times with 0.1 M hydrochloric acid and one milliliter of its was introduced to SCX-SPE. The cartridge was washed by 4.00 mL of methanol in order to elimination the matrices. The analytes were eluted with 4.00 mL of 0.5 M ammonia in ethanol. The eluate was evaporated to dryness under nitrogen at 60°C and reconstituted with 100 µL of mobile phase prior to injection into the HILIC system. The developed HILIC with SCX-SPE for urinary analysis was successfully validated using the Appendix F: Guidelines for Standard Method Performance Requirements by Association of Official Analytical Communities (AOAC) as a guideline.

The proposed method was applied to determine 16 urine samples from normal volunteers aged between 20 – 44 years old (8 males and 8 females). The results showed that the ratio of phenylalanine/creatinine (Phe/Cre) and tyrosine/creatinine (Tyr/Cre) was agreed with other researches. This proposed method can provide the Phe/Cre ratio in urine which tended to be used as diagnostic markers of phenylketonuria (PKU).

Additionally, this HILIC method was also applied to determine Phe and Tyr in dietary supplement samples. The sample preparation in dietary supplement samples for determination of Phe and Tyr including extraction solvent, agitation method and extraction time were studied. The powders of the dietary supplement were accurately weighed of 0.0250 g and extracted with 25.00 mL of 0.1 M hydrochloric acid by vortex for 3 minutes. One milliliter of the extracted solution was diluted to

10.00 mL with acetonitrile prior to injection into the HILIC system. The validation of the proposed HILIC with the extraction method for the determination of dietary supplement was achieved using the Appendix K: Guidelines for Dietary Supplements and Botanicals, Part I: AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals by Association of Official Analytical Communities (AOAC) as a guideline.

The developed method was applied to determine phenylalanine and tyrosine in eight dietary supplement samples including the single and the multi-nutrients dietary supplement. There was no significant difference at 95% confidence interval for comparing the contents of Phe and Tyr between the amount obtained from the proposed method and the nutrition facts labeled amount by paired *t*-Test.

This method was sufficiently sensitive for the simultaneous determination of Phe and Tyr in urine sample (also with Cre) and dietary supplement. In conclusion, the proposed method provided a simple (without derivatization and not required ion-pairing reagent), efficient, fast, precise and accurate method which can simultaneously determine all the analytes within 5 minutes.

Recommendations

This research successfully developed the method for the determination of phenylalanine, tyrosine and creatinine in 16 urines sample from normal volunteers aged between 20 – 44 years old collected by spot urine collection. The method provided a good performance for urinary analysis; however, the urine collection was faced with the limitation of source which lacked a number of various aged volunteers and the phenylketonuric patients. If the proposed method is applied to determine phenylalanine and tyrosine in phenylketonuric urines, it will be supported the data to find the correlation of phenylalanine and tyrosine from normal and phenylketonuric urine.

Moreover, the scope of this research could be further extended to apply the proposed method for the determination of phenylalanine and tyrosine in blood to investigate the correlation of phenylalanine and tyrosine in blood and urine of

normal and phenylketonuric patient. The data of further research may support to optimize the clinical sensitivity and specificity of the PKU diagnosis test in urine sample instead of blood analysis. This developed method can also provide Phe/Tyr ratio in urine for the diagnostic markers of PKU.

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APPENDICE

APPENDIX A

Additional HILIC chromatograms of Section 4.1.2.1

Additional HILIC chromatograms of mixed standard solution at wavelength of 225 and 234 nm in **Section 4.1.2.1 pH of buffer and mobile phase composition**.

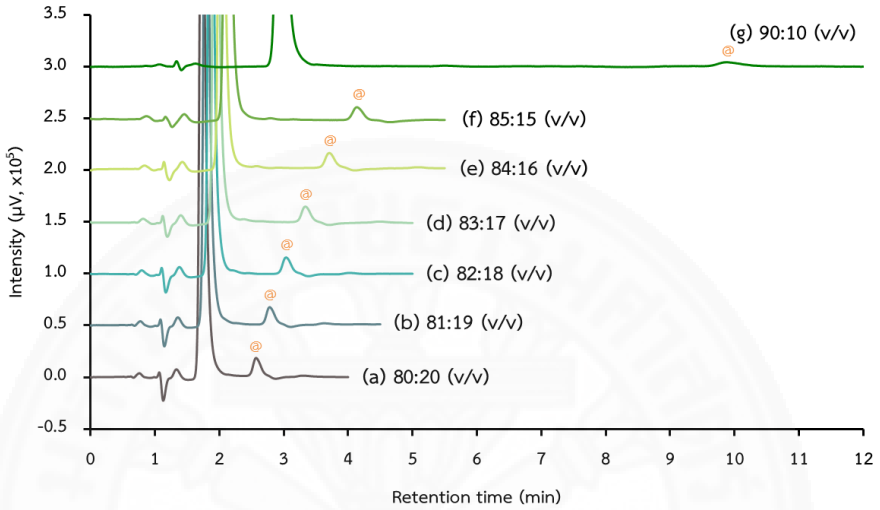


Figure A1 HILIC chromatograms of mixed standard solution at wavelength 225 nm for the determination of tyrosine (@). The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium formate pH 3.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

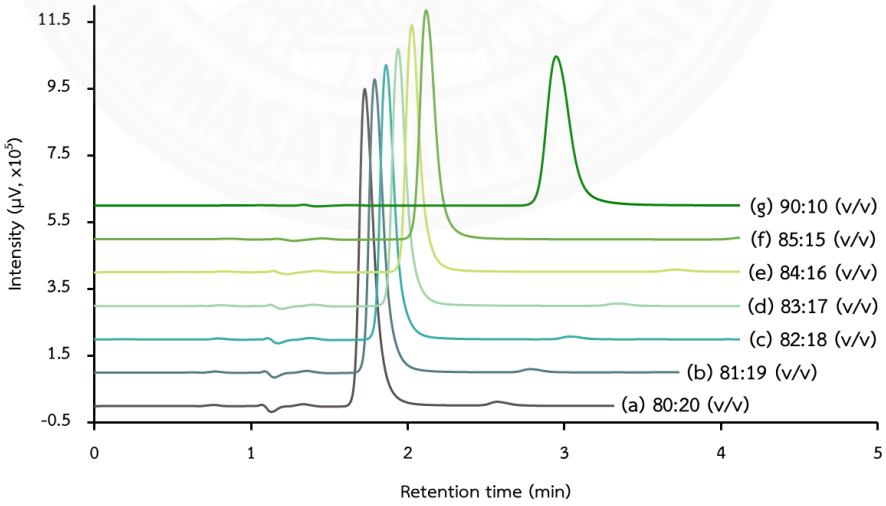


Figure A2 HILIC chromatograms of mixed standard solution at wavelength 234 nm for the determination of creatinine. The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium formate pH 3.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

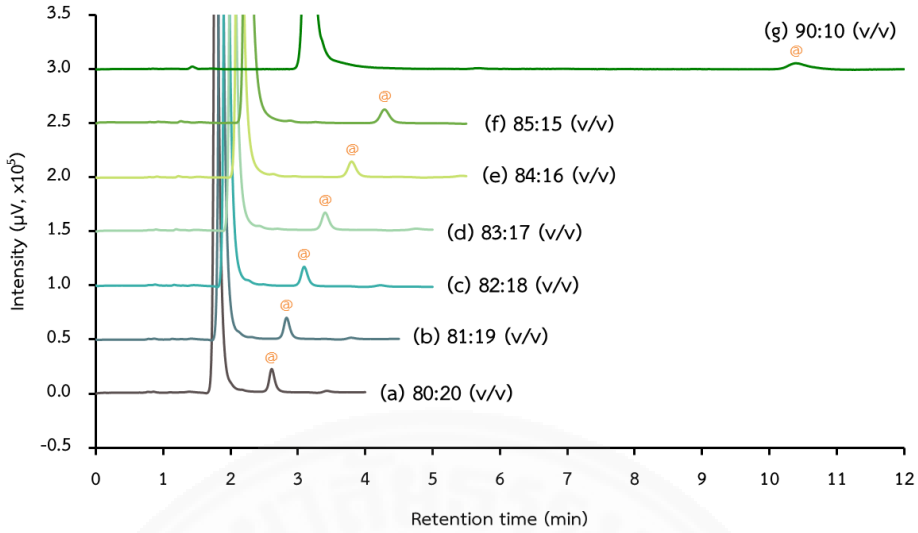


Figure A3 HILIC chromatograms of mixed standard solution at wavelength 225 nm for the determination of tyrosine (@). The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium formate pH 4.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

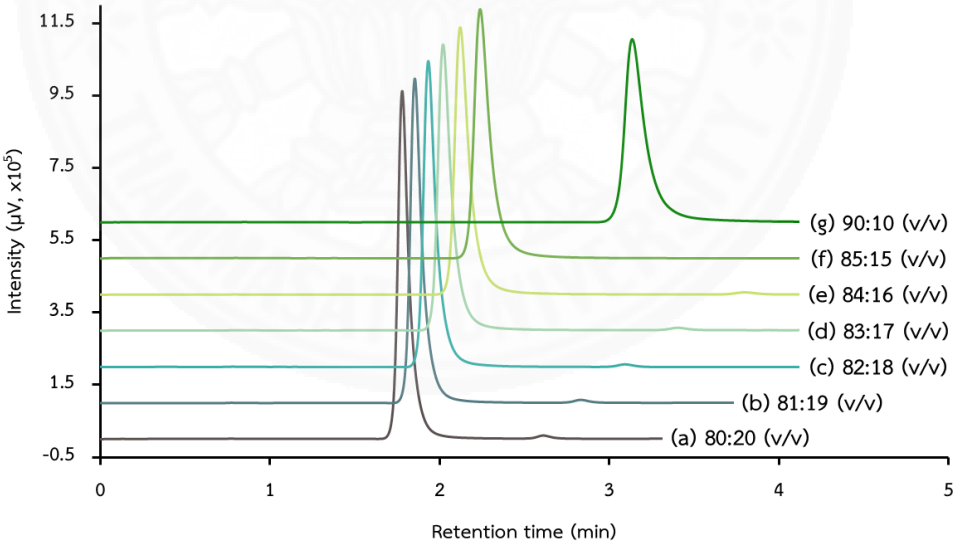


Figure A4 HILIC chromatograms of mixed standard solution at wavelength 234 nm for the determination of creatinine. The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium formate pH 4.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

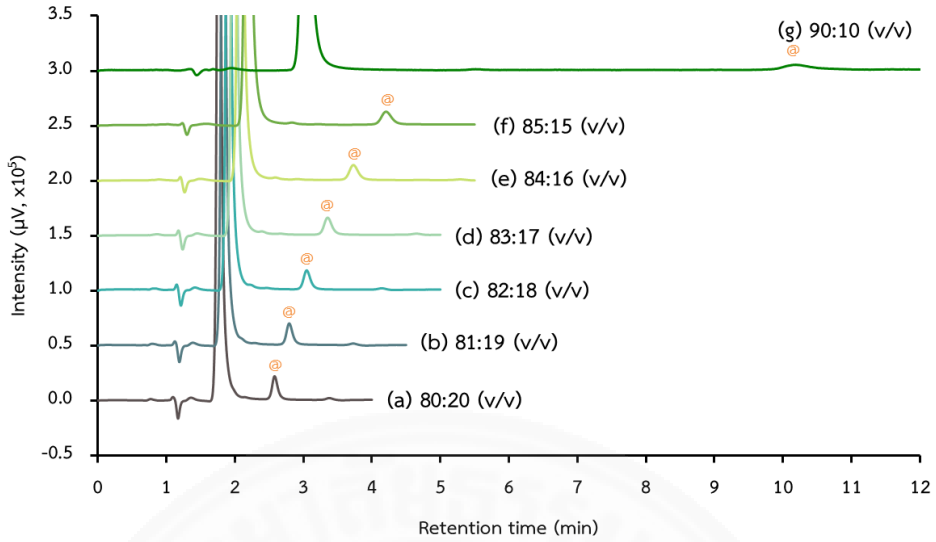


Figure A5 HILIC chromatograms of mixed standard solution at wavelength 225 nm for the determination of tyrosine (@). The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium acetate pH 4.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

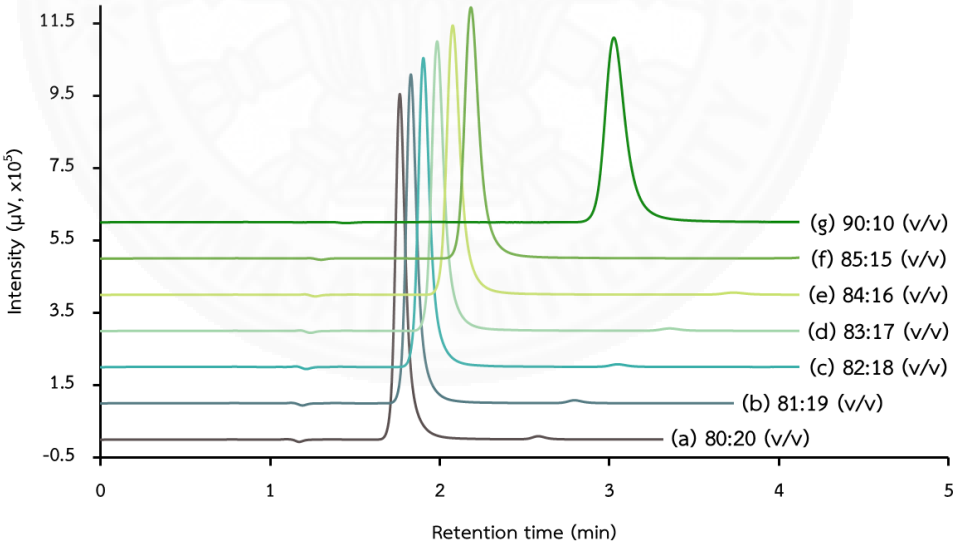


Figure A6 HILIC chromatograms of mixed standard solution at wavelength 234 nm for the determination of creatinine. The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium acetate pH 4.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

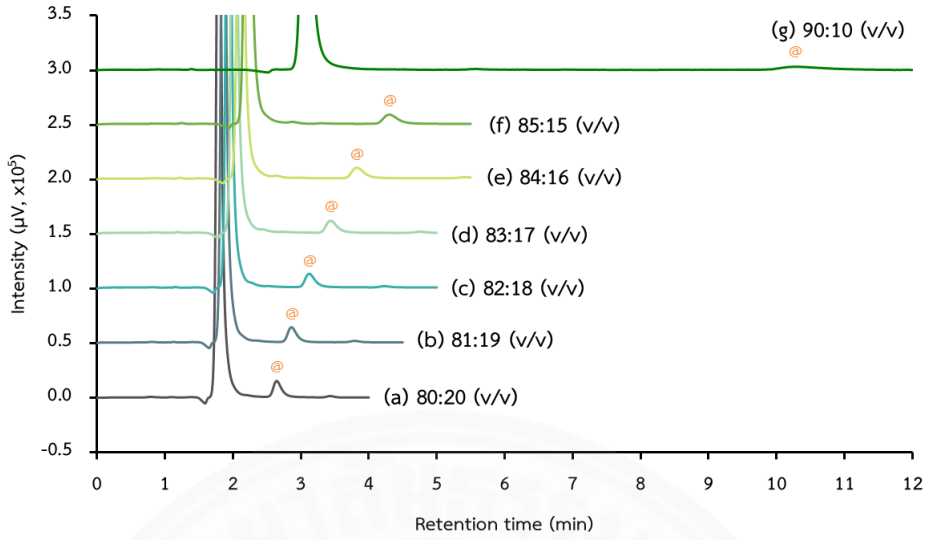


Figure A7 HILIC chromatograms of mixed standard solution at wavelength 225 nm for the determination of tyrosine (@). The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium acetate pH 5.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

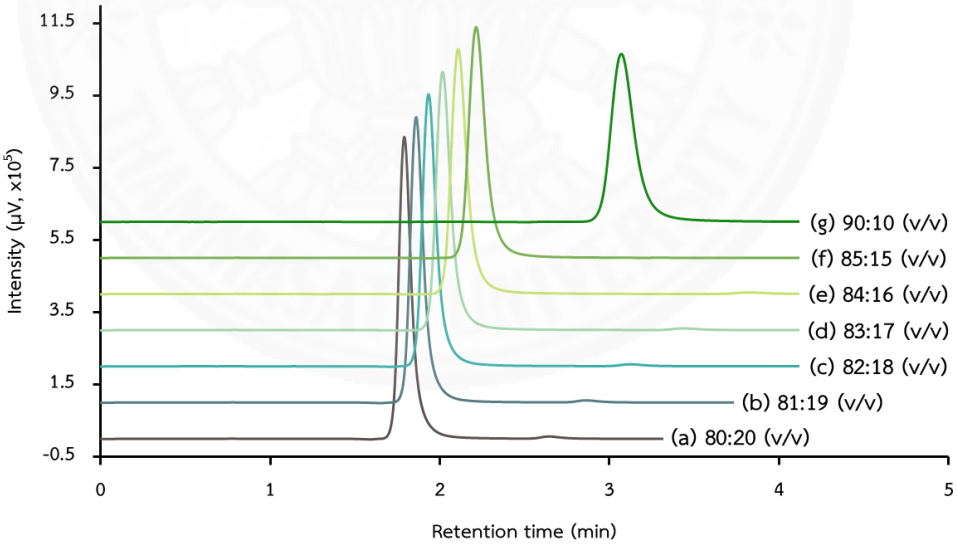


Figure A8 HILIC chromatograms of mixed standard solution at wavelength 234 nm for the determination of creatinine. The conditions of mobile phase were acetonitrile mixed with 50 mM of ammonium acetate pH 5.0 at the ratio of (a) 80:20, (b) 81:19, (c) 82:18, (d) 83:17, (e) 84:16, (f) 85:15 and (g) 90:10 (v/v).

APPENDIX B

Additional information (resolution, tailing factor of each analytes and analysis time) of Section 4.1.2.1

Additional information such as resolution (R_s) and tailing factor of each analytes with the analysis time depending on the pH and the mobile phase ratio were presented in **Table B1**.

Table B1 The resolution (R_s) and tailing factor of each analytes with the analysis time depending on the pH and the mobile phase ratio.

Mobile phase	Mobile phase composition (v/v)	Resolution				Tailing factor				Analysis time (min)
		Phe	Tyr	Cre	I.S.	Phe	Tyr	Cre	I.S.	
ACN:50 mM of ammonium formate pH 3.0	80:20	0.71	4.39	-	1.27	-	1.13	1.33	-	3.5
	81:19	0.86	5.08	-	1.66	-	1.16	1.35	-	4.0
	82:18	1.30	5.14	-	2.27	1.12	1.20	1.38	1.20	4.3
	83:17	1.42	5.90	-	2.30	1.30	1.22	1.45	1.21	4.7
	84:16	1.57	6.96	-	2.69	1.36	1.23	1.47	1.22	5.0
	85:15	1.58	8.70	-	3.23	1.36	1.25	1.50	1.22	6.0
	90:10	2.57	10.44	-	5.29	1.38	1.52	1.55	1.23	11.0

Table B1 The resolution (R_s) and tailing factor of each analytes with the analysis time depending on the pH and the mobile phase ratio (cont.).

Mobile phase	Mobile phase composition (v/v)	Resolution				Tailing factor				Analysis time (min)
		Phe	Tyr	Cre	I.S.	Phe	Tyr	Cre	I.S.	
ACN:50 mM of ammonium formate pH 4.0	80:20	1.13	6.26	-	1.75	-	1.12	1.55	-	3.5
	81:19	1.24	6.73	-	2.01	-	1.13	1.56	-	4.1
	82:18	1.35	7.36	-	2.31	-	1.16	1.58	-	4.5
	83:17	1.48	7.93	-	2.67	-	1.16	1.60	-	5.0
	84:16	1.79	8.68	-	3.24	1.22	1.17	1.62	1.15	5.5
	85:15	1.91	9.56	-	3.70	1.24	1.21	1.63	1.16	6.0
	90:10	3.10	20.49	-	9.96	1.68	1.46	1.64	1.27	12.0
ACN:50 mM of ammonium acetate pH 4.0	80:20	1.12	6.24	-	1.72	-	1.19	1.36	-	4.0
	81:19	1.21	6.75	-	2.03	-	1.19	1.39	-	4.5
	82:18	1.34	7.29	-	2.36	-	1.20	1.40	-	5.0
	83:17	1.42	7.84	-	2.70	-	1.21	1.40	-	5.5
	84:16	1.73	8.48	-	3.27	1.30	1.23	1.41	1.17	6.0
	85:15	1.84	8.62	-	3.71	1.31	1.23	1.42	1.18	6.3
	90:10	3.00	9.25	-	8.23	1.69	1.44	1.44	1.26	12.0

Table B1 The resolution (R_s) and tailing factor of each analytes with the analysis time depending on the pH and the mobile phase ratio (cont.).

Mobile phase	Mobile phase composition (v/v)	Resolution				Tailing factor				Analysis time (min)
		Phe	Tyr	Cre	I.S.	Phe	Tyr	Cre	I.S.	
ACN:50 mM of ammonium acetate pH 5.0	80:20	0.90	4.80	-	1.35	-	1.41	1.32	-	4.0
	81:19	1.01	5.24	-	1.67	-	1.41	1.35	-	4.5
	82:18	1.14	5.71	-	2.01	-	1.42	1.36	-	5.0
	83:17	1.22	6.22	-	2.34	-	1.45	1.37	-	5.3
	84:16	1.49	6.83	-	2.86	1.48	1.45	1.39	1.28	5.8
	85:15	1.55	7.39	-	3.26	1.56	1.46	1.41	1.29	6.5
	90:10	2.58	8.18	-	6.92	1.85	1.80	1.41	1.49	12.0

APPENDIX C

HILIC chromatograms of Section 4.1.2.2

HILIC chromatograms of mixed standard solution at wavelength of 210, 225 and 234 nm in **Section 4.1.2.2 Flow rate**.

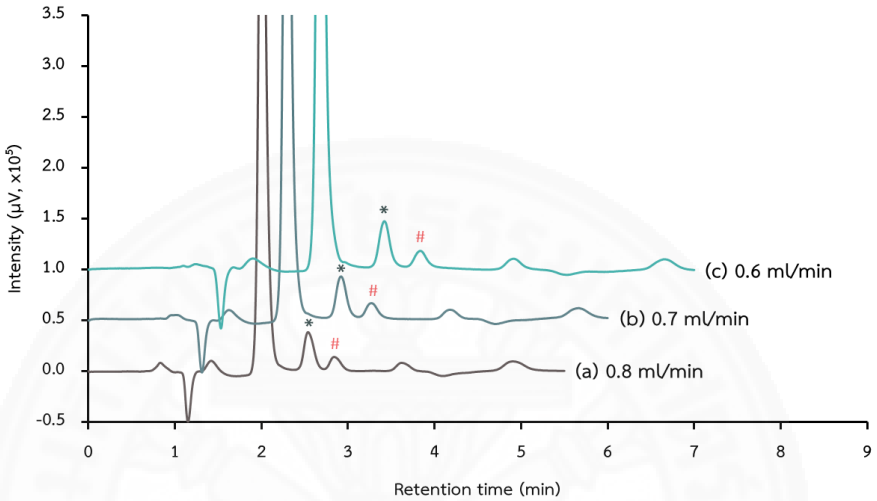


Figure C1 HILIC chromatograms of mixed standard solution at wavelength 210 nm for the determination of phenylalanine (#) and α-methyl phenylalanine (*). The conditions of mobile phase were 84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0 and injection volume of 4 μL at flow rate of (a) 0.8 mL min^{-1}, (b) 0.7 mL min^{-1} and (c) 0.6 mL min^{-1}.

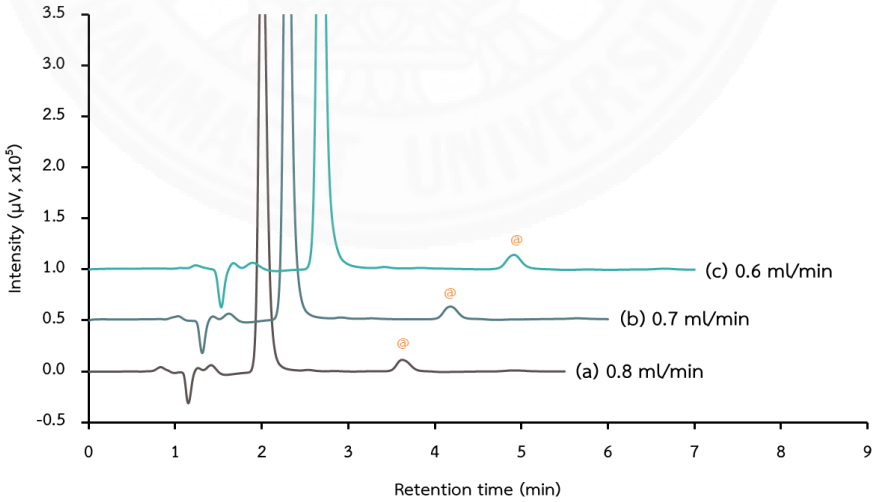


Figure C2 HILIC chromatograms of mixed standard solution at wavelength 225 nm for the determination of tyrosine (@). The conditions of mobile phase were 84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0 and injection volume of 4 μL at flow rate of (a) 0.8 mL min^{-1}, (b) 0.7 mL min^{-1} and (c) 0.6 mL min^{-1}.

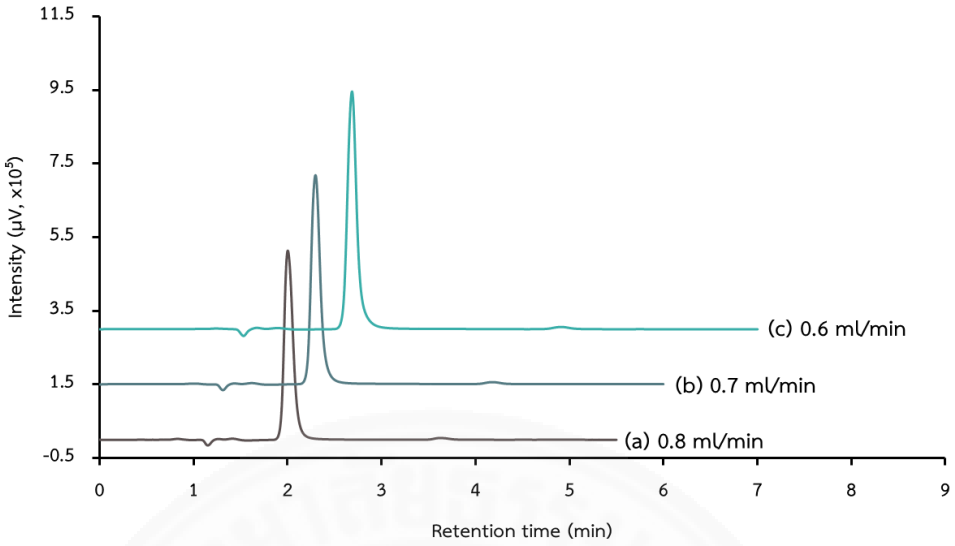


Figure C3 HILIC chromatograms of mixed standard solution at wavelength 234 nm for the determination of creatinine (O). The conditions of mobile phase were 84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0 and injection volume of 4 μL at flow rate of (a) 0.8 mL min^{-1}, (b) 0.7 mL min^{-1} and (c) 0.6 mL min^{-1}.

APPENDIX D

Additional HILIC chromatograms of Section 4.1.3

Additional HILIC chromatograms of mixed standard solution at wavelength of 225 and 234 nm in **Section 4.1.3 Injection volume**.

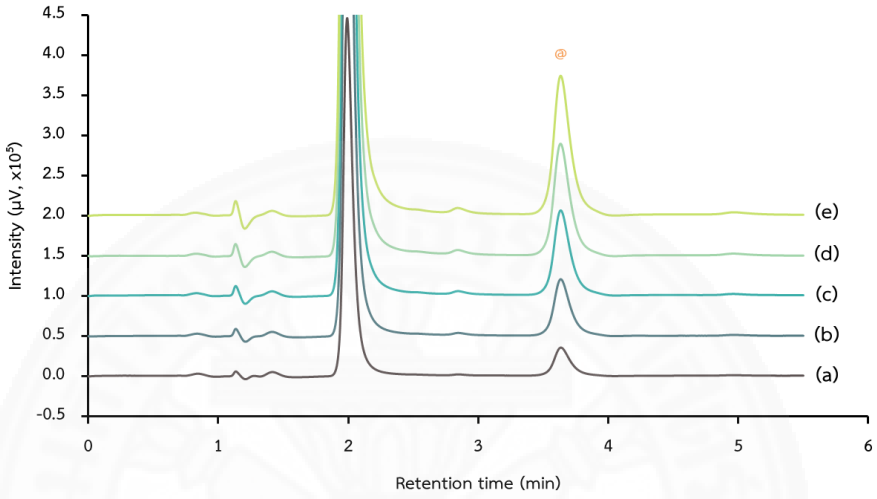


Figure D1 HILIC chromatograms of mixed standard solution at wavelength 225 nm for the determination of tyrosine (@). The conditions of mobile phase were 84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0 at flow rate of 0.8 mL min^{-1} with the injection volumes (a) 1 μL, (b) 2 μL, (c) 3 μL, (d) 4 μL and (e) 5 μL.

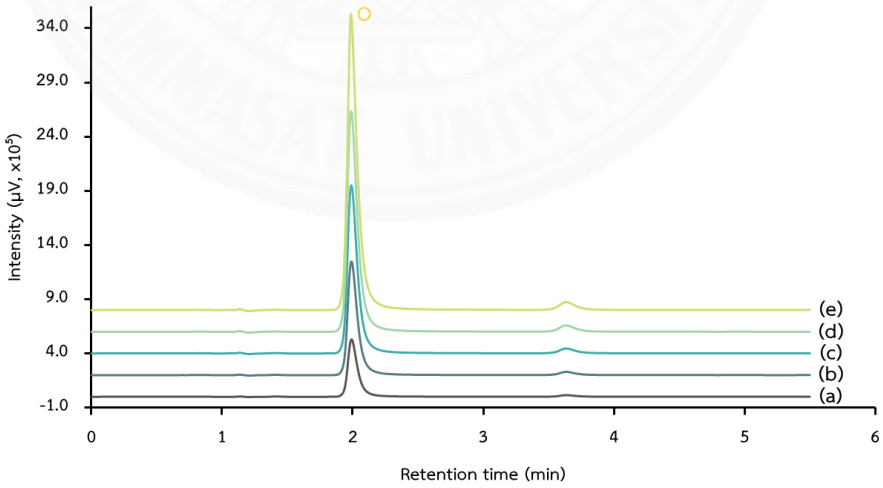
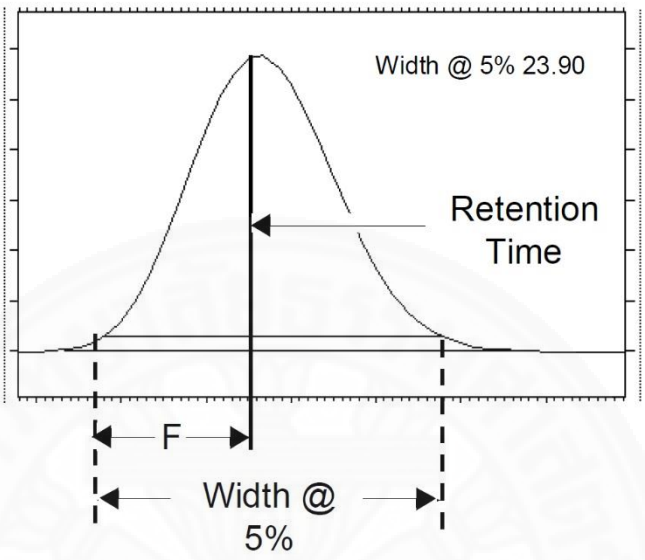


Figure D2 HILIC chromatograms of mixed standard solution at wavelength 234 nm for the determination of creatinine (O). The conditions of mobile phase were 84:16 v/v of acetonitrile:50 mM of ammonium formate pH 3.0 at flow rate of 0.8 mL min^{-1} with the injection volumes (a) 1 μL, (b) 2 μL, (c) 3 μL, (d) 4 μL and (e) 5 μL.

APPENDIX E

The definition and calculation of tailing factor from USP guideline [96]



The tailing factor establishes the maximum permissible asymmetry of the peak. For pharmaceutical purposes, the tailing factor, T, is defined as the distance between the leading edge and tailing edge of the peak at a width of 5% of the peak height divided by twice the distance, F, between the peak maximum and the leading edge of the peak at 5% of peak height.

$$T = \frac{W}{2 \times F}$$

where T = Tailing factor

W = Peak width at 5% of peak height

Rt = Retention time

F = Time from width start point at 5% of peak height to Rt

For a symmetrical peak, the tailing factor, T, is 1.0, and the value of T increases as tailing becomes more pronounced.

APPENDIX F

Additional UV absorption spectra of Section 4.2.1.2 (4.2)

Additional UV spectra of the extracted phenylalanine from the urine sample with the usage of 3 mL methanol comparing with 4 mL methanol as washing reagent at wavelength of 210 nm in **Section 4.2.1.2 (4.2) Washing volume**.

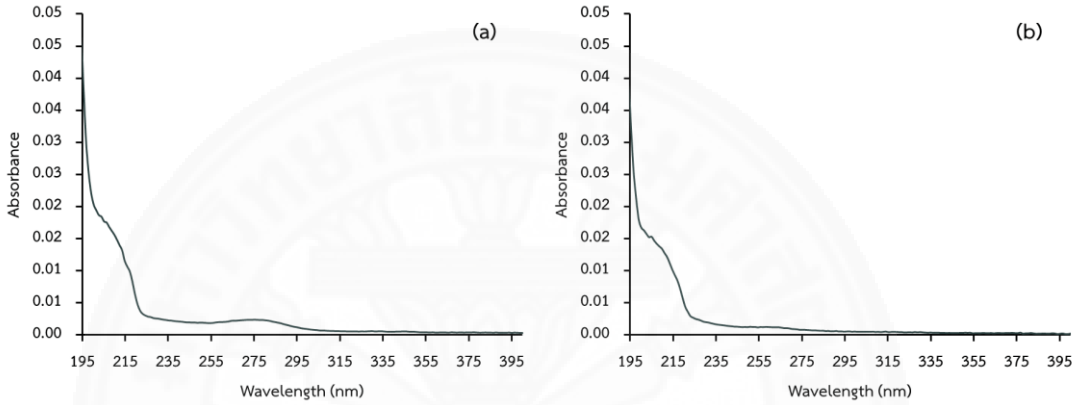


Figure F1 UV absorption spectra of (a) the extracted phenylalanine from the urine sample with the usage of 3 mL methanol as washing reagent comparing with (b) the extracted phenylalanine from the urine sample with the usage of 4 mL methanol as washing reagent.

BIOGRAPHY

Name	Mr. Woraphot Wanichalanant
Date of Birth	December 26, 1992
Educational Attainment	2015: Bachelor of Science (Chemistry), Thammasat University, Thailand
Scholarship	2015 – 2016: Scholarship for talent student to study graduate program in Faculty of Science and Technology Thammasat University
Publications	W. Wanichalanant, and W. Tiyapongpattana, Hydrophilic interaction liquid chromatography (HILIC) with diode array detection for determination of phenylalanine and tyrosine in dietary supplements. Proceedings of the Pure and Applied Chemistry International Conference 2018 (PACCON 2018), 2018. p. 126-130.
Work Experiences	2015 – Present: Teaching Assistant, Department of Chemistry, Faculty of Science and Technology, Thammasat University, Thailand