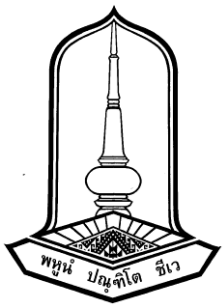

DETERMINATION OF NUCLEOSIDES BY HIGH PERFOMANCE LIQUID CHROMATOGRAPHY IN ENTOMOPATHOGENIC FUNGAL SAMPLES

KHANITTHA CORDHANAM

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry at Mahasarakham University

August 2017

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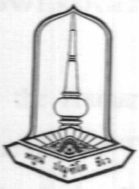

The examining committee has unanimously approved this thesis, submitted by Miss Khanittha Cordhanam, as a partial fulfillment of the requirements for the Master of Science degree in Chemistry, Mahasarakham University.

Examining Committee

R. Burakham. ..... Chairman
(Assoc.Prof. Rodjana Burakham, Ph.D) (External expert)

Uthai Sakee ..... Committee
(Assoc. Prof. Uthai Sakee, Ph.D) (Advisor)

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(Asst. Prof. Watchara Kanchanarach , Ph.D) (Co-advisor)

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(Asst.Prof. Piyanete Chantiratikul, Ph.D) (Faculty graduate committee)

W. Siriangkhawut ..... Committee
(Watsaka Siriangkhawut , Ph.D) (Faculty graduate committee)

Mahasarakham University has granted approval to accept this thesis as a partial fulfillment of the requirements for the Master of Science degree in Chemistry

Wichian Magtoon ....

(Prof. Wichian Magtoon, Ph.D)
Dean of the Faculty of Science

(Prof. Pradit Terdtoon, Ph.D.)
Dean of Graduate School
August 10, 2017

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Khanittha Cordhanam

TITLE Determination of nucleosides by high performance liquid chromatography in entomopathogenic fungal samples

CANDIDATE Miss Khanittha Cordhanam

DEGREE Master of Science **MAJOR** Chemistry

ADVISORS Assoc. Prof. Uthai Sakee, Ph.D

Asst. Prof. Watchara Kanchanarach, Ph.D

UNIVERSITY Mahasarakham University **YEAR** 2017

ABSTRACT

Cordyceps are a fastidious pathogenic fungus interesting insects and commonly used traditional Chinese medicine (TCM). Necleosides and major active component are used as a valuable chemical marker for quality control of *Cordyceps*. This study, a high performance liquid chromatography (HPLC) method with ultraviolet light detection was developed to determine five nucleosides namely uridine, inosine, guanosine, adenosine and cordycepin. Five analytes could be well separated in ACE-5 C18-AR column (4.6 mm x 250 mm i.d., 5 µm.) with gradient elution of methanol and 5 mM aqueous triethylamine. Under the optimal condition, the liner range was from 1 to 200 mg/L (R^2 > 0.995). The developed method showed good repeatability for the quantification of five investigated nucleosides in *Cordyceps* with intra-day and inter-day variation of 3.57 and 4.57%, respectively.

The validated method was successfully applied to quantify five nucleosides in natural, cultured *Cordyceps* and the *Cordyceps* contented in commercial products, which is helpful to control their quality.

Keywords: *Cordyceps*; High performance liquid chromatography; nucleosides; natural and cultured *Cordyceps* species

ชื่อเรื่อง การหาปริมาณนิวคลีโอไซด์โดยใช้เทคนิคโครมาโทกราฟีของเหลวสมรรถณะสูง ในตัวอย่างเชื้อราทำลายแมลง

ผู้วิจัย นางสาวขนิษฐา โคตรหานาม

ปริญญา วิทยาศาสตรมหาบัณฑิต **สาขาวิชา** เคมี

อาจารย์ที่ปรึกษา รองศาสตราจารย์ ดร.อุทัย สาขี

ผู้ช่วยศาสตราจารย์ ดร.วัชรา กาญจนรัช

มหาวิทยาลัย มหาวิทยาลัยมหาสารคาม **ปีที่พิมพ์** 2560

บทคัดย่อ

Cordyceps และ *Ophiocordyceps* เป็นเชื้อราที่ก่อโรคในแมลงและโดยทั่วไปการแพทย์แผนจีนโบราณใช้ในการรักษาโรคต่างๆ ซึ่งนิวคลีโอไซด์และสารออกฤทธิ์เป็นองค์ประกอบทางเคมีที่สำคัญบ่งบอกคุณภาพของสารในเชื้อราทำลายแมลง ในงานวิจัยนี้เทคนิคโครมาโทกราฟี่ของเหลวสมรรถนะสูงตรวจวัดด้วยแสงอัลตร้าไวโอเลตได้ใช้เพื่อวิเคราะห์หาปริมาณสาร ยูริดีน อินโนซีน กวานีน อะดีโนซีน และ คอดีเซฟปิน โดยสารทั้งห้าชนิดสามารถแยกได้ในคอลัมน์ ACE-5 C18-AR (4.6 มิลลิเมตร x 250 มิลลิเมตร อนุภาค 5 ไมโครเมตร) ด้วยการชะแบบเกรเดียนท์ระหว่างเมทานอลและสารละลายไตรเอทิลอะมีน 5 มิลลิโมลาร์ ภายใต้สภาวะที่เหมาะสมนี้ค่าความเป็นเส้นตรงที่ความเข้มข้น 1 ถึง 100 มิลลิกรัมต่อลิตรมีค่าสัมประสิทธิ์สหสัมพันธ์ของสารมาตรฐานทั้งห้า (R^2) มากกว่า 0.995 วิธีการที่ได้พัฒนานี้มีค่าความถูกต้องและความแม่นยำจากการทำการทดลองซ้ำภายในวันเดียวกันเท่ากับ 3.75 เปอร์เซ็นต์และ ระหว่างวัน 4.75 เปอร์เซ็นต์

วิธีการตรวจสอบนี้ประสบความสำเร็จในการนำมาประยุกต์ใช้เพื่อหาปริมาณสารนิวคลีโอไซด์ทั้ง 5 ชนิดจาก *Ophiocordyceps* ในธรรมชาติและการเพาะเลี้ยง และ *Cordyceps* จากการเพาะเลี้ยง และ ในผลิตภัณฑ์เชิงพาณิชย์ซึ่งเป็นประโยชน์ในการควบคุมคุณภาพของผลิตภัณฑ์

คำสำคัญ: เชื้อราทำลายแมลง, เทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง, นิวคลีโอไซด์

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LIST OF ABBREVIATION

ADEK	adenylate kinase
ADK	adenosine kinase
ADP	adenosine diphosphate
AIR	5-Aminoimidazole ribonucleotide
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CAIR	carboxyaminoimidazole ribonucleotide
CE	Capillary electrophoresis
cm	Centimeter
FAICAR	5-Formylaminoimidazole carboxamide ribonucleotide
FGAM	formylglycinamidine ribonucleotide
GAR	glycinamide ribonucleotide
GDP	guanine diphosphate
GMP	guanosine monophosphate
GTP	guanine triphosphate
HPLC	high performance liquid chromatography
i.d.	inner diameter
IEC	Ion-exchange chromatography
IMP	inosine monophosphate
L	liter
LC/ESI-MS	Liquid Chromatography/electron spray ionization-MS
LOD	limit of detection
LOQ	limit of quantitation
mg	Milligram
mg L^{-1}	milligram per liter
min	Minute
mL	milliliter
mm	millimeter
mol L^{-1}	mole per liter
N5TE	5′-nucleotidase

nm	nanometer
NT5E	5’-nucleotidase
OMP	orotic monophosphate
PRPP	phosphoribosyl pyrophosphate
R5P	ribose 5-phosphate
RNR	ribonucleotide reductases
RSD	relative standard deviation
SAICAR	N-succinylo-5-aminoimidazole-4-carboxamide ribonucleotide
SD	standard deviation
TCM	traditional Chinese medicines
TLC	thin layer chromatography
UMP	uridine monophosphate
UV-Vis	ultraviolet visible spectrophotometry
XMP	xanthosine monophosphate
μg	microgram
μL	microliter

CHAPTER 1

INTRODUCTION

1.1 Background

The genus *Cordyceps* has been recently divided and placed into three families and four genera; *Metacordyceps* (*Clavicipitaceae*), *Elaphocordyceps* (*Ophiocordycipitaceae*), *Ophiocordyceps* (*Ophiocordycipitaceae*) and *Cordyceps* (*Cordycipitaceae*) (Sung et al., 2007). Many *Cordyceps* species are used in traditional Chinese medicines in China, Japan, Korea and other eastern Asian countries. In Chinese, it is called as Dong Chong Xia Cao which means ‘‘winter worm summer grass’’ (Li *et al.*, 2006a; Li *et al.*, 2006b)

The range of therapeutic uses claimed for *Cordyceps* is far reaching and most of them have yet to be sufficiently investigated. In *Cordyceps* has been used to treat conditions including anti-microbial (Park SJ,1996**),** anti-bacterial (Ahn YJ *et al*., 2000), anti-viral (Ortiz *et al*.,1999; Lin and Chiang, 2008; Mueller *et al.*,1991), anti-fungal (Mao and Zhong, 2006), anti-angiogenic (Yoo *et al.*, 2004), anti-diabetic (Choi *et al.*, 2004), anti-HIV (Mueller *et al.*,1991), anti-malarial (Sugar *et al.*,1998), anti-proliferative, anti-metastatic (Liu *et al.*,1997), anti-inflammatory (Yu *et al.*, 2004; Won and Park, 2005), anti-oxidant/anti-aging (Yu *et al*., 2007; Chen *et al*., 2004), pro-sexual (Yu *et al*., 2007; Lin *et al.*, 2007), insecticidal (Mao and Zhong, 2006; Kim *et al*., 2002), steroidogenic (Shih *et al*., 2007), hypogla-caemic (Choi et al., 2004; Yu *et al*., 2007), hypolipidaemic (Yu *et al*., 2007; Shen and Chen, 2001), neuroprotective (Ribeizo, 1995; Gu *et al.*, 2007), pneumo-protective (Yu *et al*., 2007), liver-proctective (Jung *et al.*, 2004; Yu *et al*., 2007) and immunomodulatory (Sone *et al.*, 1985; Mao and Zhong, 2006; Shih *et al.*, 2007). Many also believe it to be a medicine for the treatment for impotence, acting as an aphrodisiac in both men and women. *Cordyceps* is traditionally most often used in the treatment of health issues related to or stemming from the kidneys and the lungs. (Yu *et al*., 2007).

Nucleosides are considered the main bioactive component in *Cordyceps* species. To date, more than 10 nucleosides have been isolated and identified in

Cordyceps species. These include adenine, adenosine, cytosine, cytidine, uridine, guanine, guanosine, hypoxanthine, inosine, thymine, thymidine, 2'-deoxyuridine and cordycepin (Yang *et al.*, 2009). Nucleosides have been used as a marker for the quality control of *Cordyceps* in Chinese Pharmacopoeia (Ballarin *et al*., 1987). Adenosine and cordycepin were pharmaceutically active components exhibits multiple pharmacological actions such as immune-modulatory (Kim, 2010; Yu *et al*., 2007), pharmacokinetic effects, cardio-protection, Anti-cancer effects (Yoshikawa *et al*., 2011), antileukemic (Kodama *et al*., 2000), etc.

Several techniques were available for the analysis of nucleosides in *Cordyceps* such as thin layer chromatography (TLC) (Ma *et al*., 2008), high-performance liquid chromatography (HPLC) (Song *et al*., 2008) and capillary electrophoresis (Li *et al*.,2008). Ion-pairing reversed-phase liquid chromatography (IP-RP-LC-MS) has been commonly used in other fields for the separation of nucleosides (Yang *et al*., 2010). In this work, five main nucleosides namely uridine, inosine, guanosine, adenosine, and cordycepin in samples of entomopathogenic fungal were simultaneously determined by HPLC.

1.2 Objectives of the research

The objectives of this research can be summarized as follows:

1. To validate the analytical method for determination of nucleoside in entomopathogenic fungal samples.
2. To determine the contents of nucleoside in entomopathogenic fungal samples.

1.3 Expected results obtain from the research

This research could be validation and determination the nucleoside in entomopathogenic fungal sample. It also could be lead to development of culture to the amount of nucleoside.

1.4 Scope of the research

1. Literature review.

2. The method for extraction of entomopathogenic fungal sample.

3. The condition for simultaneous determination of nucleosides in entomopathogenic fungal sample by HPLC.

4. Qualitative and quantitative determination of nucleosides in entomopathogenic fungal sample by HPLC.

CHAPTER 2

LITERATURE REVIEW

2.1 Nucleoside

A nucleoside is a nucleobase containing (purine or pyrimidine) connected to sugar (D-ribose or 2-deoxy-D-ribose) by a β-*N*-glycosidic bond. The glycosidic bond linked between C-1' (the anomeric carbon) of sugar and N-1 position of a pyrimidine or N-9 position of a purine base. While nucleotides are composed of three subunit molecules: a nucleobase, a five-carbon sugar, and at least one phosphate group. The position of the phosphoric ester is specified by the number of the carbon to which it is bonded as shown in Fig 1.1 (Paula, 2004; William *et al.*, 2012).

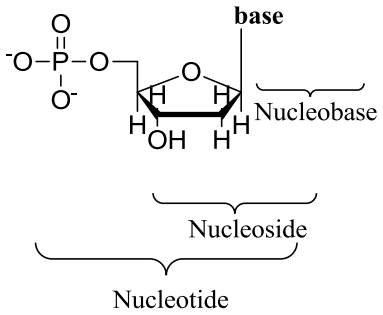


Fig.2.1 Structure of a nucleoside, nucleotide and nucleobase

2.1.1 Purines and pyrimidines

Pyrimidine and purine are the names of nucleobase. Purines consists of a six-membered and a five-membered nitrogen containing ring, fused together. While, pyrimidines have only a six-membered nitrogen ring. There are 3 pyrimidines and 3 purines that are of concern to as (Fig.2.2). Guanine is found in both RNA and DNA. Hypoxanthine and xanthine are not contained into the nucleic acid as they are being synthesized but emphasis intermediates in the synthesis and degradation of the purine nucleotide. Uracil is found only in RNA. While cytosine is founds in both RNA and DNA. Thymine is normally found in DNA (Albert G. Moat *et al.*, 2002).

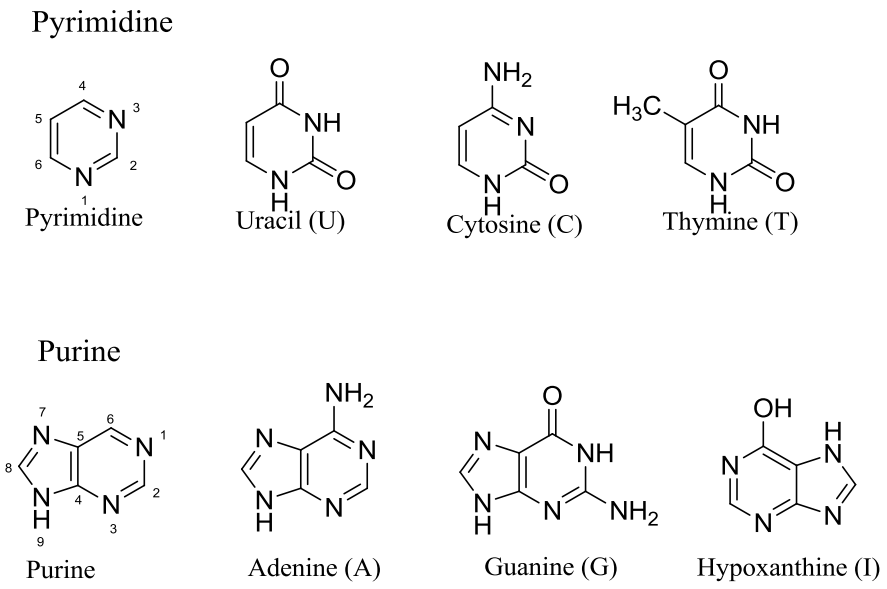


Fig.2.2 Structure of purines and pyrimidines (William *et al.*, 2012)**.**

2.2 Biosynthesis of purine and pyrimidine

Nucleotide biosynthesis in the all can be grouped in to two broad classes

i) *de-novo* synthesis (synthesis from scratch): nucleotides are synthesized new from simple precursor molecule.

ii) Salvage pathway (recycle pathway): nucleosides and nucleobases are formed during the degradation of RNA and DNA.

2.2.1 Biosynthesis of inosine monophosphate (IMP)

The *de novo* pathway for purine synthesis, the first purine product of this pathway, IMP (inosinic acid or inosine monophosphate), serves as a precursor to AMP and GMP. *Step 1:* PRPP synthesis from ribose-5-phosphate and ATP by ribose-5-phosphate pyrophosphokinase. *Step 2:* 5-Phosphoribosyl-b-1-amine synthesis from a-PRPP, glutamine, and H_2O by glutamine phosphoribosyl pyrophosphate amido-transferase. *Step 3:* Glycinamide ribonucleotide (GAR) synthesis from glycine, ATP, and 5-phosphoribosyl-b-amine by glycinamide ribonucleotide synthetase. *Step 4:* Formylglycinamide ribonucleotide synthesis from N^{10}-formyl-THF and GAR by GAR transformylase. *Step 5:* Formylglycinamidine ribonucleotide (FGAM) synthesis from FGAR, ATP, glutamine, and H_2O by FGAM synthetase (FGAR amidotransferase). The

other products are ADP, P_i, and glutamate. *Step 6:* 5-Aminoimidazole ribonucleotide (AIR) synthesis is achieved via the ATP-dependent closure of the imidazole ring, as catalyzed by FGAM cyclase (AIR synthetase). *Step 7:* Carboxyaminoimidazole ribonucleotide (CAIR) synthesis from CO_2, ATP, and AIR by AIR carboxylase. *Step 8:* N-succinylo-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR) synthesis from aspartate, CAIR, and ATP by SAICAR synthetase. *Step 9:* 5-Aminoimidazole carboxamide ribonucleotide (AICAR) formation by the nonhydrolytic removal of fumarate from SAICAR. The enzyme is adenylosuccinase. *Step 10:* 5-Formy-laminoimidazole carboxamide ribonucleotide (FAICAR) formation from AICAR and N^{10}-formyl-THF by AICAR transformylase. *Step 11:* Dehydration/ring closure yields the authentic purine ribonucleotide IMP. The enzyme is IMP synthase (Fig.2.3)

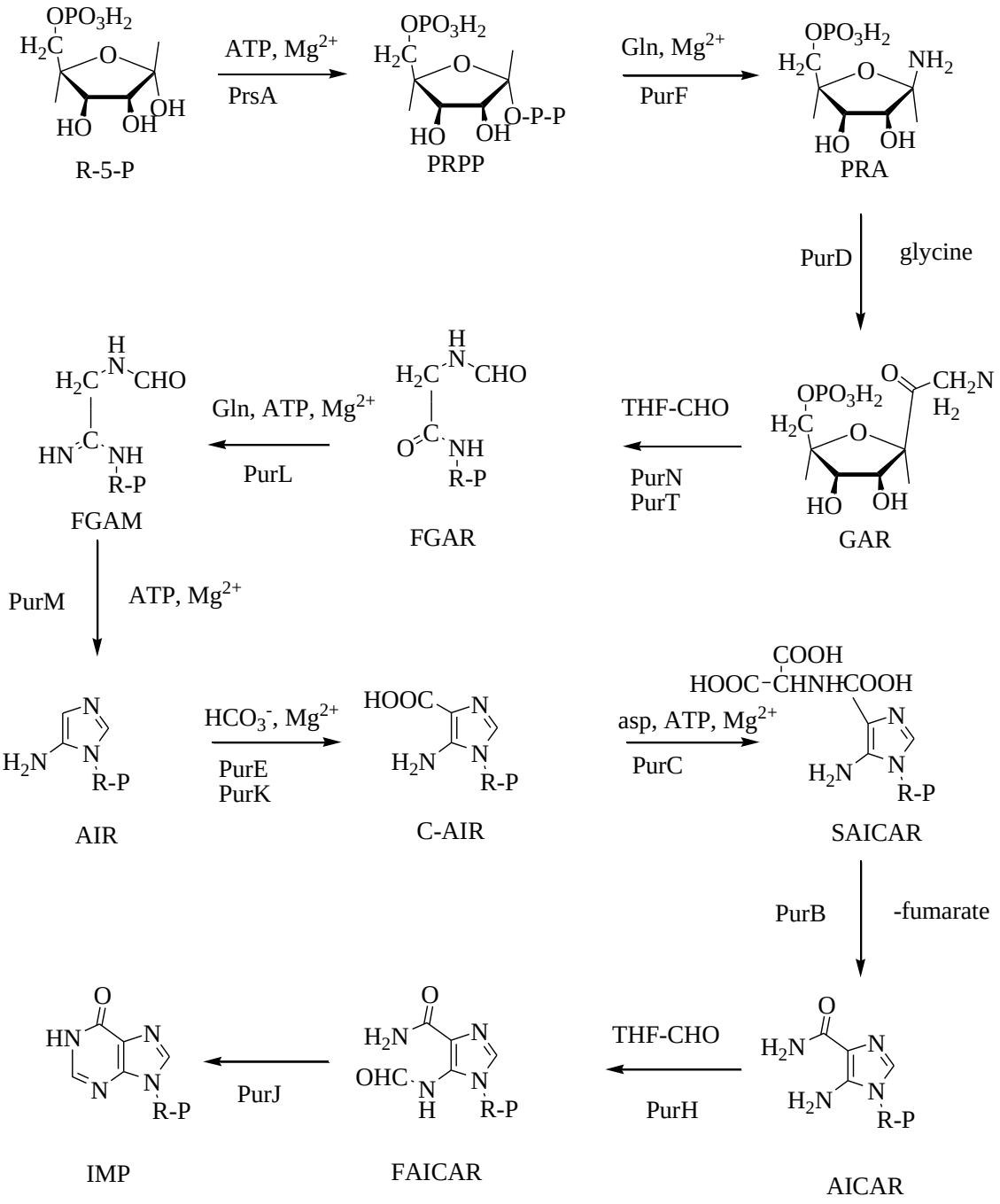


Fig.2.3 The biosynthesis pathway of IMP

2.2.2 Biosynthesis of adenosine monophosphate (AMP)

IMP serves as the branch point from which both adenine and guanine nucleotides can be produced. Adenosine monophosphate (AMP) is derived from IMP in two steps (Fig.2.4). In the first step, aspartate is added to IMP to form adenylosuccinate, a reaction similar to the one catalyzed by argininosuccinate synthetase in the urea cycle. Note how this reaction requires a high-energy bond, donated by GTP. Fumarate is then released from the adenylosuccinate by the enzyme adenylosuccinase to form AMP (Hershfield MS *et al*., 2001)

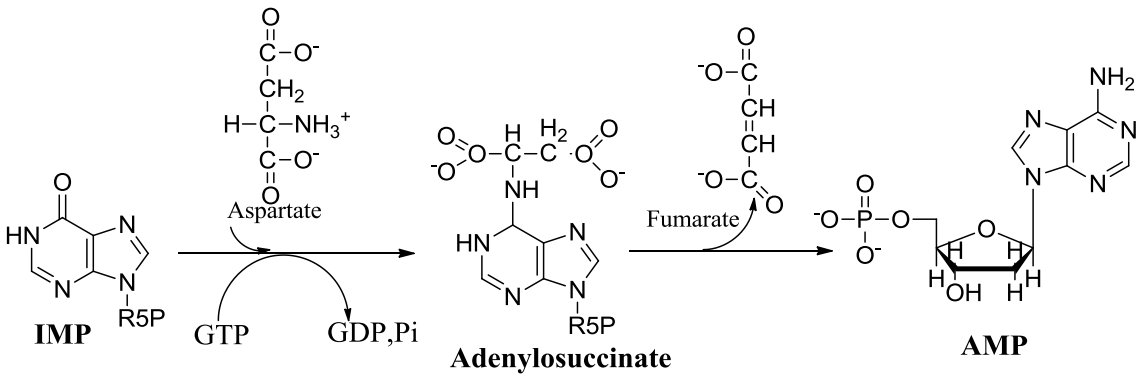


Fig.2.4 The conversion of IMP to AMP: IMP, inosine monophosphste; R5P, ribose 5-phosphate; GTP, guanine triphosphate; GDP, Guanine diphosphate; AMP, adenosine monophosphate.

2.2.3 Biosynthesis of guanosine monophosphate (GMP)

GMP is also synthesized from IMP in two steps (Fig.2.5). In the first step, the hypoxanthine base is oxidized by IMP dehydrogenase to produce the base xanthineband the nucleotide xanthosine monophosphate (XMP). Glutamine then donates the bamide nitrogen to XMP to form GMP in a reaction catalyzed by GMP synthetase. This second reaction requires energy, in the form of ATP (Hershfield MS *et al*., 2001).

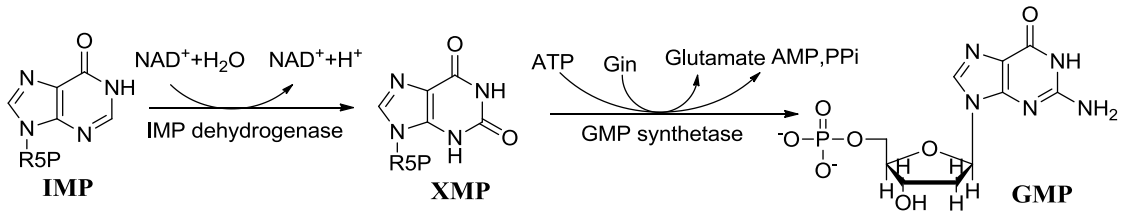


Fig.2.5 The conversion of IMP to GMP: R5P, ribose 5-phosphate; IMP, inosine monophosphste ; XMP, xanthosine monophosphate; ATP, adenosine tri phosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate.

2.2.4 Biosynthesis of uridine monophosphate (UMP)

Uridine monophosphate (UMP) synthase is a bifunctional enzyme of the *de novo* synthesis of pyrimidines (Fig.2.6). A first reaction, orotate phosphor-ribosyltransferase (OPRT), converts orotic acid into OMP, and a second, orotidine decarboxylase (ODC), decarboxylates OMP into UMP. The defect provokes a massive overproduction of orotic acid and a deficiency of pyrimidine nucleotides (Smith, 1973)

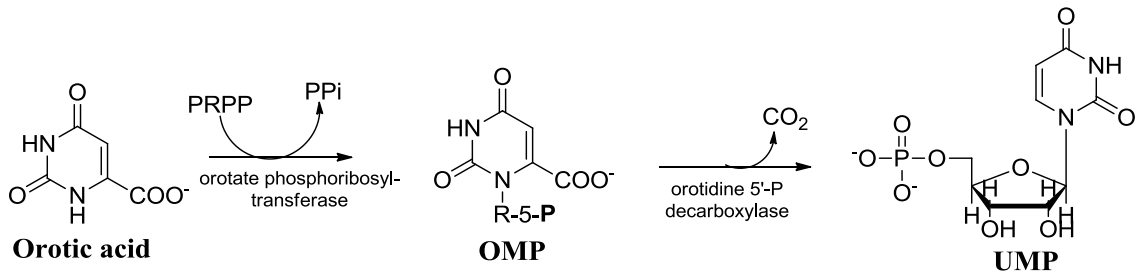


Fig.2.6 The conversion of orotic acid to UMP: PRPP, 5-phosphoribosyl-1-pyrophos-phate; OMP, orotic monophosphate; UMP, uridine monophosphate.

2.2.5 A proposed biosynthesis of cordycepin

Cordycepin (3′-deoxyadenosine), an analog of adenosine lacking the 3′-OH, is one of the main pharmaceutical active ingredients in *O. sinensis*. The biosynthesis of cordycepin in *C. militaris* has been shown to involve the reduction of adenosine (Li *et al*., 2014)

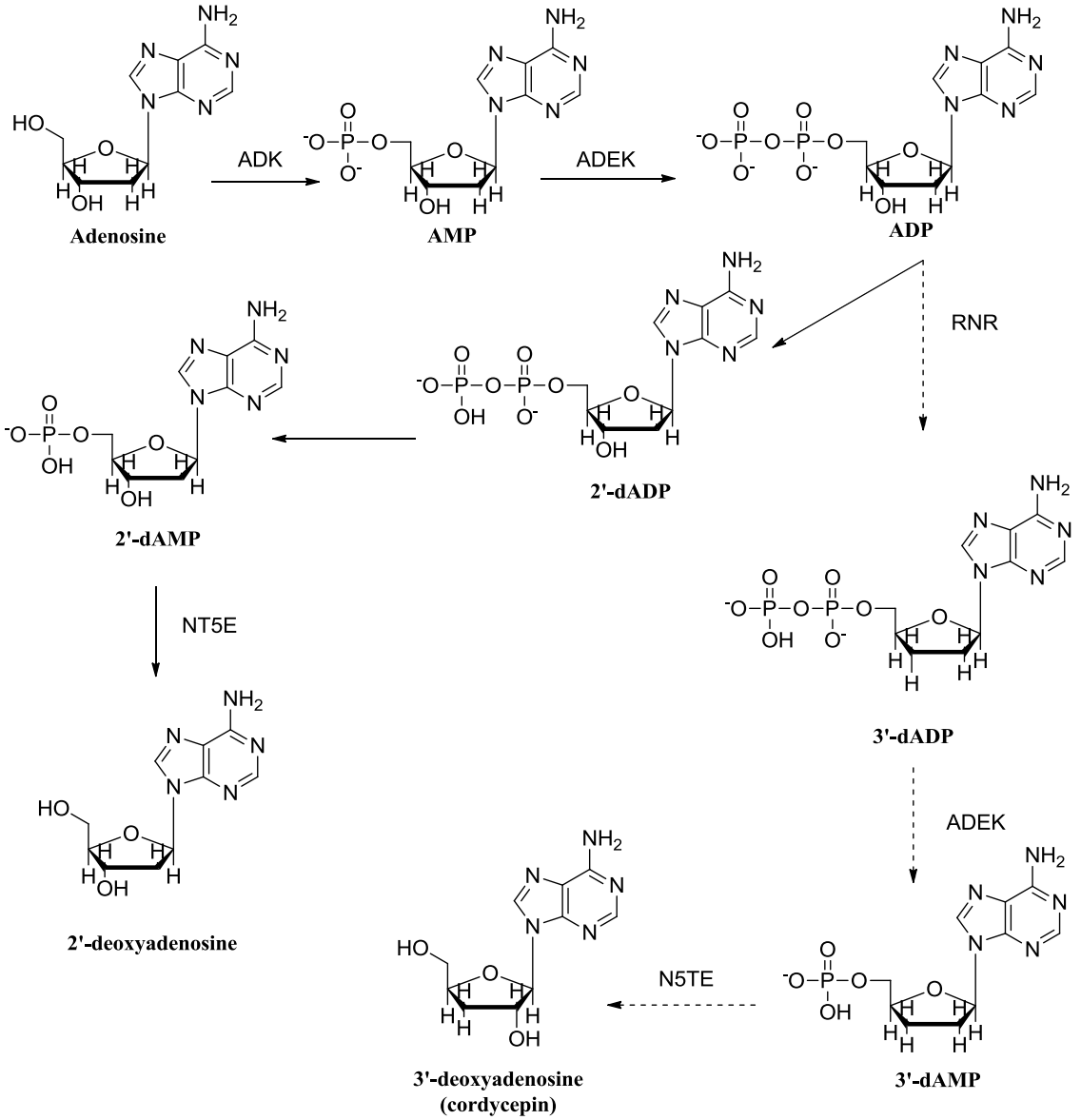


Fig.2.7 A putative biosynthesis pathway for cordycepin: ADK, adenosine kinase ; AMP, adenosine monophosphate; ADEK, adenylate kinase; ADP, adenosine diphosphate; N5TE, 5′-nucleotidase; RNR, ribonucleotide reductases; NT5E, 5’-nucleotidase.

2.3 Entomopathogenic fungi

Entomopathogenic fungi are a heterogeneous group of insect pathogens with nearly 700 known species belonging to approximately 100 orders. Most of them belong to the order *Entomophthorales* of the phylum *Glomeromycota* and to *Hypocreales* of the phylum *Ascomycota*. The genus *Cordyceps* has been recently divided and placed into three families and four genera: *Metacordyceps* (*Clavi-cipitaceae*), *Elaphocordyceps* (*Ophiocordy-cipitaceae*), *Ophiocordyceps* (*Ophio-cordycipitaceae*) and *Cordyceps* (*Cordycipitaceae*) (Sung *et al.,* 2007).

Cordyceps species are interesting macrofungi because of their characteristic parasitic habitat on the larvae and pupae of insects, and even on perfect insects. Many natural *Cordyceps* species are used in Traditional Chinese Medicine (TCM) in China, Japan, Korea, Taiwan and other eastern Asian countries (Xiao *et al.,* 2013). *Cordyceps sinensis* (Berk.) Sacc. is an entomopathogenic fungus that has long been used as a Chinese medicine and tonic. The natural *C. sinensis* product is composed of the fruiting body and its host larva (Chen *et al.,* 2001). *Cordyceps militaris* is a valuable source of useful natural components that possess diverse biological activities. Despite some similarities between *C. militaris* and *C. sinensis* they differ in their color and host. The host of *C. militaris* is *Lepidopteran* pupa and the color of its fruiting bodies is yellow or orange; while, *C. sinensis'* host is *Hepialu* larva and the color of its fruiting bodies is dark brown (Ching *et at.,* 2016). *Ophiocordyceps* is one of the teleomorphic genera in the family *Ophiocordycipitaceae* within the *Hypocreales* and is comprised of more than 100 species, so it is one of the biggest invertebrate pathogenic fungal genera next to *Cordyceps*.

Cordyceps species are rich sources of novel biologically active chemical constituents with diverse structural architectures. They mainly contain many active ingredients, including ribonucleosides, mannitol, sterols, organic acids, polysaccharides, proteins, polyamines, amino acid dipeptides, vitamins (Vit E, K and water-soluble vitamins B1, B2 and B12) and a variety of trace elements (K, Na, Ca, Mg, Fe, Cu, Mn, Zn, Pi, Se, Al, Si, Ni, Sr, Ti, Cr, Ga, V and Zr) (Zhu *et al.*, 1998).

2.4 Pharmacological actions of entomopathogenic fungal

In TCM, *Cordyceps* has been used to treat conditions including respiration and pulmonary diseases; renal, liver and cardiovascular diseases; hypo sexuality; and hyperlipidemia. It is also used in the treatment of immune disorders and as an adjunct to modern cancer therapies (chemotherapy and radiation treatment, etc.). *Cordyceps* is believed by many, particularly in and around Tibet, which is its place of origin, to be a remedy for weakness and fatigue, and it is often used as an overall rejuvenator for increased energy while recovering from a serious illness. Many also believe it to be a medicine for the treatment for impotence, acting as an aphrodisiac in both men and women. *Cordyceps* is often prescribed for the elderly to ease general aches and pains. Practitioners of TCM also recommend the regular use of *Cordyceps* to strengthen the body's resistance to infections, such as colds and flus, and to generally improve the homeostasis of the patient. *Cordyceps* is traditionally most often used in the treatment of health issues related to or stemming from the kidneys and the lungs. For example, it is used to ease a range of respiratory ailments, such as cough and phlegm, shortness of breath, bronchial discomfort, chronic obstructive pulmonary disease (COPD) and asthma. Modern science is attempting to confirm the efficacy of *Cordyceps* for most of its traditional uses; however, most medical studies regarding its efficacy remain incomplete (Zhou *et al.,* 1998; Holliday *et al.,* 2005).

Many studies, both *in vitro* and *in vivo*, have supported *Cordyceps* having diverse biological activities and pharmacological potential. The range of therapeutic uses claimed for *Cordyceps* is far reaching and most of them have yet to be sufficiently investigated. *Cordyceps* has been used with reference to various of its properties, including anti-microbial (Park, 1996), anti-bacterial (Ahn *et al*., 2000), anti-viral (Ortiz *et al*., 1999; Lin and Chiang, 2008; Mueller *et al.,* 1991), anti-fungal (Mao and Zhong, 2006), anti-angiogenic (Yoo *et al.,* 2004), anti-diabetic (Choi *et al.,* 2004), anti-HIV (Mueller *et al.,* 1991), anti-malarial (Sugar *et al.,* 1998), anti-proliferative (Liu *et al.,* 1997), anti-metastatic (Liu *et al.,* 1997), anti-inflammatory (Yu *et al.,* 2004; Won and Park, 2005), anti-oxidant/anti-aging (Yu *et al*., 2007; Chen *et al*., 2004), pro-sexual (Yu *et al*., 2007; Lin *et al.,* 2007), insecticidal (Mao and Zhong, 2006; Kim *et al*., 2002), steroidogenic (Shih *et al*., 2007), hypogla-caemic (Choi *et al.,* 2004; Yu *et al*., 2007),

hypolipidaemic (Yu *et al*., 2007; Shen and Chen, 2001), neuroprotective (Ribeizo, 1995; Gu *et al.*, 2007), pneumo-protective (Yu *et al*., 2007), liver-protective (Jung *et al.*, 2004; Yu *et al*., 2007) and immunomodulatory (Sone *et al.*, 1985; Mao and Zhong, 2006; Shih *et al.*, 2007). Many also believe it to be a medicine for the treatment of impotence, acting as an aphrodisiac in both men and women. *Cordyceps* is traditionally most often used in the treatment of health issues related to or stemming from the kidneys and the lungs (Yu *et al*., 2007).

2.5 Distribution of nucleosides and nucleobases in *Cordyceps*

Nucleosides and nucleobases are some of the major components in *Cordyceps.* To date, more than 20 nucleosides and analogs have been found in natural and cultured *Cordyceps* (Table 2.1). Following that, adenosine and cordycepin are being used as marks for quality control. The nucleoside content differs among natural and cultured *Cordyceps* (Li *et al.*, 2001). Many other nucleosides have been found, including uridine, several distinct structures of deoxyuridines, adenosine, 2'-3'-dideoxyadenosine, hydroxyethyl-adenosine, cordycepin triphosphate, guanidine and deoxyguanidine, which are not found anywhere else in nature. Adenosine and cordycepin (3'-deoxyadenosine) are pharmaceutically active components that exhibit multiple pharmacological actions, such as immunomodulatory and anti-oxidant, etc. Cordycepin was originally extracted from *C. militaris* (Chen and Chu, 1996). Other than adenosine and cordycepin, a few other nucleosides and nucleobases were reported in water extracts of stroma and caterpillars infected with *Cordyceps*, like cytosine, uracil, cytidine, uridine hypoxanthine, thymine, adenine, inosine, guanosine and thymidine (Yuan *et al*., 2008).

Table 2.1 List of nucleoside and nucleobases found in *cordyceps*.

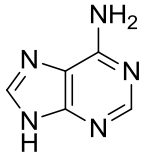
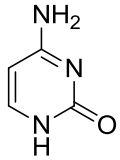
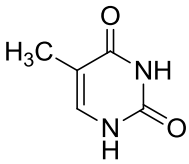
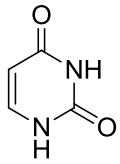
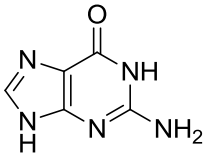
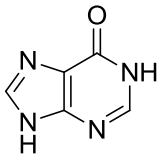
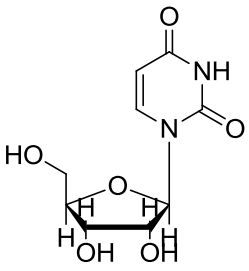
Analytes	Structure	Samples	References
Adenine		*C. sinensis*	Fan *et al.*, 2006
		C. milintaris	Huang *et al.*,2003
			Zhou *et al.*, 2009
			Huichun *et al*., 2011
Cytosine		*C. sinensis*	Fan *et al.*, 2006
			Lei *et al.*, 2006
			Ling *et al*., 2009
Thymine		*C. sinensis*	Fan *et al.*, 2006
		C. milintaris	Huang *et al.*, 2003
		Cordyceps species	Xie *et al*., 2010
Uracil		*C. sinensis*	Fan *et al.*, 2006
		C. milintaris	Guo *et al*., 2006
		Cordyceps species	Yu *et al*., 2006
Guanine		*C. sinensis*	Fan *et al.*, 2006
		C. milintaris	Guo *et al*., 2006
		Cordyceps species	Lei *et al.*, 2006
			Yu *et al*., 2006
Hypoxanthine		*C. sinensis*	Fan *et al.*, 2006
		C. milintaris	Huang *et al.*, 2003
		Cordyceps species	Lei *et al*., 2006
			Feng *et al*., 2009
Uridine		*C. sinensis*	Fan *et al.*, 2006
		C. milintaris	Hu and Li, 2007
		Cordyceps species	Huichun *et al*., 2011
			Lei *et al.*, 2006
			Ling *et al*., 2009
			Feng *et al*., 2009

Table 2.1 List of nucleoside and nucleobases found in *cordyceps*.

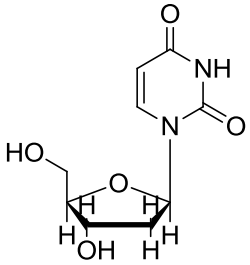
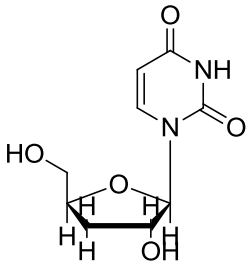
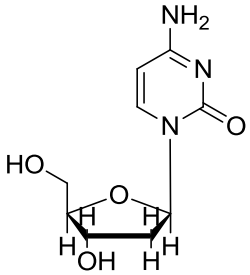
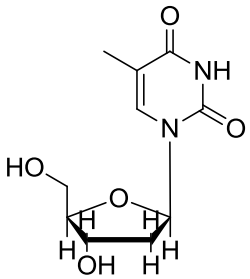
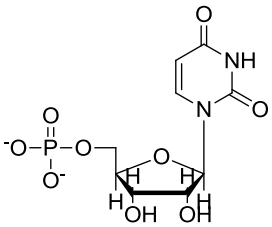
Analytes	Structure	Samples	References
2'-deoxyuridine		*C. sinensis*	Fan *et al.*, 2006 Hu and Li, 2007
3'-deoxyuridine		*C. sinensis*	Fan *et al.*, 2006 Hu and Li, 2007
Cytidine		*C. sinensis* *Cordyceps* species	Fan *et al.*, 2006 Yuan *et al.*, 2008
Thymidine		*C. sinensis* *Cordyceps* species	Fan *et al.*, 2006 Yang *et al*., 2007 Yuan *et al.*, 2008 Yu *et al*., 2006 Yang *et al*., 2007
Uridine-5'-monophosphate		*C. sinensis*	Yue *et al.*, 2013

Table 2.1 List of nucleoside and nucleobases found in *cordyceps*.

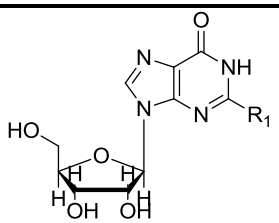
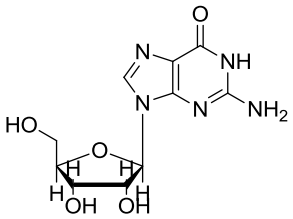
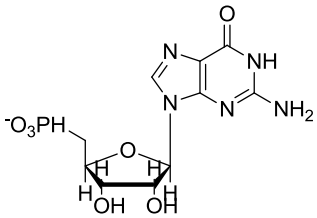
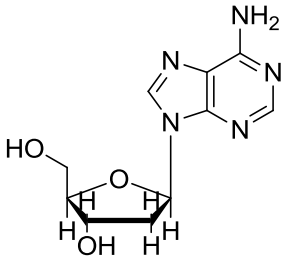
Analytes	Structure	Samples	References
Inosine		*C. sinensis* *C. milintaris* *Cordyceps* species	Fan *et al.*, 2006 Yue *et al.*, 2013 Yuan *et al.*, 2008
Guanosine		*C. sinensis* *C. milintaris* *Cordyceps* species	Fan *et al.*, 2006 Yue *et al.*, 2013 Ikeda *et al*., 2008 Yang *et al*., 2008 Lei et al., 2006 Ling *et al*., 2009 Feng *et al*., 2009 Yang *et al*., 2007 Yuan *et al.*, 2008
Guanosine-5'-monophosphate		*C. sinensis*	Fan *et al.*, 2006 Yue *et al.*, 2013
Adenosine:		*C. sinensis* *C. milintaris* *Cordyceps* species *C. kyushuensis* *C. cicadae*	Fan *et al.*, 2006 Xei *et al.*, 2010 Zhou *et al.*, 2005 Zhou *et al.*, 2005 Huang *et al.*, 2003 Xie *et al*., 2010 Zhou *et al.*, 2009 Yang *et al*., 2007 Huichun *et al*., 2011 Wang *et al.*, 2012

Table 2.1 List of nucleoside and nucleobases found in *cordyceps*.

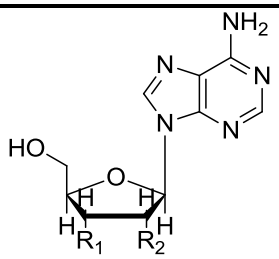
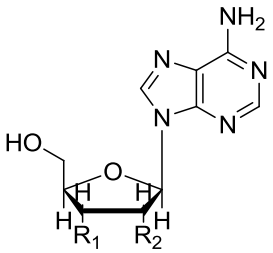
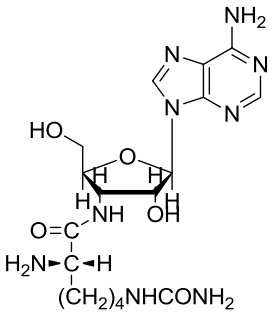
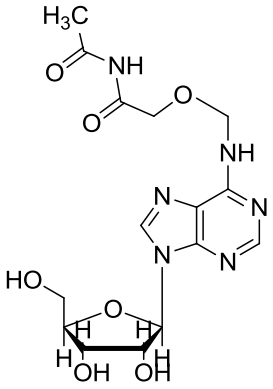
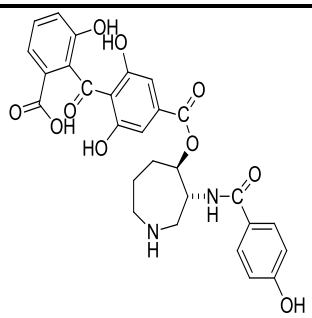
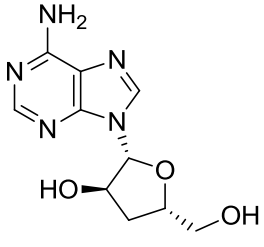
Analytes	Structure	Samples	References
2’-deoxy-adenosine R_1= OH, R_2= H 3’-deoxy-adenosine R_1= H, R_2= OH		*C. sinensis*	Fan *et al.*, 2006
3’-amino-3’-deoxyadenosine : R_1= NH_2, R_2= OH		*C. sinensis*	Fan *et al.*, 2006
3’-homo-citrullyl-amino-3’-deoxy-adenosine		*C. sinensis*	Hu and Li, 2007
N^6-[β-(acetylcaba-moyloxy)ethyl] adenosine		*C. sinensis*	Hu and Li, 2007

Table 2.1 List of nucleoside and nucleobases found in *cordyceps*.

Analytes	**Structure**	**Samples**	**References**
Ophicordin		*C. sinensis* *Cordyceps* species	Hu and Li, 2007
Cordycepin		*C. sinensis* *C. milintaris* *Cordyceps* species *C. kyushuensis*	Huang *et al.*, 2004 Huang *et al.*,2003 Xie *et al*., 2010 Zhou *et al.*, 2009 Yang *et al*., 2007

2.6 Analysis of nucleoside and nucleobase

Nucleosides and nucleobases are one of the major components in *Cordyceps*. To date, more than 20 nucleosides and analogs have been found in natural and cultured *Cordyceps*. These include adenine, adenosine, cytosine, cytidine, uridine, guanine, guanosine, hypoxanthine, inosine, thymine, 2'-deoxyuridine and cordycepin (Yang *et al.,* 2009)

2.6.1 Preparation

Several preparation methods use solvents employed for isolation of selective bio-active compounds. Extracting solvents vary based mainly on polarity, increasing polarity, ethyl acetate, ethanol, methanol and water figure amongst the most commonly used solvents for extraction although combination and sequential extractions are also common practice as shown in Table 2.1.

Water being a high polar molecule extracts polar compounds like nucleosides (Sun *et al.,* 2003). Yamaguchi *et al.* performed a simple hot water extraction using 10 g of dried fruiting bodies of cultured *C. sinensis* blanched in 200 mL of hot water at 70 ^{o}C for 5 min. This produced a brown-colored extract which was then filtered and lyophilized into a gray powder which was administered to rats to determine its effects on cholesterol levels (Yamaguchi *et al.,* 2000). Jia *et al.* seemingly followed the same extraction protocol however, at a higher temperature and longer extraction time. *C. sinensis* powder was added to water at a 1:20 ratio (w/v) and heated for 20 min to a temperature of 120^{o}C (Jia *et al.,* 2009). Koh *et al.* performed a series experiments involving hot water extract from mycelia of *C. sinensis* and effectively demonstrated physiological function as antibiotic growth promoter, antistress and antifatigue effect as well as hypocholesterolemic effect of the hot water extract. (Koh *et al.,* 2003a,b,c). Liu *et al*. prepared separate hot water and ethanol extract, which were later combined for their experiments involving the therapeutic effects of the extract on brain damage. Once, a straight forward extraction procedure was followed with temperature set to 100 ^{o}C and extraction time of 3 h. It would appear that based on the above mentioned extract preparation protocols, time of extraction appears to be of minimal value seeing as a 5 min extraction proved just as effective as a 3 h extraction (Liu *et al.,* 2010).

Methanol and ethanol are also good solvents for the extraction of bio-active principles from *Cordyceps*. These alcoholic extracts are rich in very potent bio-actives like nucleosides, polysaccharides, proteins. Ethanolic extract shows strong anti-oxidant activity (Yamaguchi *et al.,* 2000), preserve β-cell function and offer renoprotection (Kan *et al.,* 2012). Methanolic extract obtained from culture liquid of CS also showed cytotoxic effect against cancer cell lines (Jia *et al.,* 2009). More about pharmacological effects of these extracts as such or purified fractions of extracts are discussed in later section.

Ethyl acetate, although the least polar of the three aforementioned solvents. Preparation of the extracts followed a sequential order starting from non-polar to polar solvents. The dried mycelium powder was first extracted using petroleum ether, ethyl acetate, ethanol and water at a 1:10 (w/v) for each solvent. The extraction process involved the use of an orbital shaker at room temperature for 24 hour except in the case of the hot water extraction where a hot plate and stir bar were used for a period of 3h.After each round of extraction, the extract was filtered and the solvent was evaporate to dryness to afford the crude extract (Wu *et al.,* 2007; Zhang *et al.,* 2004). However, sample preparation has significant effect on the quantification of nucleosides in *Cordyceps.* Three extraction methods, including organic solvent (methanol) pressurized liquid extraction (OSPLE), boiling water extraction (BWE) and ambient temperature water extraction (ATWE), were optimized and applied to the extraction of nucleosides from natural and cultured *C. sinensis* and cultured *Cordyceps militaris*. Their contents of five nucleosides, uridine, inosine, guanosine, adenosine and cordycepin, were quantitatively evaluated and compared by HPLC with DAD (HPLC–DAD). The results showed that the effects of extraction methods on quantification of nucleosides in different types of *Cordyceps* samples were different (Yang *et al.*, 2008).

2.7 Nucleoside determination in *Cordyceps*.

Several detection method are commonly available such as thin layer chromatography; TLC (Ma *et al.,* 2008), HPLC (Song *et al.,* 2007) and capillary electrophoresis (Li *et al.,* 2008). However, limitations are encountered in the way of sensitivity, selectivity and suitability. Ion-exchange chromatography; IEC is yet another

method for separation of nucleosides however, the K^+ and Na^+ present in the mobile phase prevents further analysis and detection via mass spectrometry; MS. Ion-pairing reversed-phase liquid chromatography; IP-RP-LC-MS has been commonly used in other fields for the separation of nucleotides (Yang *et al.,* 2010). Reagents used here are critical for optimal mass spectrometry results. Another effective method of separation and detection involves liquid chromatography separation coupled with electrospray ionization-mass spectrometry; LC/ESI-MS (Xie *et al.*, 2010).

2.7.1 Capillary electrophoresis (CE) –mass-spectroscopy (MS)

CE provides an attractive method for the identification of nucleosides and nucleobases due to its simplicity of use, high separation efficiency, small sample volume and low organic solvent consumption (Yang *et al*., 2009). Optimized CE–MS conditions were used involving 100 mM formic acid containing 10% (v/v) methanol as CE electrolyte. The sheath liquid assists the transfer of the analytes from liquid phase to gas phase. Along with the CE electrolyte, this ensures stable electrospray. Optimal sheath liquid conditions were 75% (v/v) methanol containing 0.3% formic acid. Following optimal conditions, twelve nucleosides and nucleobases were detected. This includes cytosine, adenine, guanine, cytidine, cordycepin, adenosine, hypoxanthine, guanosine, inosine, 2'-deoxyuridine, uridine and thymidine in both natural and cultured *C. sinensis*. Quantitative results indicate total content of nucleosides is much higher in the cultured fungi with the exception hypoxanthine and inosine. Cordycepin, which is mostly found in *C. militaris*, was only detected in minute amounts in natural *C. sinensis* and was not detected in cultured samples (Yang *et al*., 2009).

2.7.2 Liquid Chromatography/electron spray ionization-MS (LC/ESI-MS)

The selection of mobile phase must take into consideration of the separation of the nucleosides but also the ESI component whereby the analytes must be in a volatile state. Optimization of ESI-MS involved testing in positive and negative ion mode and scanning between m/z 50–350 per second. Positive ion mode enabled the detection of thymine, adenine, adenosine, cordycepin and 2-chloroadenosine. Selective ion monitoring (SIM) mode was used to detect as well as to quantify the four main nucleosides (thymine, adenine, adenosine and cordycepin) (Xie *et al.,* 2010) used $[M+H]^+$ at m/z 127, 136, 268, 252 and 302 for monitoring. The ionization temperature was set to 400 ^{0}C. The *Cordyceps* samples from different sources were extracted using

distilled water and ultrasonicated. The vacuum dried filtrate was dissolved in methanol prior to chromatographic separation and subsequent ESI-MS detection (Guo *et al*., 1998)

2.7.3 High performance liquid chromatography (HPLC)

One of the first attempts at generating a profile of nucleosides and nucleobases for *C. sinensis* was performed by Shiao *et al*. (1994) and employed the use of reversed-phase HPLC. Gradient elution using two solvent system consisting of 2.5% MeOH and 20% MeOH in 0.01 M ammonium phosphate revealed the presence of major nucleo-sides and nitrogen bases, uracil, guanine, uridine, guanosine and adenosine (Shiao *et al*., 1994) were successful in producing RP-HPLC profiles of various *Cordyceps* species and *Paecilomyces* species providing the first step in distinguishing between the various species and the use of metabolites as markers for quality control. The use of a phosphate buffer improves chromatographic performance however can result in ion suppression for mass spectrometry detection and poor separation (Klawitter *et al*., 2007).

RP HPLC was used by Chen *et al*. (2009) to identify three anti-cancer compounds from active fractions of a crude ethyl acetate extracted *C. sinensis*. Mobile phase parameters employed in detecting these compounds varied and therefore three separate runs were performed. In the isolation of gliocladicillin A, 50–55% acetonitrile in water ran for 5 min and the concentration of acetonitrile was held constant at 55% for the remaining 25 min with peak retention time observed at 14.3 min. Gliocladicillin B was observed at a retention time of 19.5 min using 60–85% MeOH as mobile phase for 40 min. The last compound 11, 11'-dideoxyverticillin was eluted at 18.0 min using 60% acetonitrile in water for 5min followed by 60–70% acetonitrile for 25 min (Chen *et al*.,2009)

HPLC is commonly used technique for separation and analysis of nucleosides in *Cordyceps* species. A simple HPLC with UV detection (HPLC–UV) method was also proposed for the authentication of *Cordyceps* and its allies (Ikeda *et al.,* 2008) though nucleosides amount might be not available for the purpose due to the great variation in different samples with the same origin. Further study indicated that natural *C. sinensis* might contain some enzymes which could transfer the target compounds such as nucleic acids and nucleotides into uridine, inosine and guanosine or

decompose adenosine (Yang *et al.,* 2008). To date, several condition of HPLC was reported for determination of nucleoside and nucleobases as shown in Table 2.2. Therefore, identification and determination of nucleosides in *Cordyceps* are very important for controlling its quality. However, at most eight nucleosides and their bases were considered, which excluded guanosine with high content and inosine with significant pharmacological activities in *Cordyceps* (Guo *et al.,* 2006). However, qualitative and quantitative determinations of nucleosides in natural and cultured *cordyceps* are necessary for quality control of *cordcyeps*. More than 10 nucleosides and their bases were determined using HPLC (Li *et al.*, 2004).

Fast simultaneous determination of 14 nucleosides and nucleobases namely adenine, adenosine, cytosine, cytidine, uracil, uridine, guanine, guanosine, hypoxanthin, inosine, thymine, thymidine, 2-deoxyuridine and cordycepin in cultured *Cordyceps* using ultra-performance liquid chromatography were reported by Yang *et al*. (2017). The separation was performed on Waters Acquity UPLC system with Acquity UPLC BEH C18 column and gradient elution of 0.5 mM acetic acid and acetonitrile in 5 min. The peaks were detected at 254 nm. (Yang *et al*., 2007). However, simple HPLC-UV was use for determination of 6 nucleosides. The column was ODS (250 mm. x 4.6 mm. i.d 5 µm). Mobile phase was mixture of acetonitrile and water (5:95 v/v). The UV detection was set at 260 nm (Ikeda *et al.,* 2008). The high performance liquid chromatography–electrospray ionization tandem mass spectrometry was developed for qualitative and quantitative determination of nucleoside. A ZORBAX ODS-C18 guard column (12.5 mm. × 4.6 mm. i.d., 5µm) were used for separation. Solvents for the mobile phase were 5 mM aqueous ammonium acetate (A) and methanol (B). The gradient elution was: 0–10 min, linear gradient 0–5% B; 10–30 min, linear gradient 5–20% B. Peaks were detected at 254 nm positive (negative for uridine) ion mode of MS and MS/MS detection. Mass spectrometry was carried out in the scan mode from m/z 50–350 (Fan *et al.,* 2006). As noted above, we can see that HPLC is commonly used technique for separation and analysis of purine and pyrimidine in natural and cultured *cordyceps* species.

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	Sample	Preparation	Analytical method	References
Cytosine, Cytidine, Uracil, Pseudouridine, Uric acid, Guanine Hypoxanthine, 2'-deoxycytidine, Xanthine, Uridine, Adenine, Thymine, 2'-deoxyuridine, Adenosine, Inosine, Cordycepin, Guanosine, Thymidine, 2'deoxyguanosine	Natural *C. sinensis* Cultured *Cordyceps*	1. extracted with distilled water 2. The aqueous extracts were passed though cartridge columns (reversed phase) 3. After eluting with distilled water, the cartridge column was eluted with a mixture of methanol-water (1:1, v/v) to recover samples.	**Column**: Cosmosil $5C_{18}$ (250mm×4.6 mm,i.d.), 5 µm **Mobile phase:** Gradient elution with Solvent A (2.5% MeOH in 0.01 mmol/L (NH4)H2PO4) and solvent B (20% MeOH in 0.01 mmol/L $(NH_4)H_2PO_4$) **Detection**: UV 260 nm.	Shiao *et al.*, 1994

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	**Sample**	**Preparation**	**Analytical method**	**References**
Adenosine	Cultured *Cordyceps*	Ultrasonic with distilled water	**Column**: Dupont C18 (250mm×4.6 mm,i.d.) **Mobile phase:** Methanol–0.06 mol/L monopotassium phosphate–THF (10:150:1.5) **Detection:** UV 260 nm.	Gong *et al.*, 1999
Adenine Hypoxanthine Adenosine Cordycepin Uracil Uridine Thymine Guanine	Natural *C. sinensis* Cultured *C. militaris*	Ultrasonic with distilled water	**Column**: Shimadzu VP-ODS column **Mobile phase:** Water–methanol–formic acid (85:14:1, v/v/v) **Detection:** DAD-ESI-MS	Huang *et al.*, 2003
Adenosine Cordycepin	Natural *C. sinensis*	Soxhlet with methanol	**Column**: Zorbax–ODS (250mm×4.6 mm, i.d.), 5 μm **Mobile phase:** Methanol **Detection:** UV 260 nm.	Huang *et al.*, 2004

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	**Sample**	**Preparation**	**Analytical method**	**References**
Adenosine, Adenine, Cordycepin, Cytosine, Cytidine, Guanine, Uracil Guanosine, Thymidine, Uridine, 2'-deoxyuridine, Thymine	Natural *C. sinensis* Cultured *Cordyceps* Cultured *C. militaris*	Pressurized liquid extraction	**Column**: Zorbax NH_2 (250mm×4.6 mm, i.d.), 5 µm **Mobile phase:** Gradient elution with acetonitrile and 10 mmol/L ammonium acetate water **Detection:** UV 254 nm.	Li *et al.*, 2004
Uridine Adenosine	Natural *C. sinensis*, Cultured *Cordyceps* Cultured *C. militaris*	Soxhlet with methanol	**Column**: Shimadzu VP-ODS column **Mobile phase:** Water-methanol-formic acid (90:9:1) **Detection:** UV 260 nm.	Zhou *et al.*, 2005

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	Sample	Preparation	Analytical method	References
Uracil Adenosine Hypoxanthine Uridine Thymine Guanine Adenine Cordycepin 2-chloro- adenosine	*C. sinensis*	Extraction with distilled water	**Column**: Shimadzu VP-ODS **Mobile phase:** Gradient elution of ammonium acetate and methanol **Detection:** UV 260 nm. MS	Guo *et al*.,2006
Cordycepin	Natural and cultured *C.* species	Extraction with methanol	**Column**: Waters ODS C-18 **Mobile phase:** Water–methanol (72:28) **Detection:** UV 260 nm.	Meena *et al.*, 2010
	C. militaris	Extraction with with distilled water	**Column**: Mightysil RP-18 **Mobile phase:** Methanol–20 mM phosphoric acid (15:85) **Detection:** UV 260 nm.	Rao *et al.*, 2006

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	Sample	Preparation	Analytical method	References
Adenine Adenosine Cytosine Cytidine Cracil	Natural and cultured *C.*species	Extraction with distilled water	**Column**: Symmetry Shield Rp18 **Mobile phase:** Gradient elution of acetonitrile and water **Detection:** UV 260 nm.	Lei *et al.*, 2006
Uridine Guanine Guanosine Hypoxanthin Inosine Thymine Thymidine 2-deoxyuridine Cordycepin	cultured *C.*species	PLE with methanol	**Column**: Acquity UPLC BEH C18 **Mobile phase:** Gradient elution of 0.5 mM acetic acid and acetonitrile **Detection:** UV 254 nm.	Yang *et al*., 2007
Adenine Cytosine Guanine Hypoxanthine Thymine Uracil	Natural and cultured *C.*species	Acid hydrolysis pH 7 2 mol/NaOH	**Column**: Zobax SB-AQ **Mobile phase:** Gradient elution of 5 mM aqueous TEA and methanol **Detection:** UV 254 nm.	Fan *et al*., 2007

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	**Sample**	**Preparation**	**Analytical method**	**References**
Adenosine Guanosine Uridine Inosine Cytidine Thymidine Uracil Cordycepin Adenine Guanine, Hypoxanthine, Cytosine, Thymine	*C. sinensis*	Reflux with water	**Column**: ZORBAX SB-Aq **Mobile phase:** Gradient elution of 0.25 mM PDFOA in water and acetonitrile **Detection:** UV 260 nm.	Yang *et al*., 2007
Cordycepin	Cultured *C. militaris*	Ultrasonic with distilled water	**Column**: Eclipse XDB-C18 **Mobile phase:** Gradient elution of water and acetonitrile **Detection:** UV 260 nm.	Song *et al*., 2007

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	**Sample**	**Preparation**	**Analytical method**	**References**
Cordycepin	*C. militaris*	HSCCC acetate: butanol: water (3:2:5)	**Column**: Alltima C18 **Mobile phase:** Methanol–water (17:83) **Detection:** UV 260 nm.	Ju *et al.*, 2009
Adenosine Cordycepin Cytidine Guanosine	*C. kyushuensis*	Extraction with distilled water	**Column**: Kromasil 100-C18 **Mobile phase:** Phosphate buffer-methanol (17:3) **Detection:** UV 260 nm.	Ling *et al*., 2009
Inosine Uridine, Thymidine Cytosine Guanine Thymine Uracil	Natural and cultured *C.*species	Extraction with distilled water	**Column**: Zorbax 300SB C18 **Mobile phase:** Gradient elution of water and methanol **Detection:** UV 260 nm. MS	Yu *et al*., 2006
Adenosine Cordycepin Guanosine Uridine	*C. sinensis* Cultured *C. militaris*	Ultrasonic with distilled water	**Column**: Daisopak 120-5-ODS-BP **Mobile phase:** Acetonitrile–water (5:95) **Detection:** UV 260 nm.	Ikeda *et al*.,2008

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	Sample	Preparation	Analytical method	References
Uridine inosine Guanosine Adenosine Cordycepin	*C. sinensis* Cultured *C. militaris*	PLE with methanol	**Column**: Zobax SB-AQ **Mobile phase:** Gradient elution of 10 mM aqueous TEA and methanol **Detection:** UV 254 nm.	Yang *et al*.,2008
Uracil Uridine Hypoxanthine Inosine Guanosine Adenosine, Adenine, Cordycepin	*C.*species	Socked with 75% ethanol	**Column**: Zobax SB-AQ **Mobile phase:** Gradient elution of water and acetonitrile **Detection:** UV 260 nm.	Feng *et al*., 2009
Cordycepin Adenosine	*C. sinensis* Cultured *C. militaris*	Ultrasonic with distilled water	**Column**: Waters Symmetry Shield RP 18 **Mobile phase:** Water–methanol (92:8) **Detection:** UV 254 nm.	Huang *et al.*,2009

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	**Sample**	**Preparation**	**Analytical method**	**References**
Adenine Adenosine Cordycepin 2-chloro- adenosine	*C. sinensis*	Extraction with methanol	**Column**: Shimadzu VP-ODS **Mobile phase:** Water–methanol– formic acid (92:7:1) **Detection:** ESI-MS	Zhou *et al.*,2009
Adenosine Cordycepin	*C. sinensis*	Extraction with methanol	**Column**: Water Xterra MS C18 **Mobile phase:** Water–methanol– formic acid (89:10.5:1) **Detection**: ESI-MS	Yang *et al.*,2007
Thymine Adenine Adenosine Cordycepin 2-chloro- adenosine	*C. sinensis*	Extraction with distilled water	**Column**: Shimadzu VP-ODS **Mobile phase:** Gradient elution of ammonium acetate and methanol **Detection:** UV 260 nm. ESI-MS	Xie *et al.*,2010

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	**Sample**	**Preparation**	**Analytical method**	**References**
Uridine Adenine Adenosine Cordycepin	*C. militaris*	Extraction with distilled water	**Column**: Phenomenex C18 **Mobile phase:** Methanol–water (15:85) **Detection:** UV 260 nm.	Huichun *et al*., 2011
Adenosine Cordycepin Cytidine Guanosine Thymidine Uridine Inosine Adenine Cytosine	Cultured *C. militaris*	Ultrasonic with distilled water	**Column**: ZORBAX Eclipse XDB-C18 **Mobile phase:** Gradient elution of 20 mM phosphate buffer and methanol **Detection:** UV 260 nm.	Yuan *et al.*, 2008
Adenosine Cordycepin	Cultured *C. cicadae*	Extraction with distilled water	**Column**: YMC-Pack C8 **Mobile phase:** Methyl alcohol–water (85:15) **Detection:** UV 258 nm.	Wang *et al.*, 2012

Table2.2. Comparison to different methods of HPLC for determination of nucleosides.

Analyes	Sample	Preparation	Analytical method	References
Cordycepin	*C. sinensis*	Extraction with distilled water	**Column**: YMC-packed C18 **Mobile phase:** Methanol–water (20:80) **Detection:** UV 260 nm.	Ni *et al.*, 2009
		Ultrasonic with distilled water	**Column**: ZORBAX SB-Aq **Mobile phase:** Acetonitrile–water (10:90) **Detection:** UV 260 nm.	Mao *et al.*, 2010
Adenosine Cordycepin	*C.*species	Microwave assisted with water	**Column**: Eclipse XDB-CN **Mobile phase:** Methanol–water (7:93) **Detection:** UV 260 nm.	Li *et al.*, 2012

CHAPTER 3

METHODOLOGY

3.1 Reagents and standards

Uridine, inosine, guanosine and adenosine were purchased from Acros (New Jersey, USA). Cordycepin was purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol form Carol Erba Reagent SpA. Triethylamine form Carol Erba Reagent SpA, deionized water. A stock standard solution was prepared in methanol.

The individual stock standard solutions were prepared in methanol at concentration of 200 mg L^{-1} and stored at 4 °C in the refrigerator. The standard working solutions were daily prepared by dilution of stock standard solution with methanol to the required concentrations. All chemical were prepared in glass apparatus and stored in a glass bottle.

3.2 Microorganism

Natural *Cordyceps* samples were obtained from different resources of Thailand (4 samples). The samples were cultured by Asst. Prof. Dr.Watchara Kanchanarach, department of Biology, faculty of Science Mahasarakham university (11 samples). The commercial available products of *Cordyceps* (13 samples) were purchased from different markets and online markets located in Thailand (Table 3.1 - 3.3).

Table 3.1 Cultured *Cordyceps* samples.

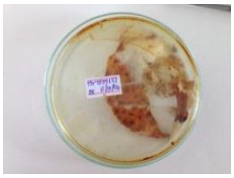
Samples	Name	Pictures
***Cordyceps* sp.**		
C-01	MY 7341(2) 28	
C-02	MSU 017(3) 28	
C-03	MY 7337(2) 28	
C-04	MY 7339(3) 28	
C-05	MSU 027(2) 28	
C-06	MY 7287(2) 28	
C-07	MY 7340(3) 28	
C-08	MY 7337	
C-09	MY 7337/2	

Table 3.1 Cultured Cordyceps samples.

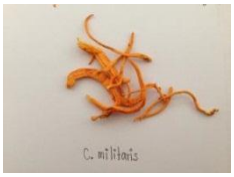
Samples	Name	Pictures
C-10	MY 007/06	
C-11	MY 007/06/2	
C-12	*C. militaris*	
***Ophiocordyceps* sp.**		
C-13	ophiocordyceps	
C-14	MSU NO.1	
C-15	MSU NO.3	
C-16	MSU NO.4	
C-17	MSU NO.8	
C-18	WO	
C-19	017	
C-20	034	

Table 3.2 The commercial available products of *Cordyceps*.

Samples	**Brand name**	**Ingredients**	Pictures	
C-21	Mister mushroom	*C. militaris* 100 %		
C-22	ปราชญา	*C. militaris* 100 %		
C-23	พอใจฟาร์ม	*C. militaris* 100 %		
C-24	Giffarine	*C. sinensis* 82.817% Calcium Ascorbate 1.923%		
C-25	SAND-M Handy Herb	*C. sinensis* 299 mg. Ginseng extract 100 mg. Reishi extract 100 mg.		
C-26	Finn	*C. sinensis* 330 mg. Panax ginseng 170 mg.		
C-27	ถั่งเช่า 168	*C. militaris* 100 %		

Table 3.2 The commercial available products of *Cordyceps*.

Samples	**Brand name**	**Ingredients**	Pictures	
C-28	Zen Cordyceps	*C. sinensis* 400 mg. Ginseng extract 100 mg. L-arginine 200 mg.		
C-29	Cordyceps-plus (herbal one)	*C. sinensis* 299 mg. Ginseng extract 100 mg. Reishi extract 100 mg.		
C-30	Mister mushroom	*C. militaris* 100 %		
C-31	ถั่งเช่า 168	*C. militaris* 100 %		

Table 3.3 Natural *Cordyceps* samples.

Code	Pictures	
N-01		
N-02		
N-03		
N-04		

3.3 Sample preparation for extraction

The dried *Cordyceps* samples were grounded at first. Then, about 0.5 g of samples was exactly weighed and added to 20 ml of methanol. The sample-methanol mixture was placed into an ultrasonic bath for 30 min. Then the sample mixture was filtered and the filtrate was evaporated. The crude extract was dissolved with methanol and injected into HPLC.

3.4 Analysis

3.4.1 Validation of the HPLC methods

3.4.1.1 The linear calibration

The linearity was evaluated through the relationship between peak area (y) and the concentration (x) on the calibration curves of uridine, inosine, guanosine, adenosine and cordycepin. The calibration curves were plotted by the standard mixtures at various concentrations ranging from 1 to 100 mg L^{-1} of each standard. The coefficient of determinations (r^2) was calculated by mean of the least-square analysis.

3.4.1.2 Precision

The precision of the proposed method was presented as the repeatability and reproducibility of retention time and peak area. The relative standard deviation (RSD) is target value for quantitation. The repeatability (intra-day precision) was from five times per day (n=5) and reproducibility (inter-day precision) was calculate from three times a day for three consecutive days (n=3×3). The mixture of standard solutions was used at concentration of 10 mg L^{-1}, and calculated as RSD as shown in equation 3.1.

$$\%RSD = \frac{SD}{\bar{X}} \times 100 \qquad (3.1)$$

% RSD = percentage relative standard deviation

SD = standard deviation

$\bar{X}$ = mean average

3.4.1.3 Accuracy

The accuracy of the proposed method as percentage recoveries were studied by spiking of 1.0 mg L^{-1} of standard solutions into sample under the optimum experimental conditions. Nucleoside concentrations were calculated from the linear calibration graph and the accuracy can be evaluated as % recovery using equation 3.2

$$\%\text{Recovery} = \frac{(\text{peak area of spike sample} - \text{peak area of sample})}{\text{peak area of standard solution}} \times 100 \quad (3.2)$$

3.4.1.4 Sensitivity

The limit of detection (LOD) and limit of quantitation (LOQ) under the optimum chromatographic conditions were calculated as the analyte concentration giving a signal to signal to noise ratios (*S/N*) of 3 and 10, respectively.

3.4.2 Determination of nucleoside contents

3.4.2.1 Instrumentation

Extraction was performed on Bandelin sonorex digitec. Analysis was performed on Shimadzu LC - 20AC liquid, a chromatograph, equipped with diode array detection (DAD) system. An ACE-5 C18-AR column (4.6 mm. x 250 mm., i.d. 5 µm.) and an ACE-5 C18 - AR guard column (4.6 mm. x 12.5 mm., i.d. 5 µm.). The analytes were monitored at 254 nm.

3.4.2.2 Conditions of HPLC

Conditions were optimized for simultaneous determination of five nucleosides. Solvents that constituted the mobile phase were used A (5 mM aqueous TEA) and B (methanol). The separation was achieved using gradient elution as shown in Table 3.4. The injection volume of 20 µL was used. The flow rate was 1 mL min^{-1}. The analytes were monitored at 254 nm. (Fan *et at*., 2006, 2007; Yang *et al*., 2008; Gao *et al*., 2007)

Table 3.4 Condition of gradient mobile phase

Time (min)	% MeOH	% 5 mM aqueous TEA
0	0	100
2	0	100
25	25	75
30	100	0
35	0	100
45	0	100

CHAPTER 4

RESULT AND DISCUSSION

4.1 Validation of the HPLC methods

In order to evaluate the present method for quantitative determination, linearity, LOD, LOQ, precision and recovery were investigated. The results obtained were shown as following.

4.1.1 Linearity

According to the proposed method, the calibration curve for determination of uridine, inosine, guanosine, adenosine and cordycepin were constructed under the optimum conditions. The linear range for mix standard was from 0 to 100 mg L^{-1}. The linear regression equation and coefficient determination (r^2) of mix standard were in the range of 0.995 - 0.999, as summarized in Table 4.1. The calibration curves of mix standards were shown in Fig 4.1.

Table 4.1 Linear regression equation and coefficient determination (r^2) of mix standard.

Analytes	**Linear range (mg L^{-1})**	**regression equation**	**r^2**
Uridine	1.00-100.00	y = 3854.3x + 8109.3	0.998
Inosine	1.00-100.00	y = 2350.5x - 6442.7	0.995
Guanosine	1.00-100.00	y = 3166.5x + 2420.6	0.996
Adenosine	1.00-100.00	y = 8241.3x + 21969	0.997
Cordycepin	1.00-200.00	y = 25488x + 22671	0.999

4.1.2 Precision

The repeatability and reproducibility were investigated in terms of relative standard deviation (RSD) of peak area. The repeatability was performed from five replicates within one day (intra-day precision, n = 5) and reproducibility was calculated from the experiment in three days (inter-day precision, n = 3 × 3). The RSDs of peak areas of standard in terms of repeatability and reproducibility were summarized in Table 4.2. The repeatability present as the relative standard deviation for intra-day and inter-day variations of less than 3.57 and 4.57%.

Table 4.2 Repeatability and reproducibility of analytes.

Analytes	intra-day (%RSD)	inter-day (%RSD)
Uridine	1.71	2.10
Inosine	0.76	3.53
Guanosine	3.57	1.53
Adenosine	2.82	4.57
Cordycepin	3.42	2.39

4.1.3 Accuracy

The accuracy of the proposed method of HPLC as percentage recoveries were studied by spiking of 1.0 $mg.L^{-1}$ of standard solutions into sample under the optimum experimental conditions. The recoveries of uridine, inosine, guanosine, adenosine and cordycepin were 109.09, 107.57, 102.20, 102.49 and 94.74%, respectively, as shown in Table 4.3.

Table 4.3 Percentage recovery of five nucleosides

Analytes	%Recovery
Uridine	109.09 ±0.06
Inosine	107.57±0.10
Guanosine	102.20±0.03
Adenosine	102.49±0.13
Cordycepin	94.74±0.08

4.1.4 Sensitivity

The limit of detection (LOD) and limit of quantitation (LOQ) under the optimum chromatographic conditions were calculated as the analytes concentration giving a signal to signal to noise ratios (*S/N*) of 3 and 10, respectively. The limits of detection and quantification were in the range of 0.15 - 2.50 $mg.L^{-1}$ and 4.50 - 20.00 $mg.L^{-1}$, respectively as show in Table 4.4.

Table 4.4 Sensitivity of mix standard by HPLC.

Analytes	Limit of detection (LOD , mg L^{-1})	Limit of quantitative (LOQ, mg L^{-1})
Uridine	1.75	11.80
Inosine	2.50	20.00
Guanosine	1.50	10.50
Adenosine	0.50	5.00
Cordycepin	0.15	4.50

4.2 Qualitative determination of nucleoside

Chromatograms of nucleoside of *Cordyceps* were shown in Fig.4.1, 4.2 and 4.3. The investigated compounds were identified by comparison of their retention times with those obtained on injecting standards under the same conditions or by spiking *Cordyceps* samples with stock standard solutions.

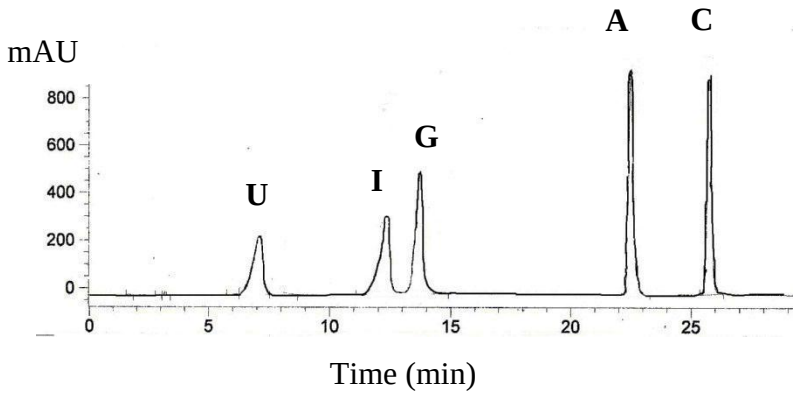


Fig.4.1 HPLC profiles of mixed nucleosides standards constantants 20 $mg.L^{-1}$. U; Uridine, I; Inosine, G; Guanosine, A; Adenosine, C; Cordycepin

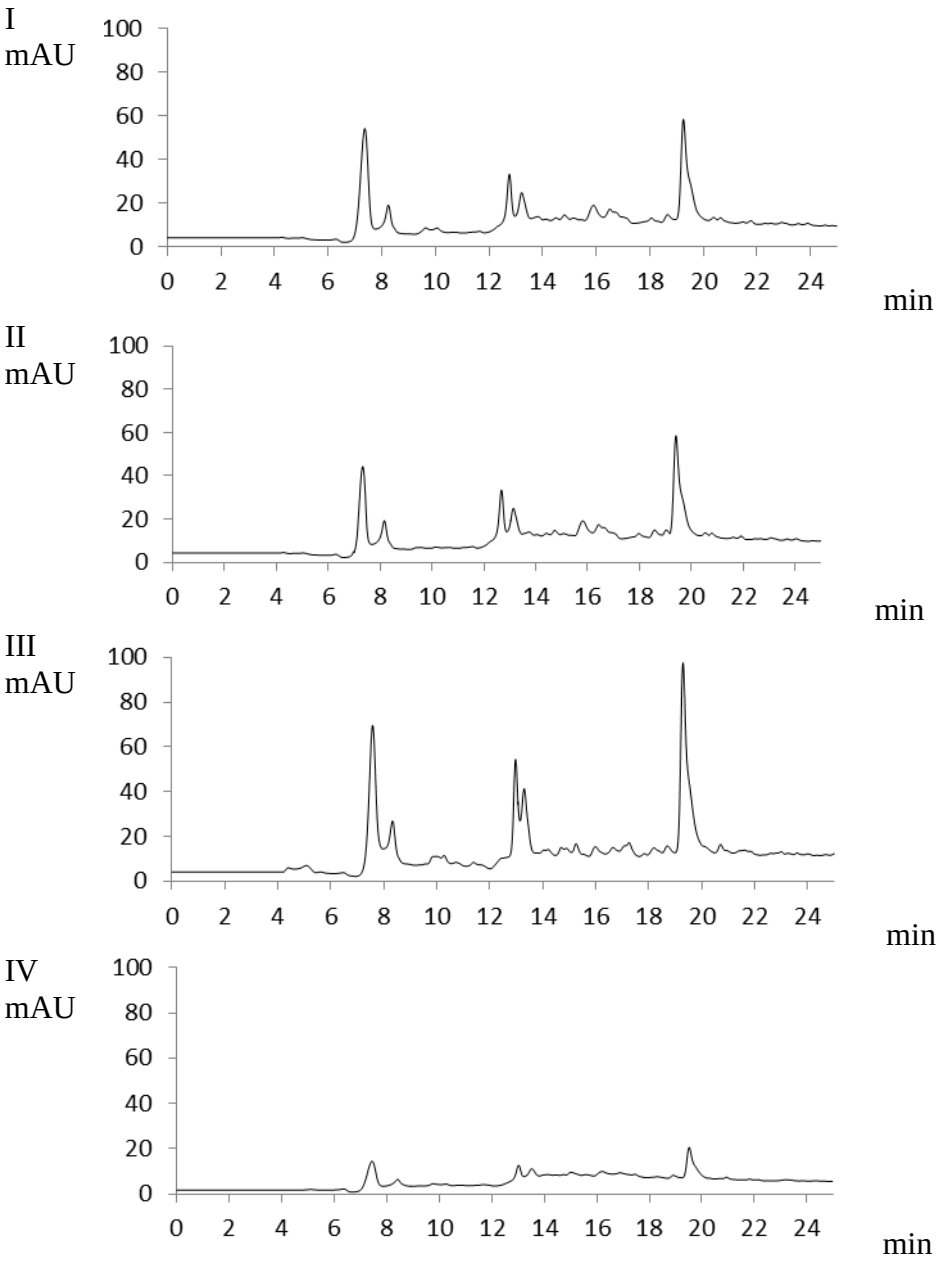


Fig. 4.2 HPLC profiles of cultured *Cordyceps* species: I; C-01, II; C-02, III; C-03, IV; C-04

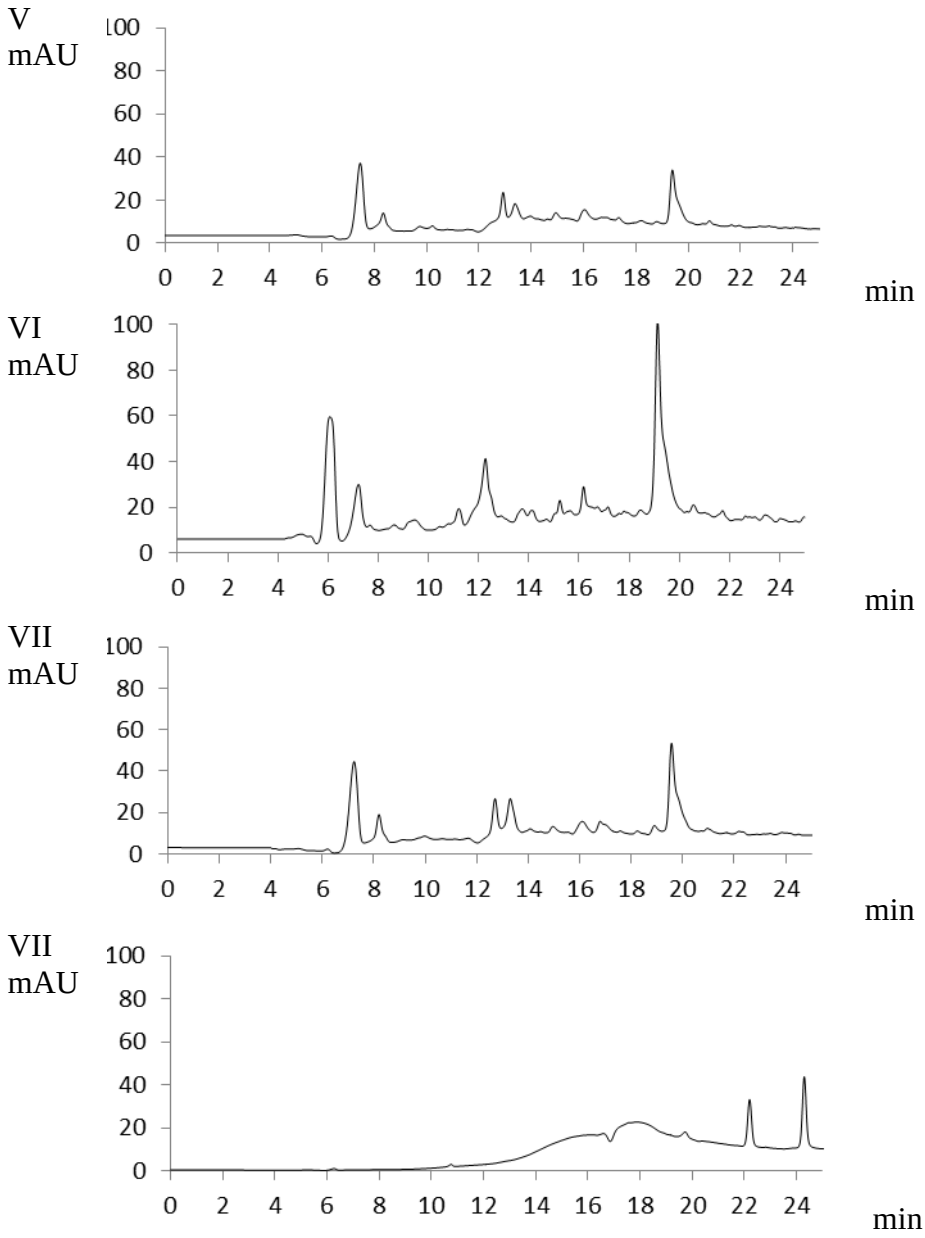


Fig. 4.2 (Continued): V; C-05, VI; C-06, VII; C-07, VIII; C-12

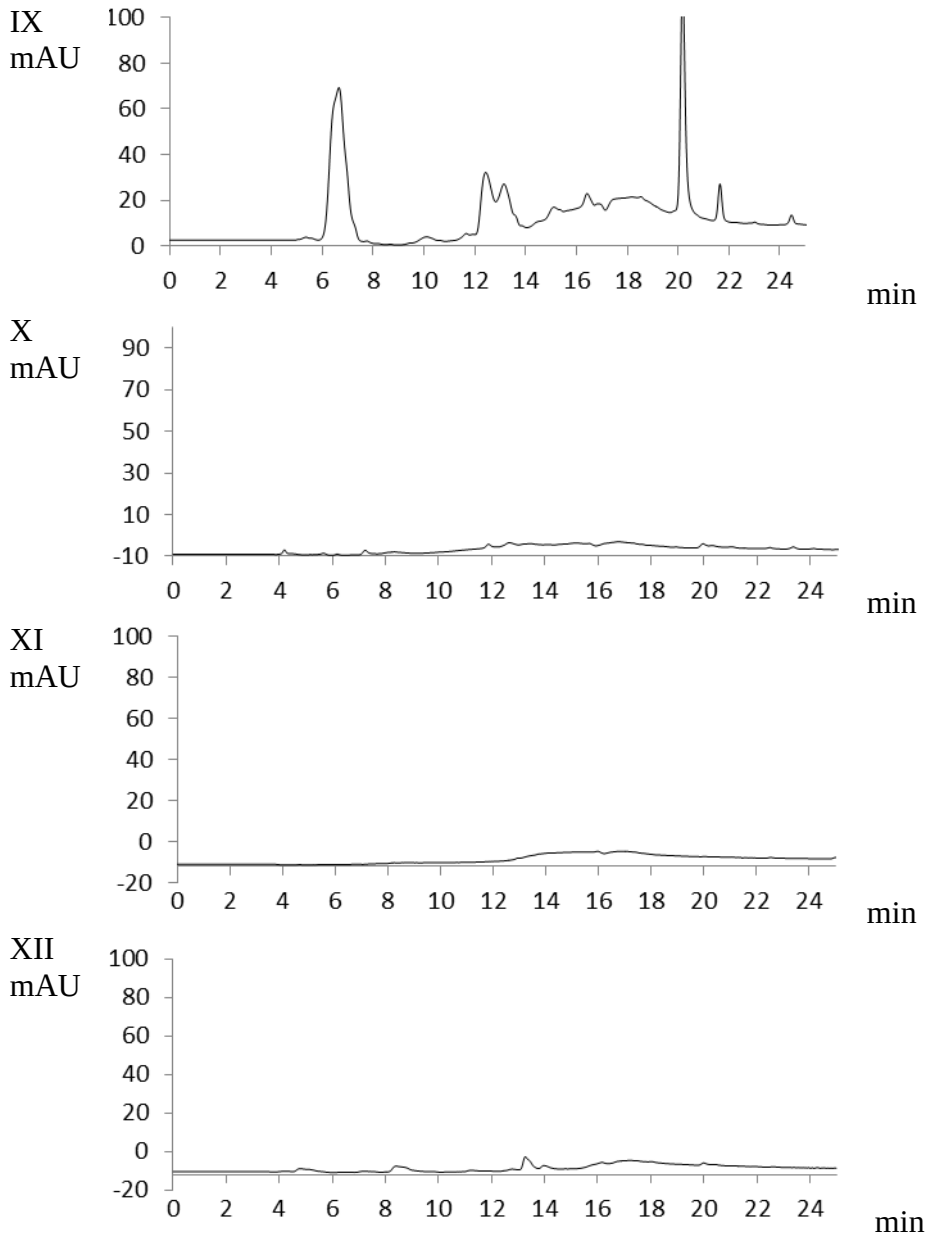


Fig. 4.2 (Continued): IX; C-13, X; C-14, XII; C-15, XII; C-17

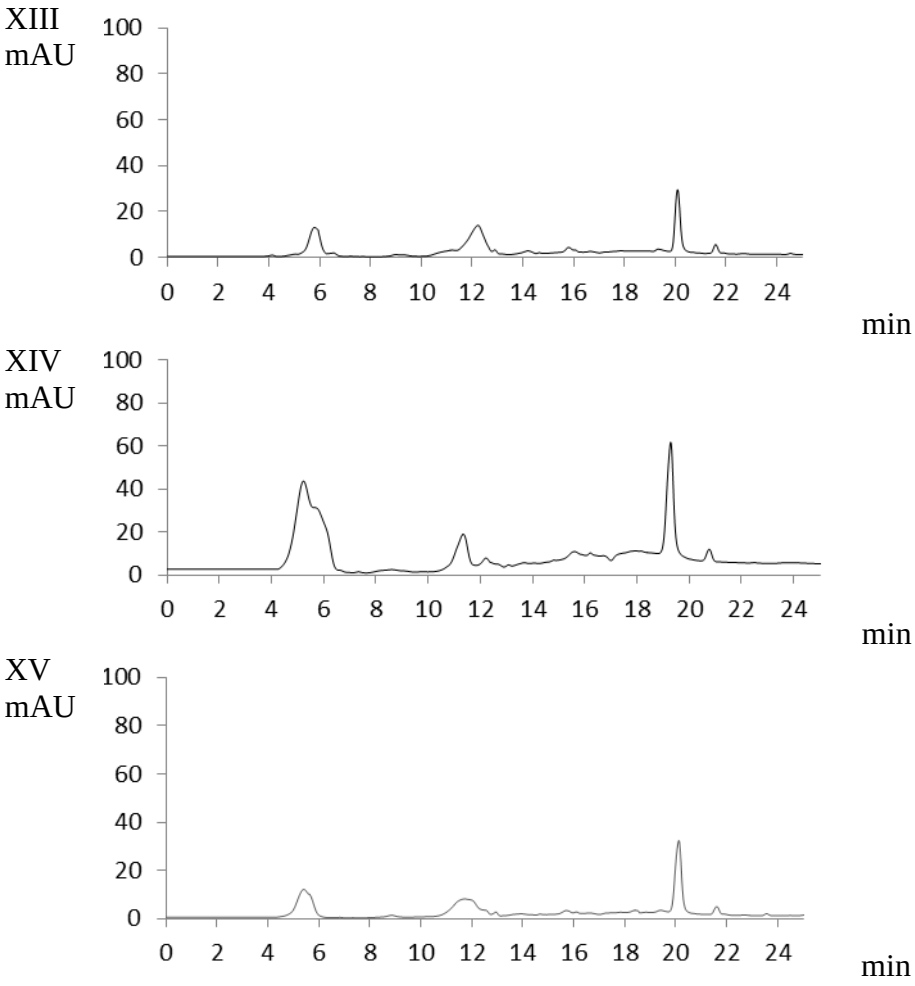


Fig. 4.2 (Continued): XIII; C-18, XIV; C-19, XV; C-20

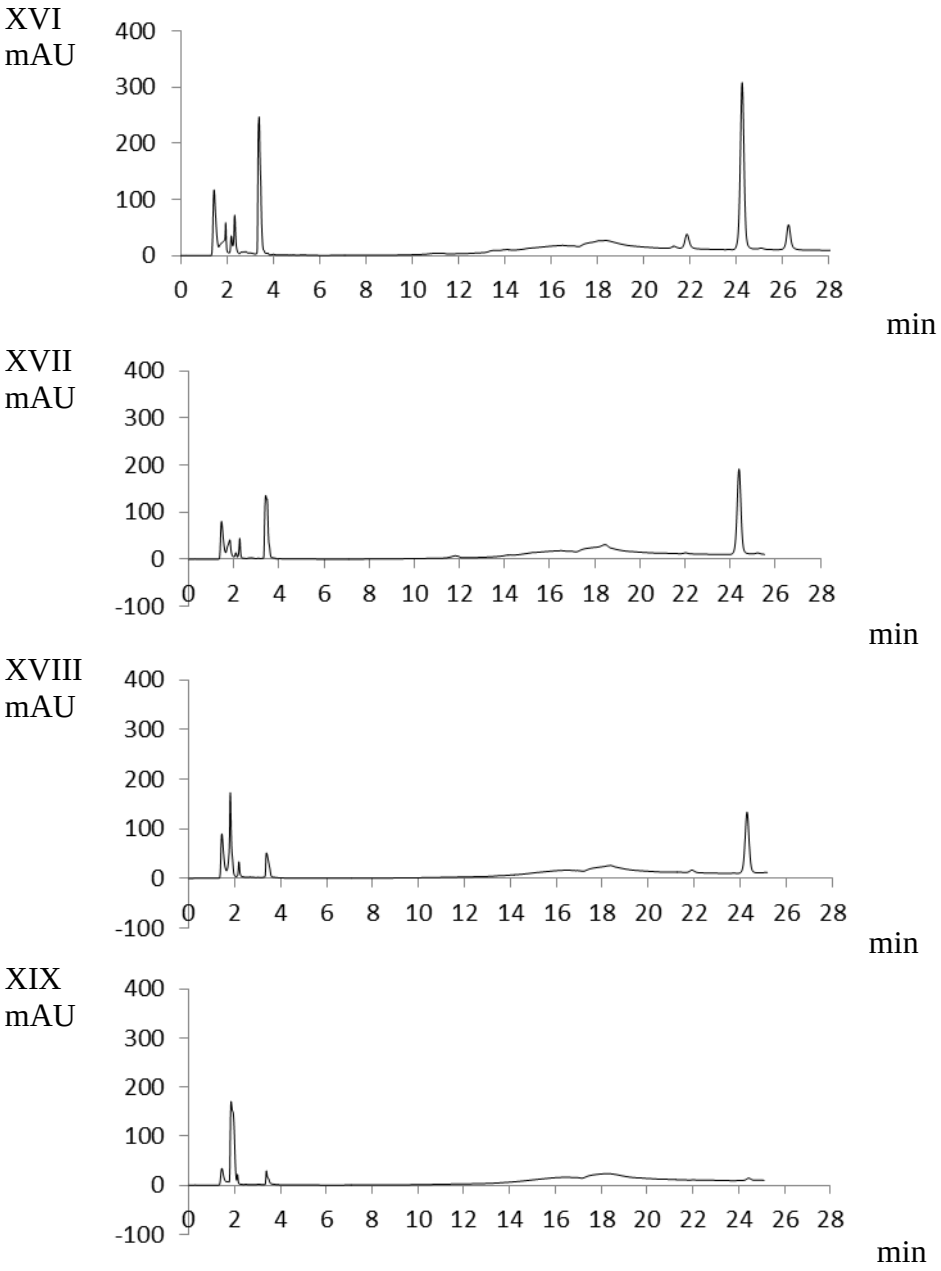


Fig. 4.2 (Continued): XVI; C-21, XVII; C-22, XVIII; C-23, XIX; C-24

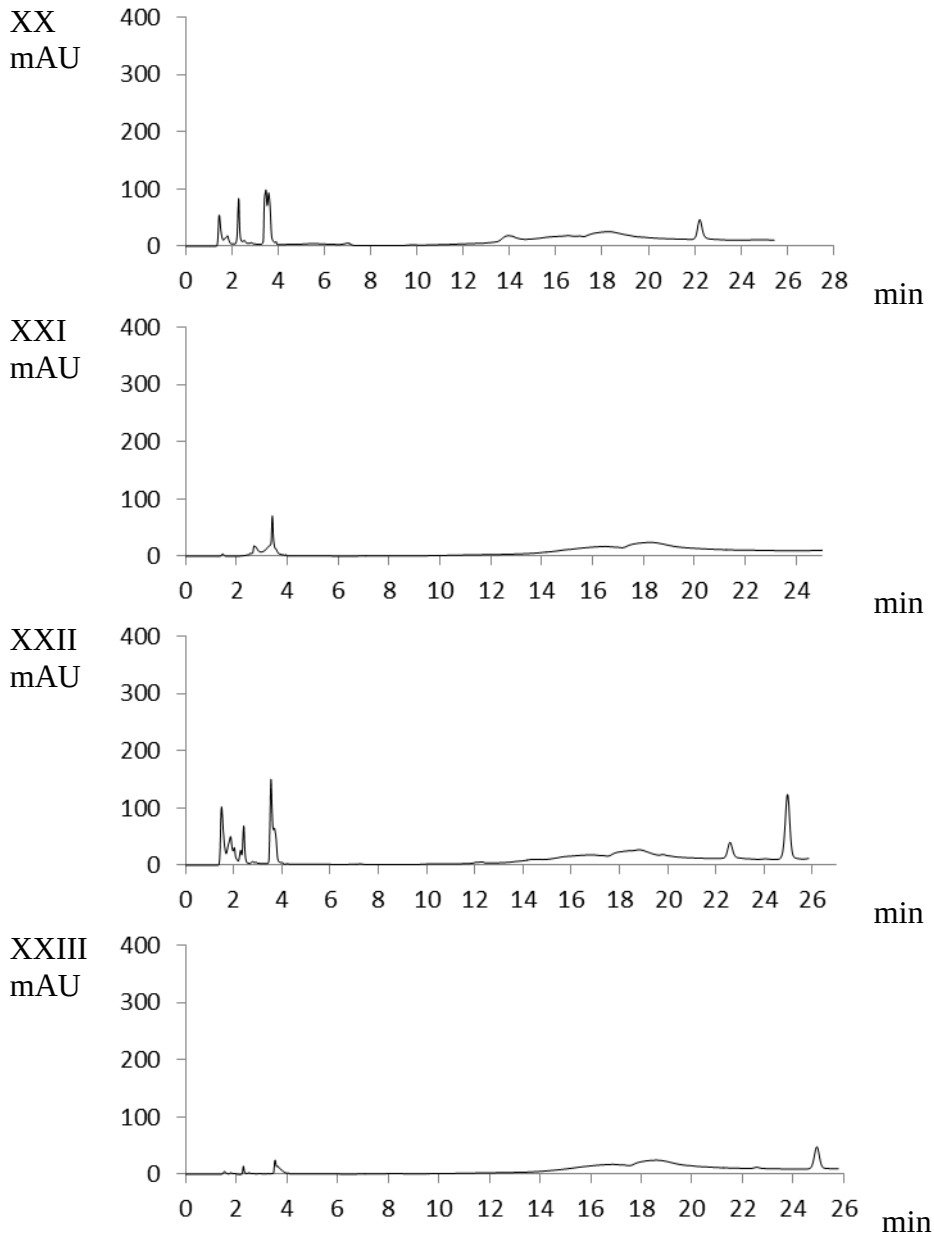


Fig. 4.2 (Continued): XX; C-25, XXI; C-26, XXII; C-27, XXIII; C-28

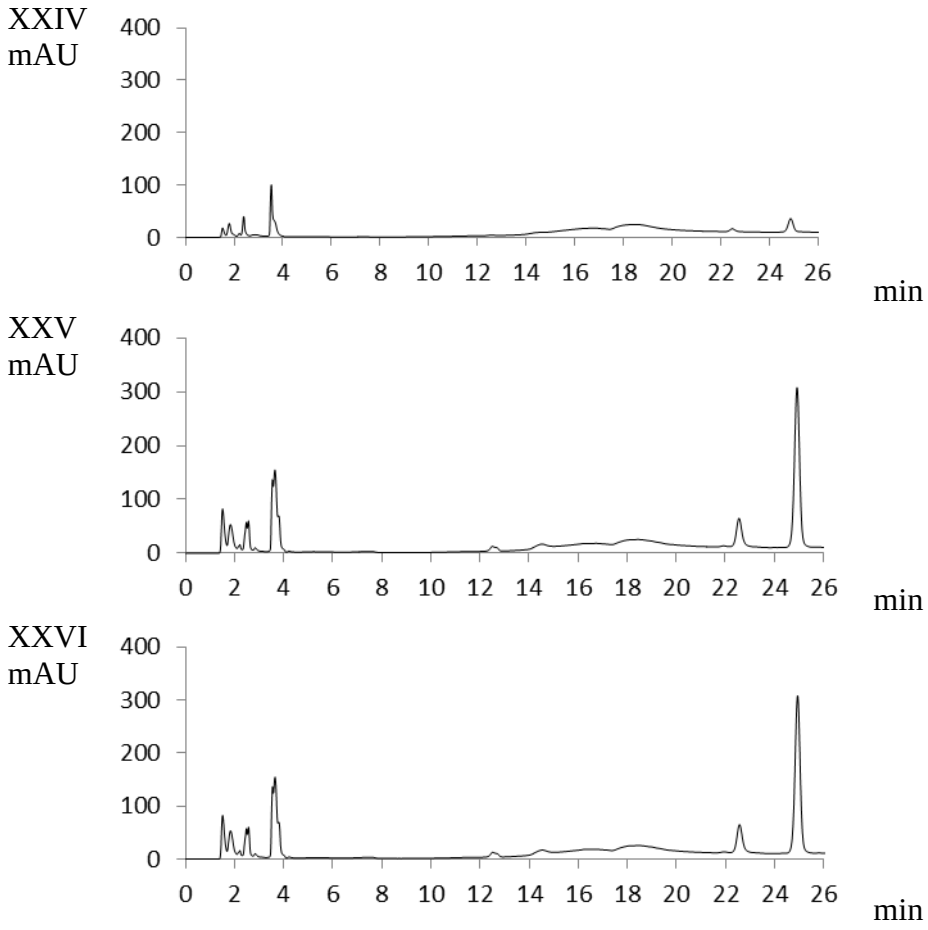


Fig. 4.2 (Continued): XXIV; C-29, XXV; C-30, XXVI; C-31

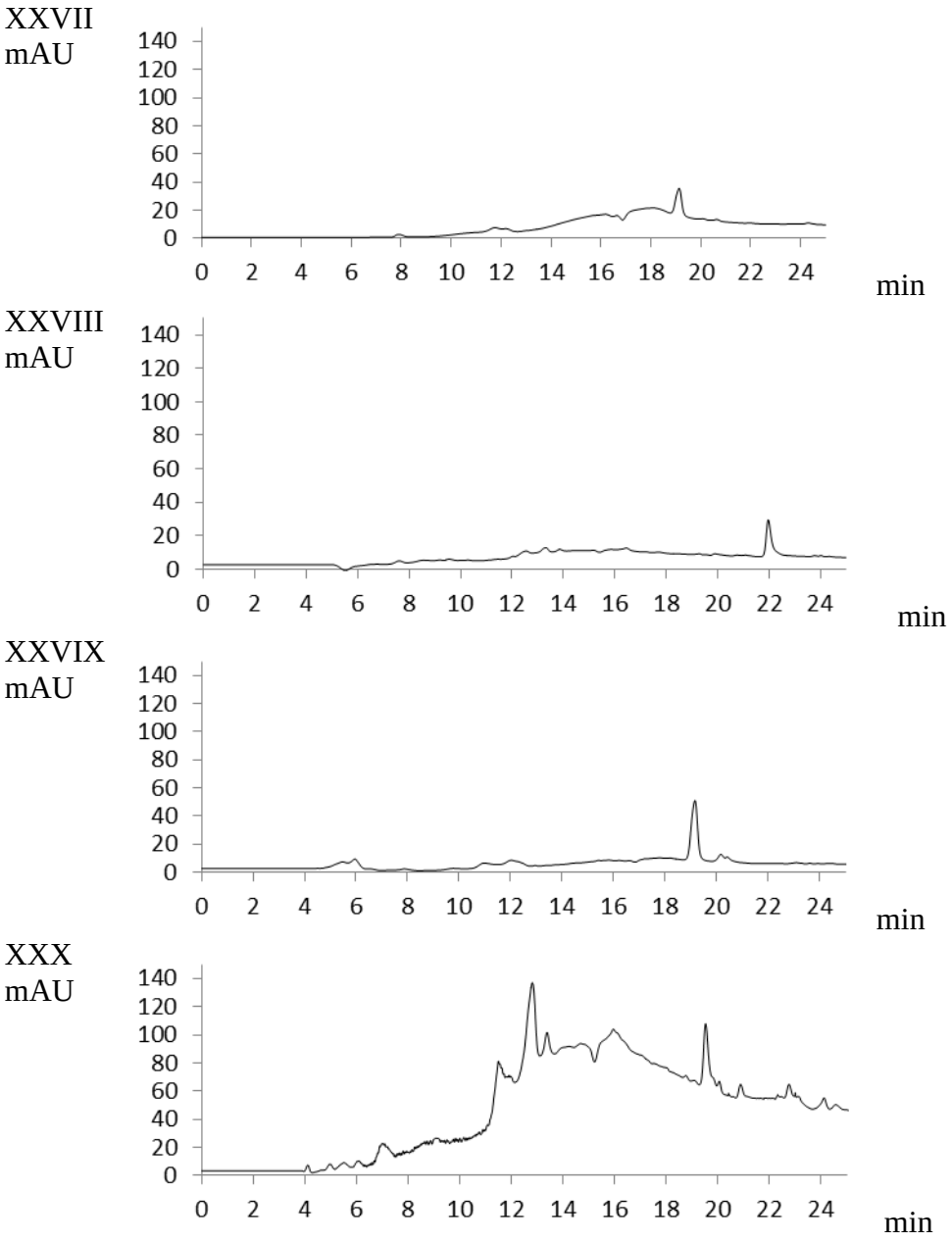


Fig. 4.3 HPLC profiles of natural *Cordyceps*: XXVII; N-01, XXVIII; N-02, XXIX; N-03, XXX; N-04

4.3 Quantitative determination of nucleoside in *Cordyceps* samples.

4.3.1 Analysis of nucleoside in cultured *Cordyceps*.

Contents of five analytes are summarized in Table 4.5

Table 4.5 The contents (mg g^{-1}) of five investigated compounds in samples.

Samples	cont. (mg g^{-1}) Uridine	Inosine	Guanosine	Adenosine	Cordycepin
C-01	28.59±1.38	nd	8.05±0.119	14.11±0.02	nd
C-02	23.26±0.24	nd	6.91±0.06	12.56±0.01	nd
C-03	35.12±1.38	nd	17.12±0.24	25.24±0.07	nd
C-04	7.54±0.22	nd	0.43±0.10	2.85±0.03	nd
C-05	18.17±0.12	nd	6.43±0.13	6.35±0.02	nd
C-06	13.46±0.15	2.92±0.06	20.93±0.03	31.03±0.15	nd
C-07	15.42±0.12	nd	11.34±0.04	16.25±0.47	nd
C-08	nd	nd	nd	nd	nd
C-09	nd	nd	nd	nd	nd
C-10	nd	nd	nd	nd	nd
C-11	nd	nd	nd	nd	nd
C-12	1.02 ±1.10	1.83±0.28	nd	20.59±0.03	22.72±0.05
C-13	57.16±0.01	11.08±0.03	nd	26.94±0.22	5.35±0.05
C-14	0.81±0.02	1.58±0.01	nd	0.76±0.03	nd
C-15	nd	nd	nd	nd	nd
C-16	nd	1.08±0.06	nd	nd	nd
C-17	nd	nd	nd	1.05±0.02	nd
C-18	8.64±0.12	nd	17.96±1.24	39.42±0.30	1.26±0.01
C-19	72.46±0.39	33.24±1.12	7.35±1.32	28.15±0.02	1.81±0.01
C-20	5.48±0.62	nd	nd	39.59±0.04	0.70±0.03
C-21	nd	nd	nd	362.47±0.90	349.04±0.34
C-22	nd	nd	nd	nd	188.76±0.20
C-23	nd	nd	nd	1.70±0.10	109.95±0.30

nd = not detected

Table 4.5 The contents (mg g-1) of five investigated compounds in samples.

Samples	cont. (mg g^{-1})				
	Uridine	Inosine	Guanosine	Adenosine	Cordycepin
C-24	nd	nd	nd	nd	5.21±0.16
C-25	nd	nd	nd	132.73±0.63	nd
C-26	nd	nd	nd	nd	nd
C-27	nd	nd	nd	76.82±1.04	167.03±0.63
C-28	nd	nd	nd	18.53±0.41	39.71±0.22
C-29	nd	nd	nd	9.85±0.43	31.33±0.24
C-30	11.82±0.92	48.94±0.25	nd	434.86±0.91	411.65±0.37
C-31	nd	nd	nd	34.93±0.47	176.56±0.11

nd = not detected

The samples were divided into two parts: the culture and the natural. The samples from the culture were partitioned into laboratory (C-01 to C-20) and commercial samples (C-21 to C-31). Table 4.5 shown that cordycepin was not found in *Cordyceps* species (C-01 to C-12). While, *Ophiocordyceps* species (C-13 to C-20) were found, which is similar to another previous reported (Yu *et al*., 2006; Fan *et al.*, 2006; Yang *et al.*, 2007). The contents of cordycepin were in the range 0.70-22.7 mg g^{-1}. Adenosine and uridine were found both of *Cordyceps* species and *Ophiocordyceps* species in the range of 0.76 – 39.59 mg g^{-1} and 0.81 – 72.46 mg g^{-1}, respectively.

The commercial available products of *Cordyceps* from the culture were divided into *Cordyceps militaris* (C-21, C-22, C-23, C-27, C-30 and C-31) and *Cordyceps sinensis* (C-24, C-25, C-26, C-28 and C-29). The contents of adenosine and cordycepin in samples were higher than other investigated compound. In commercial available products of *Cordyceps militaris*, the contents of adenosine and cordycepin were higher than those in samples from *Cordyceps sinensis*, which is similar to another previous report (Yu *et al*., 2006; Fan *et al.*, 2006; Yang *et al.*, 2007). In the commercial available products of *Cordyceps* the contents of analytes were 1.70-434.86 mg g^{-1} (adenosine) and 5.21-411.65 mg g^{-1} (cordycepin), only C-30 were 11.82 mg g^{-1} (uridine) and

48.94 mg g^{-1} (inosine). It means that the contents of adenosine and cordycepin were important for commercial product.

4.3.2 Analysis of nucleoside in natural *Cordyceps*

Natural *Cordyceps* samples were extracted by soaked the samples with methanol. The concentration of five investigated compounds in natural *Cordyceps* samples as shown in Table 4.6. Cordycepin were found only N-02 in natural *Cordyceps* while uridine, inosine, guanosine and adenosine were found.

Table 4.6 The concentration of five investigated compounds in natural *Cordyceps* samples

Samples	cont. (mg g^{-1})				
	Uridine	Inosine	Guanosine	Adenosine	Cordycepin
N-01	4.06±0.91	nd	4.88±0.93	9.92±0.59	nd
N-02	5.10±0.48	16.6±2.19	28.03±4.11	nd	11.6±0.68
N-03	5.69±0.93	10.20±0.45	9.31±0.56	27.01±0.69	nd
N-04	7.25±0.78	15.64±0.63	17.86±0.70	17.82±0.41	nd

nd = not detected

CHAPTER 5

CONCLUSION

A simple sample preparation and conventional high performance liquid chromatography (HPLC) was developed for the determination of five nucleosides namely uridine, inosine, guanosine, adenosine and cordycepin in cultured, natural and commercially available products of *Cordyceps*. The extraction was performed by ultrasonication and soaked with methanol. The separation was performed on an ACE-5 C18-AR column (4.6 mm. x 250 mm., i.d. 5 μm.) and gradient elution of methanol and 5 mM aqueous triethylamine, and UV detection at 254 nm. The correlation coefficients of five analytes were good ($r^2 > 0.995$) within test ranges. The overall RSDs for intra-day and inter-day for five analytes were less than 3.57 and 4.57%, respectively.

The results shown that uridine, inosine, guanosine, adenosine and cordycepin were found in the cultured *Cordyceps*. In this study, the commercial available products of *Cordyceps* were found adenosine and cordycepin in samples were higher than other investigated compound. In commercial available products of *Cordyceps militaris*, the contents of adenosine and cordycepin higher than those in samples from *Cordyceps sinensis*. The proposed method might be useful for quality control and authentication of *Cordyceps* samples. Simultaneous determination of five analytes in *Cordyceps* samples were performed, which provides a sample and sensitive method with good linearity, precision and recovery. The method was applied for the analysis of nucleosides in cultured, natural and commercial by available products of *Cordyceps*.

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BIOGRAPHY

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Name	Khanittha Cordhanam
Date of brith	July 21, 1989
Place of birth	Sakonnakhon
Institution attended	
2011	Bachelor of Science degree (B.Sc.) in Chemistry, Mahasarakham University, Maha Sarakham, Thailand
2017	Master of Science degree (M.Sc.) in Chemistry, Mahasarakham University, Maha Sarakham, Thailand
Contact address	
	152 Moo 3, Tambon Yodchat, Ampher Wangyang, Nakornpanom Province, 48130
Resarch grants & awards	
	Human Resource Development in Science Project (Science Achievement Scholarship of Thailand; SAST)

Research output

Khanittha Cordhanam, Uthai Sakee and Watchara Kanchanarach (2016) HPLC Analysis of nucleosides and nucleobasses from the commercial by available products of *Cordyceps*. In: Sombat Kotchasit, Akachai Sang-in and Theathanick Siriwoharn (eds.) *The 41 st National and International Graduate Research Conference (NIGRC41, 2016),* December 8-9, 2016. Valaya Alongkorn Rajabhat University under Royal Patronage, *Thailand*, 1.