Purification of Coenzyme Q_{10} from *Artemia* by Chromatography and Preservation

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Thesis Title Purification of Coenzyme Q_{10} from *Artemia* by Chromatography and Preservation

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ชื่อวิทยานิพนธ์ : การแยกโคเอนไซม์คิวเทนจากอาร์ทีเมียโดยเทคนิคโครมาโทกราฟีและการรักษาสภาพ

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บทคัดย่อ

ได้ทำการพัฒนาวิธีการสกัดสารโคเอนไซม์คิวเทนจากอาร์ทีเมียหรือไรน้ำเค็มโดยการทำให้เซลล์แตกด้วยกรดอินทรีย์ร่วมกับการสกัดด้วยตัวทำละลาย แล้ววิเคราะห์ปริมาณโคเอนไซม์คิวเทนด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงที่มีไดโอดอาเรย์เป็นตัวตรวจวัด โคเอนไซม์คิวเทนจะถูกแยกด้วยคอลัมน์รีเวอร์สเฟสชนิดคาร์บอน 18 และชะด้วยตัวทำละลายผสมระหว่างเอทานอลและเมทานอล อัตราส่วน 95:5 โดยปริมาตร ทำการตรวจวัดการดูดกลืนแสงที่ความยาวคลื่น 275 นาโนเมตร

วิธีการที่เหมาะสมในการสกัดโคเอนไซม์คิวเทนจากอาร์ทีเมียเตรียมได้โดยบ่มตัวอย่าง 1 กรัม ในกรดแอซีติกร้อยละ 75 โดยน้ำหนักปริมาตร 5 มิลลิลิตร ที่อุณหภูมิห้องเป็นเวลา 24 ชั่วโมง หลังจากนั้นสกัดด้วยเฮกเซนปริมาตร 5 มิลลิลิตร ร่วมกับการเติมเอทานอลปริมาตร 5 มิลลิลิตร ทำการสกัดทั้งหมด 3 ครั้ง กราฟมาตรฐานของการวิเคราะห์โคเอนไซม์คิวเทนในช่วงความเข้มข้น 1.0 ถึง 50.0 ไมโครกรัมต่อมิลลิลิตร เป็นเส้นตรงโดยมีสมการแสดงความสัมพันธ์เชิงเส้นตรงเป็น $y = 20.224(\pm0.041)x - 0.551(\pm1.017)$ และมีค่าสัมประสิทธิ์ความเป็นเส้นตรงมากกว่า 0.999 ขีดการตรวจวัดต่ำสุดและปริมาณต่ำสุดที่ตรวจวัดได้เท่ากับ 0.3 และ 1.1 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ค่าการได้กลับคืนเฉลี่ยของการสกัดโคเอนไซม์คิวเทนจากอาร์ทีเมียอยู่ในช่วงร้อยละ 94 ถึง 100 และมีค่าสัมประสิทธิ์การแปรปรวนต่ำกว่าร้อยละ 10 ปริมาณโคเอนไซม์คิวเทนในอาร์ทีเมียที่ได้จากวิธีการสกัดที่เหมาะสมมีค่าอยู่ในช่วง 177 ถึง 303 ไมโครกรัมต่อกรัมน้ำหนักแห้งของอาร์ทีเมีย

จากการสกัดโคเอนไซม์คิวเทนจากอาร์ทีเมีย พบว่า สารสกัดหยาบมีความบริสุทธิ์ต่ำ ซึ่งไม่เหมาะในการนำไปใช้ในอุตสาหกรรม ดังนั้นจึงได้พัฒนาวิธีการทำให้โคเอนไซม์คิวเทนในสารสกัดหยาบบริสุทธิ์ด้วยเทคนิคคอลัมน์โครมาโทรกราฟีโดยอาศัยซิลิการเจลเป็นตัวดูดซับ โดยชะโคเอนไซม์คิวเทนด้วยตัวทำละลายผสมของเฮกเซนและอะซีโตนในอัตราส่วน 95:5 โดยปริมาตร หลังจากนั้นตกผลึกด้วยเฮกเซนและเมทานอลและตกผลึกซ้ำอีกครั้งด้วยเอทานอลที่อุณหภูมิ 4 องศาเซลเซียส โครงสร้างของผลึกโคเอนไซม์คิวเทนที่ได้ยืนยันด้วยเทคนิคโปรตอนนิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโทรสโคปี และแมสสเปกโทรเมทรี ประสิทธิภาพการแยกพบว่า ได้โคเอนไซม์คิวเทนที่มีความบริสุทธิ์เพิ่มขึ้นจากร้อยละ 0.07 เป็นร้อยละ 65 และไม่พบสารอื่น ๆ รบกวนเมื่อวิเคราะห์ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง

การรักษาสภาพของโคเอนไซม์คิวเทนจากแสง ทำได้โดยการทำให้โคเอนไซม์คิวเทนเกิดเป็นสารเชิงซ้อนกับสารเบต้าไซโคลเดกตรินซ์ และทำการทดสอบความคงตัวของสารเชิงซ้อนต่อแสงด้วยแสงยูวีเอ เป็นเวลา 8 ชั่วโมง พบว่า โคเอนไซม์คิวเทนที่ผสมกับเบต้าไซโคลเดกตรินซ์มีความเสถียรต่อแสงถึง 3 เท่าเมื่อเปรียบเทียบกับโคเอนไซม์คิวเทนอย่างเดียว

จากการศึกษาโดยรวม พบว่า วิธีการสกัดที่พัฒนาขึ้นพร้อมกับวิธีการทำให้สารบริสุทธิ์และการรักษาสภาพเป็นวิธีที่ง่าย ใช้สารเคมีที่ปลอดภัย ลดการใช้พลังงาน ให้วิธีการสกัดที่มีประสิทธิภาพ และสามารถนำไปประยุกต์ใช้ในระดับอุตสาหกรรมได้

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Abstract

The developed method for extraction of coenzyme Q_{10} from *Artemia* or brine shrimp was carried by using an organic acid for cell lysis coupled with solvent extraction. The amount of CoQ_{10} was analyzed by high-performance liquid chromatography with diode array detection. The separation of CoQ_{10} was performed on reversed phase C18 column with a mixture of ethanol and methanol (95:5 v/v) as the mobile phase. The detection was set at 275 nm. The optimal conditions for extraction of CoQ_{10} was obtained by the incubation of one gram of sample with 5.00 mL of 75% (w/w) acetic acid at room temperature for 24 hours, followed by the extraction three times with 5.00 mL of hexane and 5.00 mL of ethanol. The calibration curve of CoQ_{10} in the range of 1.0 to 50.0 μg mL^{-1} was linear with a linear equation of $y = 20.224(\pm0.041)x - 0.551(\pm1.017)$ and the correlation coefficient (r) higher than 0.999. The method has no matrix effect. The limit of detection and quantification were achieved at 0.3 and 1.1 μg mL^{-1}, respectively. The mean recovery of CoQ_{10} extraction from *Artemia* was found in the range of 94 to 100% with the coefficient of variation below 10%. Under these optimal conditions, the highest CoQ_{10} yield of 177-303 μg g^{-1} dry weight of *Artemia* was obtained.

After the extraction of CoQ_{10} from *Artemia*, the crude extract contained the low purity of CoQ_{10} which would not be appropriate for industrial use. Thus, the purification of CoQ_{10} in the crude *Artemia* extract was investigated by silica gel column chromatography with the elution of the mixture of acetone and hexane (5:95, v/v). Subsequently, the crystallization of CoQ_{10} was performed using hexane and methanol with the re-crystallization of CoQ_{10} attained by ethanol at 4°C. The structure of CoQ_{10} crystal was elucidated by proton nuclear magnetic resonance spectroscopy and mass spectrometry. The purification yielded 0.4 mg of CoQ_{10} with

the increase in the purity from 0.07 to 65% in purified CoQ_{10} and the HPLC purity of 100%.

The preservation of CoQ_{10} from light was investigated by inclusion complex of CoQ_{10} with *β*-cyclodextrin. The photostability test of inclusion complex and free CoQ_{10} was evaluated by under UVA light for 8 hours. It was found that CoQ_{10} prepared using *β*-Cyclodextrin had three times stability under UVA light when compared to free CoQ_{10}.

Overall, the developed extraction method, purification and preservation were found to be simple, low toxic solvent use, low energy consuming and efficient for preparation of CoQ_{10} from *Artemia*. This developed process could be used as a practical method for industrial application.

Contents

Contents (continued)

Contents (continued)

Contents (continued)

Contents (continued)

List of Tables

List of Figures

List of Figures (continued)

List of Abbreviations

ADI	Acceptable daily intake
AOAC	Association of Official Analytical Chemists
ASE	Accelerated solvent extraction
ATP	Adenosine 5′ triphosphate
CDs	Cyclodextrins
CoQ_{10}	Coenzyme Q_{10}
$CoQ_{10}H_2$	Ubiquinol-10
EP	European Pharmacopoeia
ETC	Electron transport chain
HPLC-DAD	High-performance liquid chromatography with diode array detection
HSCCC	High-speed counter-current chromatography
NADH	Nicotinamide adenine dinucleotide
NOAEL	no-observed-adverse-effect level

CHAPTER 1
INTRODUCTION

1.1 Background and Rationale

Coenzyme Q_{10} or the other names, CoQ_{10} or ubiquinone-10 is a vitamin-like substance. CoQ_{10} involves with the production of energy (ATP) in mitochondrial electron transport chain and promotes antioxidant properties (Lenaz *et al*., 2007 and Bentinger *et al*., 2007). CoQ_{10} is also used for the treatment of various diseases including cardiac disease, hypertension, Meniere-like syndrome and Parkinson's disease (Shults, 2005 and Kumar *et al*., 2009) and the prevention for aging (Zhou, 2004 and Sohal *et al*., 2007). It is synthesized naturally in human body, but its rate of production decreases with age. Therefore, humans can gain CoQ_{10} from food or nutraceutical dietary supplement (Rao *et al*., 2008) with the acceptable daily intake (ADI) of 12 mg kg^{-1} day^{-1} (Hidaka *et al*., 2008). CoQ_{10} was found in microorganisms such as photosynthetic bacteria *Rhodospirillum rubrum* ATCC 25852 at 9,210 µg g^{-1} dry cell weight, *Agrobacterium tumefaciens* KCCM 10413 at 8,540 µg g^{-1} dry cell weight, *Sphingomonas* sp. ZUTEO3 at 32,500 µg g^{-1} dry cell weight and also found in pelagic fish in the range from 105 to 148 µg g^{-1} fresh tissue, bee pollen at 192.8 µg g^{-1}, litchi pericarp at 135.3 µg g^{-1} dry pericarp and tobacco leaves in the range from 9.9 to 15.1 µg g^{-1} (Zu *et al*., 2006; Ha *et al*., 2007; Souchet and Laplante, 2007; Zhong *et al*., 2009; Tian *et al*., 2010a; Rao *et al*., 2011 and Xue *et al*., 2012).

CoQ_{10} was found to be the highest in bacteria. However, the separation and extraction method of bacteria cell are more complicated than *Artemia*. *Artemia* is served as a carrier for components such as essential nutrients, pigments, prophylactics and therapeutics by bioencapsulating and introducing to the consumer organisms (Persoone and Wells, 1987). *Artemia* is used for the fish nursery and its food is bacteria. Since CoQ_{10} has been reported in some types of bacteria, it is probably found in *Artemia*.

Artemia or brine shrimp is the invertebrate animal identified as Crustacea class like shrimp, crayfish and crab, but its body is soft and it has no shell.

Artemia has the short life cycle and its food is microorganisms such as bacteria and yeasts. *Artemia* is commonly used for food of larval organisms due to high nutrients related to its diets. The nutritional values of adults of *Artemia* such as protein, lipid and carbohydrate were found at 56.4, 11.8 and 12.1%, respectively (Léger *et al*., 1987). Moreover, the encysted embryos of *Artemia salina* contained P^1, P^4-diguanosine 5´-tetraphosphate *asymmetrical*-pyrophosphohydrolase (*asym*-di-GDPase) which gives high energy intermediate source in biochemical process (Warner, 1964).

Extraction of CoQ_{10} from *Artemia* has been rarely reported. Many analytical methods for extraction of CoQ_{10} from different samples were reported including the saponification process of *Sphingomonas* sp. ZUTE03 before solvent extraction, ultrasonic extraction of tobacco leaves or litchi pericarp before solvent extraction, the accelerated solvent extraction (ASE) of bee pollen with ethanol and the lysing cell step by the acid-heat treatment before solvent extraction (Zu *et al*., 2006; Zhong *et al*., 2009; Tian *et al*., 2010b; Rao *et al*., 2011 and Xue *et al*., 2012). The partial CoQ_{10} could be damaged by an alkali condition used in the saponification process (Souchet and Laplante, 2007). Moreover, the ASE and ultrasonication method are not suitable for the large scale preparation for industrial scale. Therefore, the cell lysis before solvent extraction has been developed for extraction of CoQ_{10} from *Artemia*.

Generally, the natural crude extract contains complex matrices, thus, the purification step before manufacturing is necessary. High-speed counter-current chromatography (HSCCC) and adsorption chromatography were reported for the purification method of CoQ_{10} (Hagerman *et al*., 2001; Cao *et al*., 2006 and Rodríguez-Acuna *et al*., 2008). However, HSCCC is the complicated instrument. The adsorption chromatography with silica gel as an adsorbent has been widely used for the purification of CoQ_{10} since silica gel has many advantages such as simple operation and ability to separate chemicals with similar character and structure (Zhang *et al*., 2012). CoQ_{10} is degraded easily because it is sensitive to light (EP 4, 2002 and Yang and Song, 2006). Therefore, it is necessary to preserve CoQ_{10} before manufacturing or during shipping and storage. β-Cyclodextrin is able to form

inclusion complexes with several compounds. Therefore, the encapsulation of material with β-cyclodextrin was recommended in food and pharmaceutical industry for stabilization of flavours, the improvement of the physical and chemical properties of drugs, or the enhancement of the bioavailability of poorly soluble drugs (Del Valle, 2004 and Astray *et al*., 2009).

Bacteria *Agrobacterium tumefaciens* and photosynthetic bacteria *Rhodospirillum rubrum* were found to contain high amount of CoQ_{10} at 8,540 µg g^{-1} dry cell weight and 9,210 µg g^{-1} dry cell weight, respectively (Ha *et al*., 2007; Zhong *et al*., 2009 and Tian *et al*., 2010a). However, the process including centrifugation of 10,000 rpm at 4°C and freeze-drying increased the cost of a harvest of bacteria (Tian *et al*., 2010b). Up to date, the source of CoQ_{10} as *Artemia* which can be cultivated within 2 weeks and easier to be harvested than bacteria is never reported, and no suitable preparation method for CoQ_{10} from *Artemia* has been established.

Therefore, the objective of this research was to develop method of the extraction of CoQ_{10} from *Artemia*. The extraction efficiency was investigated by means of high-performance liquid chromatography with diode array detection (HPLC-DAD). Furthermore, the purification of CoQ_{10} with column chromatography using silica gel as an adsorbent was studied due to the low concentration of CoQ_{10} in crude extract from *Artemia* and the study of preservation of CoQ_{10} to improve photostability with inclusion complex of CoQ_{10} with β-cyclodextrin was performed.

1.2 *Artemia*

Artemia or brine shrimp is the invertebrate animal identified as Crustacea class like shrimp, crayfish and crab, but its body is soft and it has no shell. *Artemia* can be cultured at salinities of 5 up to 150% salt concentration (Persoone and Wells, 1987). The taxonomy of *Artemia* was described as below (Anand *et al*., 1993):

Phylum	Arthropoda
Class	Crustacea
Order	Anostraca
Family	Artemiidae
Genus	Artemia Leach

The morphology of *Artemia* is shown in Figure 1.1. An *Artemia* adult reproduced from nauplii larva is usually about 7.0-15.0 mm and the life cycle of *Artemia* (Figure 1.2) is 7-15 days. *Artemia* is commonly used for food of larval organisms because it contains high nutrients related to its diets. *Artemia* is served as food for cultured marine and freshwater organisms with appropriate size of *Artemia* from nauplii to adults. The possibility of using *Artemia* is also served as a carrier for components such as essential nutrients, pigments, prophylactics and therapeutics by bioencapsulating and introducing to the consumer organism (Persoone and Wells, 1987). Moreover, *Artemia* was used for the aquatic toxicology including the investigation of sources of toxicity in chemical mixtures and environmental samples (Persoone and Wells, 1987). The nutrition of *Artemia*, *i.e.*, protein, lipid and carbohydrate can be shown in Table 1.1 (Léger *et al*., 1987). Moreover, the encysted embryos of *Artemia salina* contained P^1, P^4-diguanosine 5´-tetraphosphate *asymmetrical*-pyrophosphohydrolase (*asym*-di-GDPase) which is a compound giving a high energy intermediate source in biochemical process (Warner, 1964).

The foods of *Artemia* are microorganisms such as diatom, green algae, photosynthetic bacteria, blue-green algae, yeast, plants and animals (Anan *et al*, 1993).

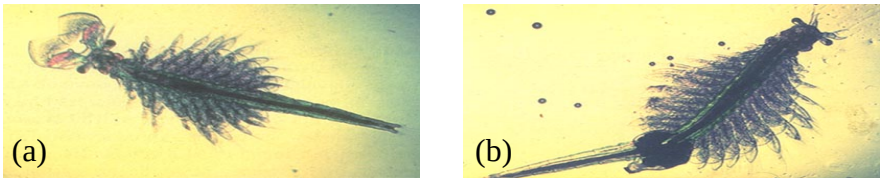


Figure 1.1 The appearance of (a) adult male *Artemia* and (b) adult female *Artemia* (http://www.fao.org/DOCREP/003/W3732E/w3732e0t.jpg, accessed May 19, 2012)

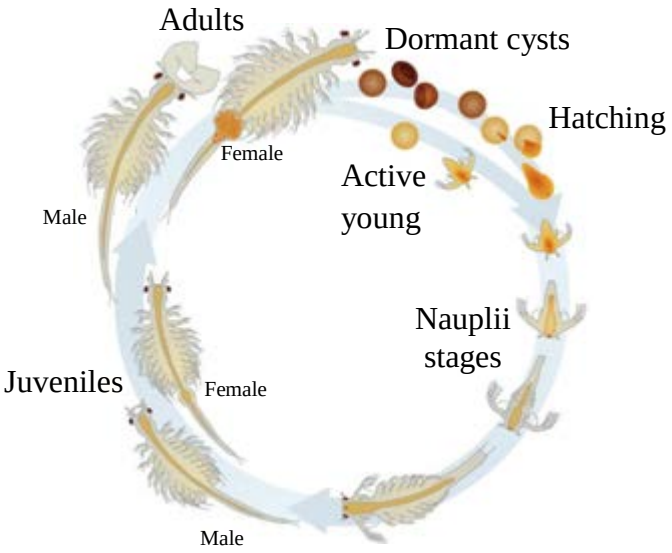


Figure 1.2 The life cycle of *Artemia* (http://www.bridgerlandaudubon.org/wildaboututah/110106brineshrimp.html, accessed September 23, 2012)

Table 1.1 Average proximate composite (% ± SD) of *Artemia* nauplii and adults

	Nauplii	**Adults**
Protein	52.2±8.8	56.4±5.6
Lipid	18.9±4.5	11.8±5.0
Carbohydrate	14.8±4.8	12.1±4.4
Ash	9.7±4.6	17.4±6.3

1.3 Coenzyme Q_{10}

1.3.1 Physical and chemical properties of CoQ_{10}

Coenzyme Q_{10} is a yellow-orange colored crystalline powder. Its chemical name is a 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone and it is also called CoQ_{10}, ubidecarenone or ubiquinone-10. Molecular formula of CoQ_{10} is $C_{59}H_{90}O_4$ (molecular weight 863.34 g mol^{-1}). Its structure composes of a benzoquinone ring and 10 isoprenoid units in the side chain (Figure 1.3a). It gradually decomposes and darkens when exposed to the light. The melting point of CoQ_{10} is 47.5-48.5°C (Ikeda and Kagei, 1979 and EP 4, 2002) and CoQ_{10} is readily soluble in

organic solvents and lipids and practically insoluble in water (Nepal *et al*., 2010). The solubility of CoQ_{10} in organic solvents depends on the polarity of solvents and nature of CoQ_{10} with a summary in Table 1.2 (Ondarroa *et al*., 1986). CoQ_{10} is very soluble in benzene and chloroform but these solvents are high toxic. Therefore, hexane and acetone were chosen for the elution of CoQ_{10} in column chromatography.

The redox states of CoQ_{10} are fully oxidized (ubiquinone-10), semiquinone intermediate (ubisemiquinone) and fully reduced (ubiquinol-10) (Figure 1.3). Ubiquinone-10 is the key for the important functions of CoQ_{10} in the body, as essential electron carriers in the mitochondrial respiratory chain and as antioxidant properties (Lenaz *et al*., 2007). Ubiquinone-10 can be oxidized to ubiquinol-10 by using ferric chloride while sodium dithionite ($Na_2S_2O_4$) or sodium borohydride ($NaBH_4$) as reducing agents can change ubiquinol-10 to ubiquinone-10 (Menke *et al*., 2000; Ruiz-Jiménez *et al*., 2007 and Shimada *et al*., 2007).

Table 1.2 Solubility limits of ubiquinone-10 and ubiquinol-10 in some solvents

Solvent	Polarity	Solubility at 20°C (mmol dm^{-3})	
		Ubiquinone-10	Ubiquinol-10
n-Hexane	0.1	105	105
Benzene	2.7	>270	240
Diethyl ether	2.8	230	-
Chloroform	4.1	>390	320
Ethanol	4.3	5.8	20
Acetone	5.1	40	100
Water	10.2	Insoluble	Insoluble

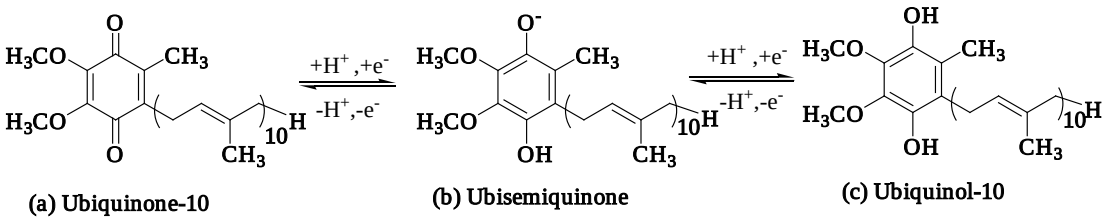


Figure 1.3 The redox states of CoQ_{10}.

1.3.2 Roles of CoQ_{10}

CoQ_{10} involves with the production of energy (ATP) in mitochondrial electron transport chain. CoQ_{10} in mitochondria serves as the electron acceptor for NADH-ubiquinone reductase and succinate-ubiquinone reductase of the electron transport chain. In the electron transport chain, the CoQ_{10} as the oxidized form can change to the ubiquinol-10 ($CoQ_{10}H_2$) which shows antioxidant properties (Shults, 2005 and Lenaz *et al*., 2007). Ubiquinol-10 can protect against free radicals and oxidative damage caused by a chain reaction of oxidation of proteins and lipids (Bentinger *et al*., 2007). Moreover, ubiquinol-10 is used for the treatment of arteries disease (atherosclerosis). It can protect low-density lipoproteins (LDL) lipid peroxidation by scavenging lipid peroxyl radicals ($LOO^{\bullet}$) and the peroxidation initiating peroxyl radical ($ROO^{\bullet}$). The radical scavenging activity of ubiquinol-10 is shown in Equation 1.1 and 1.2 (Thomas and stocker, 2001).

$$CoQ_{10}H_2 + LOO^{\bullet} \rightarrow CoQ_{10}H^{\bullet} + LOOH \qquad (1.1)$$

$$CoQ_{10}H_2 + ROO^{\bullet} \rightarrow CoQ_{10}H^{\bullet} + ROOH \qquad (1.2)$$

CoQ_{10} is used as a nutritional supplement for the treatment a parkinson's disease, in which it is able to reduce damage of the nigrostriatal dopaminergic system (Shults, 2005). CoQ_{10} is also used for the therapeutic purposes such as cardiac disease, hypertension, Meniere-like syndrome (Kumar *et al*., 2009) and pediatric cardiomyopathy (Bhagavan and Chopra, 2005). The acceptable daily intake (ADI) of CoQ_{10} is 12 mg kg^{-1}which is calculated from the no-observed-adverse-effect level (NOAEL) data (Hidaka *et al*., 2008).

1.3.3 Sources of CoQ_{10}

CoQ or ubiquinone is a natural coenzyme formed from the conjugation of a benzoquinone ring with a variety of hydrophobic isoprenoid chains, depending on species. It is synthesized from the biosynthesis pathway by precursors including 4-hydroxybenzoate derived from shikimate pathway and polyisoprene diphosphate

produced by mevalonate pathway for eukaryotes and non-mevalonate pathway for prokaryotes or plant chloroplasts (Clarke *et al*., 2000 and Kawamukai, 2002). CoQ_{10} is a main ubiquinone species in humans (Cluis *et al*., 2007 and Barshop and Gangoiti, 2007). It distributes in living organisms and is found in the multiple-layer structure of the inner mitochondrial of the eukaryotes cells and outer membrane of microorganisms (Lenaz *et al*., 2007 and Tian *et al*., 2010b). Recently, CoQ_{10} has been used as medicines and food supplements (Sasaki *et al*., 2005). It was reported that the main sources of CoQ_{10} were microorganisms, plants and animals. CoQ_{10} was produced from microorganisms including *Agrobacterium tumefaciens* (Ha *et al*., 2007 and Tian *et al*., 2010b), *Sphingomonas* sp. ZUTEO3 (Zhong *et al*., 2009) and photosynthetic bacteria such as *Rhodospirillum rubrum* (Tian *et al*., 2010c). CoQ_{10} in plant were from soyabean, peanut and *Ruta* (Ikeda and Kagei, 1979 and Rodríguez-Acuna *et al*., 2008), palm oil (Han *et al*., 2006), tobacco leaves (Zu *et al*., 2006), litchi pericarp (Rao *et al*., 2011) and bee pollen (Xue *et al*., 2012). CoQ_{10} was found in animals such as pelagic fish, beef and pork heart (Mattila *et al*., 2000; Purchas *et al*., 2004 and Souchet and Laplante, 2007). Pyo and Oh (2011) reported that the fermentation process of food contained shrimp by diverse microorganisms during manufacture might influence the production of CoQ_{10}.

1.3.4 The extraction method of coenzyme Q_{10}

The reported extraction methods of CoQ_{10} from different samples were summarized in Table 1.3. The extraction method of CoQ_{10} included sample pretreatment steps before solvent extraction. The sample pretreatment can be divided into three methods, *i.e.*, saponification method, ultrasonication and chemical cell lysis. The saponification method was carried out by adding pyrogallic acid into a sample with an alkali conditions such as sodium hydroxide or potassium hydroxide and methanol and then heating in a water bath for 30 min, followed by a solvent extraction (Zhong *et al*., 2009 and Pyo and Oh, 2011). The saponification method can remove the oily impurities such as triglycerides. Since CoQ_{10} is bound with protein in cell membrane, the saponification will ensure the complete extraction. However, a partial CoQ_{10} could be damaged by an alkali condition with the substitution of quinone ring

with ethoxy to ubichromenol (Souchet and Laplante, 2007). The cell lysis of CoQ_{10} from bacteria with the acid-heat treatment before solvent extraction was interesting due to the simple operation method and could be applied to the industrial scale (Tian *et al*., 2010b). The ultrasonic extraction and accelerated solvent extraction of CoQ_{10} seem to be not suitable for the large scale.

1.3.4.1 Liquid-liquid extraction (LLE)

Liquid-liquid extraction is based on the partition of analyte between two immiscible liquid phases. Concentration of analyte in two phases can be described in Equation 1.3. The selection of organic solvents for the extraction is based on "Like dissolves like" (Moldoveanu and David, 2002). The yield of extraction in the solvent extraction is achieved by using the multiple extractions (Skoog *et al*., 1996).

$$K_D = \frac{[X]_B}{[X]_A} \qquad (1.3)$$

Where K_D is the distribution coefficient

$[X]_A$ is the equilibrium concentration of analyte in aqueous phase

$[X]_B$ is the equilibrium concentration of analyte in solvent phase

When using solid or semi-solid samples, it is called solid-liquid extraction. This method is commonly used for the extraction of CoQ_{10} from different samples and can use the large variety of solvents with broad solubility and selectivity ranges.

Table 1.3 Reported studies of CoQ_{10} extraction from various samples

Sample	Sample pretreatment	Solvent extraction	Reference
- Plant cell culture	-	Chloroform:methanol (2:1, v/v)	Ikeda and Kagei, 1979
- Meat and pelagic fish	Cell disrupting by sodium chloride and sodium dedecyl sulfate	Hexane:anhydrous ethanol (5:2, v/v)	Purchas *et al*., 2004 and Souchet and Laplante, 2007
- *Agrobacterium tumefaciens* strain ATCC4452	-	Light petroleum:ethanol (4:1, v/v)	Cao *et al*., 2006
- Fresh tobacco leaves and litchi pericarp	Ultrasonication with ethanol	Hexane	Zu *et al*., 2006 and Rao *et al*., 2011
- Soft capsule supplement	-	Hexane:ethanol (5:1.5, v/v)	Kettawan *et al*., 2007
- Dietary supplement	-	Hexane	Shimada *et al*., 2007
- *Sphingomonas* sp. ZUTE03 and fermented food	Saponification method	Hexane	Zhong *et al*., 2009 and Pyo and Oh, 2011
- *Rhodospirillum rubrum*	Cell lysis by celLytic B	Hexane:propanol (2:1, v/v)	Tian *et al*., 2010a
- *Agrobacterium tumefaciens*	Cell lysis with acid-heat treatment	Petroleum ether	Tian *et al*., 2010b
- Bee pollen	-	Accelerated solvent extraction with ethanol	Xue *et al*., 2012

1.3.4.2 Ultrasonic extraction

The ultrasonic extraction is commonly employed for cell disintegration on the principle of mass transfer between immiscible phases through super agitation. The mechanism of ultrasonic extraction involves two processes of physical activity: the dissolution of the analyte near the particle surface and the diffusion from the solid particles to the bulk of the liquid extract (Rao *et al*., 2011). Zu *et al*. (2006) reported that the ultrasonic extraction required a shorter time compared to the reflux or soxhlet extraction of CoQ_{10} from fresh tobacco leaves. However, Tian *et al*. (2010b) found the low effective extraction of CoQ_{10} with ultrasonic extraction when compared to acid-heat treatment. This result was because the ultrasonic extraction method generated microscopic bubbles which these temporary cavities probably created high-shear gradients by microstreaming, with most cavity effects only observed close to the vibrating surface effect of cell, resulting in the incomplete extraction.

1.3.4.3 Accelerated solvent extraction (ASE)

Accelerated solvent extraction (ASE) is the first developed method performed by Dionex in 1996 since it is automated extraction system and uses low amount of solvents (Xue *et al*., 2012). The extraction yield of this technique depends on temperature and pressure (Breithaupt, 2004). It provides more advantages with requiring smaller volume of solvent and a short time of extraction and can be used a wide range of solvents (Schäfer, 1998). ASE was applied for the CoQ_{10} extraction in bee pollen with environmentally acceptable organic solvents. However, this technique was only suitable for the laboratory scale which was considered as a routine analysis method (Xue *et al*., 2012).

1.3.5 Analysis techniques of coenzyme Q_{10}

1.3.5.1 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography is based on the partition of analyte between bonded-phase on stationary phase and liquid mobile phase. The analytes are strongly retained by the stationary phase and eluted slowly with the flow of mobile phase. If analytes likes to dissolve in mobile phase, they will elute rapidly.

These differences in mobility can be analyzed qualitatively or quantitatively (Skoog and Leary, 1992). The analysis technique of CoQ_{10} is mostly performed by reversed-phase high performance liquid chromatography (RP-HPLC). CoQ_{10} was separated on a C18 column with the elution of many mobile phase systems such as methanol:ethanol (5:95, v/v) (Cao *et al*., 2006), 2-propanol:ethanol:methanol (35:24:41, v/v/v) (Souchet and Laplante, 2007) and methanol:hexane (83:17, v/v) (Zhong *et al*., 2009). Many detection methods of CoQ_{10} for HPLC system have been reported including mass spectrometric detector (Zu *et al*., 2006 and Ruiz-Jiménez *et al*., 2007), electrochemical detector (Menke *et al*., 2000 and Kubo *et al*., 2008) and diode array detector (Mattila *et al*., 2000; Karpinska *et al*., 2006 and Lunetta and Roman, 2008). The HPLC method coupled with mass spectrometry or electrochemical detection have been widely used for the determination of CoQ_{10} in human plasma (Menke *et al*., 2000 and Ruiz-Jiménez *et al*., 2007) with the high sensitivity and can simultaneously detect both of reduced and oxidized forms of CoQ_{10} (Tang *et al*., 2001 and Kubo *et al*., 2008). However, these techniques requires special skill of operation and high cost of maintenance of instrument (Ruiz-Jiménez *et al*., 2007). The diode array detection of CoQ_{10} set at 275 nm has commonly used in various samples with high selectivity and simplicity (Purchas *et al*., 2004; Cao *et al*., 2006; Souchet and Laplante, 2007; Ha *et al*., 2007; Zhong *et al*., 2009; Tian *et al*., 2010b; Tian *et al*., 2010c and Suman *et al*., 2011).

The chromatographic terms relating to the evaluation of separation system are, for example, peak resolution and peak symmetry. Peak resolution (R_s) is the measurement of the separation between two peaks (Figure 1.4) and can be calculated from Equation 1.4 (Agilent ChemStation B.03, 2007). If the peak resolution equals to 1, the good resolution of peaks was obtained without any baseline or space between the two peaks (Skoog *et al*., 1996).

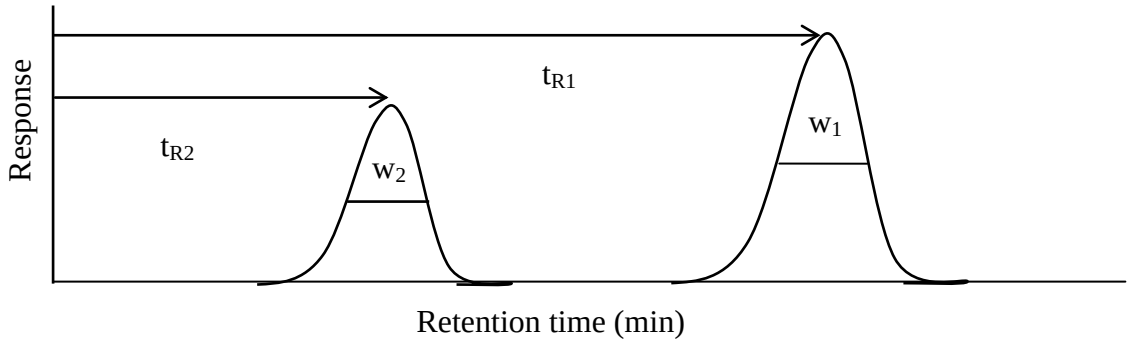


Figure 1.4 Definition of the peak resolution (derived from Agilent ChemStation B.03, 2007)

$$R_s = 2.35/2\left(\frac{t_{R2} - t_{R1}}{w_1 + w_2}\right) \qquad (1.4)$$

Where t_{R1}, t_{R2} is the retention time of the first and second peak, respectively

w_1, w_2 is the half peak height of the first and second peak, respectively

Peak symmetry can describe the sorption characteristic of solute which often changes at high concentration. If no changes occur, the relationship of the concentration of solute in the mobile phase and that in the stationary phase at constant temperature would be linear, resulting in the symmetrical profile of solute concentration. The tailing and fronting peaks of the concentration profiles lead to poor separations and unreliable quantitative analysis. Peak symmetry can be described by asymmetry factor (A_s), as shown in Figure 1.5 and Equation 1.5 (Weiss, 2004).

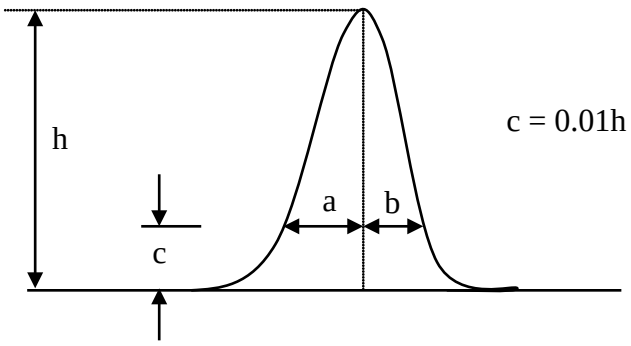


Figure 1.5 Definition of the asymmetry factor (derived from Weiss, 2004)

$$A_s = \frac{b}{a} \qquad (1.5)$$

1.4 Purification of CoQ_{10}

The methods of purification of CoQ_{10} have been reported including the high-speed counter-current chromatography (HSCCC), silica gel column chromatography and solid phase extraction (SPE). Cao *et al*. (2006) used HSCCC to separate CoQ_{10} from bacteria fermentation broth extract. The purification was performed by using two solvent phases with the elution system of heptane:acetonitrile:dichloromethane (12:7:3.5, v/v/v). Separation efficiencies such as %purity, %recovery or yield of CoQ_{10} obtained from HSCCC were higher than these obtained from column chromatography. However, HSCCC used the toxic solvents such as dichloromethane and was performed by using a special instrument. SPE is a method for clean up and preconcentration of analyte. Rodriguez-Acuna *et al*. (2008) reported the purification of CoQ_9 and CoQ_{10} from vegetable oil by using amino-propyl SPE cartridges with the elution of heptanes:ethyl ether (80:20, v/v). This technique can remove triacylglycerides. Hagerman *et al*. (2001) reported the solid-phase purification of CoQ_6 from *Saccharomyces cerevisiae* (baker's yeast) by using silica gel as an adsorbent and the mixture of dichloromethane and hexane (2.5:1, v/v) as an eluent. This SPE could remove ergosterol and ergosterol derivatives and reduced the retention time of analysis from 70 min to 15 min. Silica gel has also been reported for the purification of ubiquinone homologues as CoQ_9 and CoQ_{10} in plant cell culture after extraction with chloroform and methanol (Ikeda and Kagei, 1979) and CoQ_9 and CoQ_{10} were eluted by the gradient elution of hexane and ethyl acetate.

Column chromatography is often used with the advantage of the purification of trace amounts of organic mixture. Its principle is based on the partition of analyte between the mobile phase and stationary phase and can be described as Equation 1.6 (Skoog *et al*., 1996).

$$K_D = \frac{[X]_B}{[X]_A} \qquad (1.6)$$

Where K_D is the partition coefficient

$[X]_A$ is the equilibrium concentration of analyte in mobile phase

$[X]_B$ is the equilibrium concentration of analyte in stationary phase

The efficiency of separation depends on the types of adsorbent and solvents. For choosing a solvent used as an eluent, it is necessary to consider the solubility of the analyte in eluent, and the ease of subsequent removal. The collected fractions after the elution are usually monitored by UV or visible light and thin layer chromatograph is used for identifying the components.

1.5 Preservation of CoQ_{10}

CoQ_{10} is sensitive to light (EP 4, 2002 and Yang and Song, 2006). Thus, the preservation of CoQ_{10} would protect it from light effect during shipping and storage. In order to improve the solubility, photostability and thermal stability of CoQ_{10}, many methods has been reported including the encapsulation of CoQ_{10} in carbohydrate matrices such as gum arabic, *β*-cyclodextrin and γ –cyclodextrin (Yang and Song, 2006; Fir *et al*., 2009 and Bule *et al*., 2010), the incorporation of CoQ_{10} with poly(methyl methacrylate) nanoparticles (Kwon *et al*., 2002) and solid self-emulsifying drug delivery system of CoQ_{10} (Onoue *et al*., 2012). The microencapsulation of CoQ_{10} in gum arabic was undertaken with coconut oil and sodium stearoyl lactylate used as an emulsifier while the encapsulation of CoQ_{10} with cyclodextrins was prepared by inclusion complex with water. Solid self-emulsifying drug delivery system of CoQ_{10} was prepared by spray-drying using an emulsifier containing of CoQ_{10}, medium-chain triglyceride, sucrose ester of fatty acid and hydroxypropyl cellulose. The improvement for photostability of CoQ_{10} with poly(methyl methacrylate) nanoparticles was performed under microfluidization and solvent evaporation method. The mean diameter of the nanoparticles was highly influenced by the types of surfactants used and the recycling number of the microfluidization process. The use of cyclodextrin as a stabilizer seems to be the promising method to preserve CoQ_{10} due to its simple and ease to operation.

1.5.1 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligomers of α-D-glucopyranose that can be produced from the transformation of starch by bacterias. They consist of six, seven or eight α-(1,4)-linked glucopyranose units (Figure 1.6) which exhibits a hydrophobic central cavity and a hydrophilic outer surface. The properties of CDs can be summarized as shown in Table 1.4 (Del Valle, 2004). Cyclodextrins are able to form inclusion complexes with several compounds, thus, they are important for food and pharmaceutical applications (Tønnesen *et al*., 2002; Mourtzinos *et al*., 2007; Mourtzinos *et al*., 2008; Fir *et al*., 2009; Kalogeropoulos *et al*., 2010 and El-Kemary *et al*., 2011).

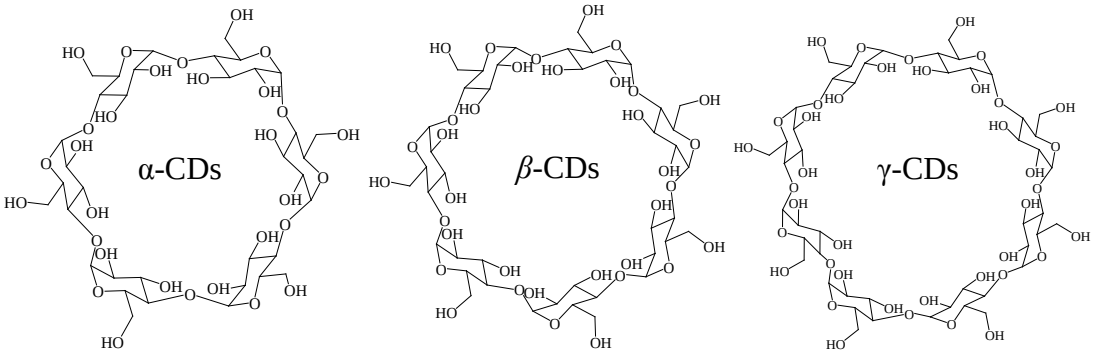


Figure 1.6 Chemical structures of α-cyclodextrin, *β*-cyclodextrin and γ-cyclodextrin (derived from Del Valle, 2004)

Table 1.4 Cyclodextrins properties (Del Valle, 2004)

Property	**α-Cyclodextrin**	***β*-Cyclodextrin**	**γ-Cyclodextrin**
Molecular weight (g mol^{-1})	972	1135	1297
Solubility in water at 25°C (%, w/v)	14.5	1.85	23.2
Outer diameter (Å)	14.6	15.4	17.5
Cavity diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (Å)	7.9	7.9	7.9
Cavity volume ($Å^3$)	174	262	427

1.5.2 Inclusion complex of CoQ_{10}

Cyclodextrins acts as empty capsules of a certain molecular size and can include many molecules in this cavity. This is called “inclusion complex”. The inclusion complex comprises two or more molecules which one of molecule is “host” includes totally or partly “the guest molecule” by physical forces (Astray *et al*., 2009). Thus, cyclodextrins are host molecules. The inclusion of a guest in cyclodextrins cavity comprises of the substitution of the included water molecules by the less polar guest. The types of bond established between guest and host are van der Waals forces, hydrophobic interaction and hydrogen bond (Astray *et al*., 2009). The ability of cyclodextrins to form an inclusion complex with a guest molecule depends on many factors such as the relative size of cyclodextrins to the size of the guest molecule (Astray *et al*., 2010), the properties of the guest (Gao and Zhao, 2005) and the amount of water (Cevallos *et al*., 2010). Some solvents such as ethanol are added to water to improve the solubility of cyclodextrin solution and more or less non-polar poorly water-soluble compounds and frequently applied for the preparation of *β*-cyclodextrin and CoQ_{10} (Yang and Song, 2006). *β*-cyclodextrin can include aromatics or heterocyclic molecules (Del Valle, 2004). The dynamic equilibrium process can be shown by the Equation 1.7 and 1.8.

$$CD+G \underset{k_D}{\overset{k_R}{\rightleftharpoons}} CD\text{-}G \qquad (1.7)$$

$$K = \frac{k_R}{k_D} \qquad (1.8)$$

Where CD is the cyclodextrin

G is the guest molecule

CD-G is the inclusion complex

K is the stability of the inclusion complex

k_R is the recombination constant

k_D is dissociation constant

1.5.3 Applications of *β*- cyclodextrin

The chemical and physical properties of the guest molecules can be improved by *β*-Cyclodextrin. *β*-Cyclodextrin have been recommended for applications in food processing or pharmaceutical industry with various objectives such as to stabilize fragrance, flavours or vitamins against unwanted changes, to improve the physical and chemical properties of drugs, to enhance the bioavailability of poorly soluble drugs or to protect lipophillic food components against oxidation degradation and heat or light induced changes (Del Valle, 2004 and Astray *et al*., 2009). The applications of *β*-cyclodextrin can be summarized in Table 1.5. In Thailand, the recommendation of Ministry of Public health for a maximum level of *β*-cyclodextrin is 20,000 mg kg^{-1} in chewing gum and 500 mg kg^{-1} in electrolyte beverage (Notification of Ministry of Public health, 2004).

1.5.4 Photostability test

The objective of the photostability test is to study the degradation of materials which are sensitive to light with accelerated conditions of light. This test can be used for the method development to evaluate the overall photosensitivity of materials (Tønnesen, 2004). The exposure conditions for photostability test include the use of various light sources such as xenon arc lamp (Onoue *et al*., 2012), fluorescent lamps emitting long wave UVA radiation between 350-400 nm (Kommuru *et al*., 1999 and Kwon *et al*., 2002) or 254 nm (Fir *et al*., 2009 and Bule *et al*., 2010) and the sunlight at Earth's surface which gives greater the amount of solar in UVA than UVB (region from 280-320 nm) or UVC (region from 200-280 nm) (Moore, 2004). The photostability test can be performed under the sunlight or in the laboratory with using the transparent containers and a control system wrapped with aluminum foil to evaluate the contribution of thermally induced change to the total observed change. The period of exposure time is studied until the extensive decomposition of substances occurs (ICH Q1B, 2006).

At the end of the exposure time, the remaining concentration of analyte is examined and the remaining concentration and exposure time is plotted to evaluate the rate of photo-degradation performed by using the order of reaction (Oxtoby *et al*.,

1999). The zeroth order kinetics comes from the linear regression between remaining concentration and exposure time. The rate constant of zeroth order kinetics and the half-life are expressed in Equation 1.9 and 1.10, respectively.

$$[A] = [A]_0 - kt \qquad (1.9)$$

$$t_{1/2} = \frac{[A]_0}{2k} \qquad (1.10)$$

Where [A] is the concentration of A at t time

$[A]_0$ is the initial concentration of A

k is the rate constant of the zeroth order kinetics

t is the exposure time

$t_{1/2}$ is the half-life as the time required for the reduction of a value to 50% of its initial concentration

The verification of first-order kinetics is also obtained when a plot of the logarithm of the remaining concentration of analyte and exposure time is found to be linear. The slope of linear equals to (-k) of the rate constant of first-order reactions. The linear equation and half-life can be expressed as Equation 1.11 and 1.12, respectively.

$$\ln[A] = \ln[A]_0 - kt \qquad (1.11)$$

$$t_{1/2} = \frac{0.693}{k} \qquad (1.12)$$

Where k is the rate constant of the first-order kinetics

The rate constant (k) of the second-order kinetics is obtained from the linear regression plotted between 1/the remaining concentration of analyte against exposure time. The linear equation and half-life can be expressed as Equation 1.13 and 1.14, respectively.

$$\frac{1}{[A]} = 2kt + \frac{1}{[A]_0} \qquad (1.13)$$

$$t_{1/2} = \frac{1}{2k[A]_0} \qquad (1.14)$$

Where k is the rate constant of the second-order kinetics

Table 1.5 Literature reviews of *β*-cyclodextrin application

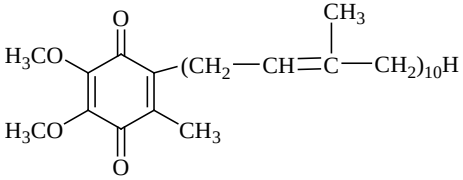
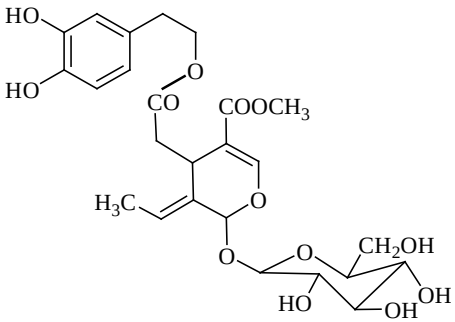
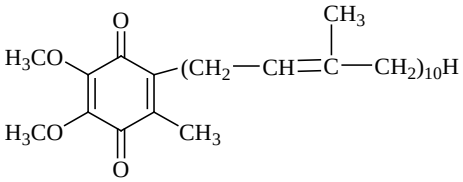
Analyte	Structure of analyte	Advantage	Its use	Reference
CoQ_{10}		Improve photostability and sensitivity	Analysis of CoQ_{10}	Yang and Song, 2006
Oleuropein		Improve thermal stability and increase water solubility	Fortify food Food supplement	Mourtzinos *et al*., 2007
CoQ_{10}		Improve thermal stability, photostability and increase water solubility	Pharmaceutical ingredient (soft-capsules and syrup) Food additive (milk, yogurt, kefir, jam, marmalade and honey)	Fir *et al*., 2009

Table 1.5 Literature reviews of *β*-cyclodextrin application (continued)

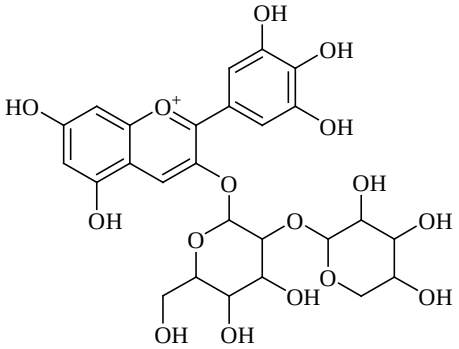
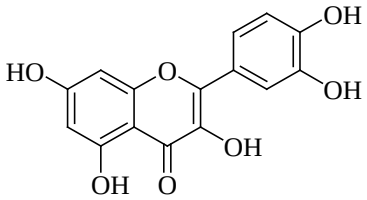
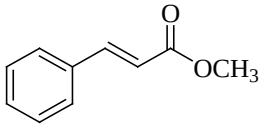
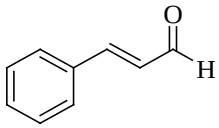
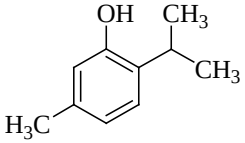
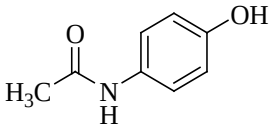
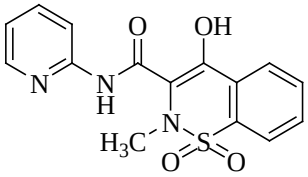
Analyte	Structure of analyte	Advantage	Its use	Reference
Anthocyanin		Improve thermal stability	Food ingredient	Mourtzinos *et al*., 2008
Flavonoid based on quercetin		Improve thermal stability	Food supplement	Kalogeropoulos *et al*., 2010
Methyl cinnamate		Stabilize flavors	Food application	Astray *et al*., 2010
Cinnamaldehyde		Stabilize flavors	Food application	Cevallos *et al*., 2010

Table 1.5 Literature reviews of *β*-cyclodextrin application (continued)

Analyte	Structure of analyte	Advantage	Its use	Reference
Thymol		Stabilize flavors	Food application	Cevallos *et al*., 2010
Paracetamol		Improve photostability	Pharmaceuticals	El-Kemary *et al*., 2011
Piroxicam		Improve drug solubility	Pharmaceuticals	Grandelli *et al*., 2012

1.6 Method Validation

1.6.1 Calibration curve

The calibration curve is a relationship between the measured signal of analyte and the series of different analyte concentration covered the range of its concentration in real sample. The evaluation of linear calibration curve is performed by using the regression line which is constructed by plotting the concentration of analyte on x-axis and signal of analyte on y-axis. The linear equation as described in Equation 1.15 is obtained from the regression line and the unknown concentration can be estimated from linear regression analysis which is often called a “least squares analysis”. The correlation coefficient (r) of a straight line should be greater than 0.99 (Miller and Miller, 2005).

$$y = bx + a \qquad (1.15)$$

Where y is the instrument signal of analyte

x is the concentration of analyte

b is the slope of the regression line or the method’s sensitivity

a is an intercept of the regression line

The random errors of the slope and intercept values are important and can be estimated by using the standard deviation of the regression line as described in Equation 1.16. The standard deviation of the slope and intercept can be calculated from Equation 1.17 and 1.18, respectively.

$$S_{y/x} = \sqrt{\frac{\sum_i \left(y_i - \hat{y}_i\right)^2}{n-2}} \qquad (1.16)$$

$$S_b = \frac{S_{y/x}}{\sqrt{\sum_i \left(x_i - \bar{x}\right)^2}} \qquad (1.17)$$

$$S_a = S_{y/x}\sqrt{\frac{\sum_i x_i^2}{n\sum_i (x_i - \bar{x})^2}} \qquad (1.18)$$

Where $S_{y/x}$ is the standard deviation calculated statically from the linear regression

n is the number of concentration point of calibration line

y_i is the signal of analyte

$\hat{y}_i$ is the signal values on the fitted y_i values, corresponding to the individual analyte concentration (x_i)

S_b is the standard deviation of slope

S_a is the standard deviation of intercept

$\bar{x}$ is mean of the concentration of analyte

The values of S_b and S_a can be used to estimate confidence limits for the slope and intercept. These are given by:

The confidence limits for the slope of the line $= b \pm t_{(n-2)} S_b$

The confidence limits for the intercept of the line $= a \pm t_{(n-2)} S_a$

Where a is an intercept of the regression line

b is a slope of the regression line

$t_{(n-2)}$ is a *t*-value taken at 95% confidence level

n is the number of calibration point on the regression line

n-2 is degrees of freedom

1.6.2 Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) is the analyte concentration which gives an instrument signal (y) significantly different from the blank or background signal. LOD is calculated from the linear least squares line procedure as shown in Equation 1.19.

$$y_L = y_B + kS_B \qquad (1.19)$$

Where y_L is the signal at the limit of detection

y_B is the blank signal

S_B is the standard deviation of the blank

k is the constant. According to IUPAC, k=3 for LOD and k=10 for LOQ. These values will be used in this work.

y_B and S_B can be obtained from the blank experiment of 20 to 30 measurements (Skoog and Leary, 1992). For the other ways, the assumption of the unweighted least-squares method is that each point on the plot (including the point representing the blank) has a normal distribution of variation. Thus, y_B and S_B are estimated as an intercept (a) and $S_{y/x}$, respectively (Miller and Miller, 2005). $S_{y/x}$ can be calculated from the Equation 1.16. The calculation of LOD can be summarized in Figure 1.7.

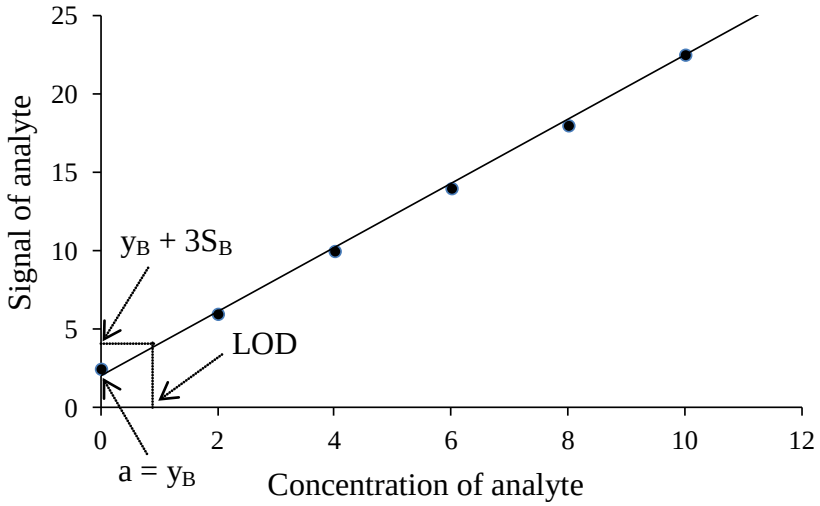


Figure 1.7 The calculation of LOD (derived from Miller and Miller, 2005)

From the Equation 1.19,

$$y_L = a + 3S_{y/x} \qquad (1.20)$$

Substitute Equation 1.20 to Equation 1.15 for a concentration of analyte at LOD (x_{LOD}),

$$x_{LOD} = \frac{\left[a + 3S_{y/x} \right] - a}{b} \qquad (1.21)$$

Therefore, the concentration of LOD (x_{LOD}) can be given by the Equation 1.22.

$$x_{LOD} = \frac{3S_{y/x}}{b} \qquad (1.22)$$

For the concentration of analyte at limit of quantification (x_{LOQ}),

$$x_{LOQ} = \frac{10S_{y/x}}{b} \qquad (1.23)$$

1.6.3 Matrix effect

The external standardization allows a series of samples to be analyzed using a calibration curve and it is assumed that there is no difference between the standard's matrix and sample's matrix. However, a proportional determinate error is introduced when differences between the two matrices cannot be ignored, as example shown in Figure 1.8. Thus, the negative determination error will occur when using a calibration curve.

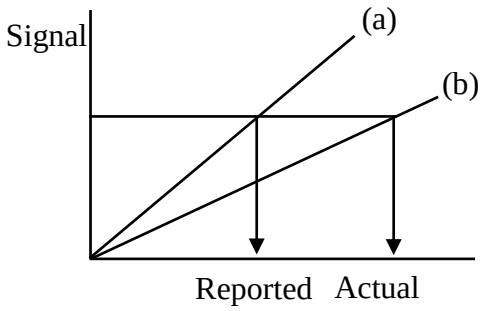


Figure 1.8 The concentration of analyte from (a) calibration curve obtained in standard's matrix and (b) calibration curve obtained in sample's matrix (derived from Harvey, 2000)

Therefore, in order to evaluate the matrix effect, the method of standard addition is commonly used and carried out by spiking aliquots of a standard solution to the sample. Equal volumes of the sample solution are taken and all but one are separately added with known different concentration of analyte. All are then diluted to the same values. The method of standard addition is shown in Figure 1.9. The negative intercept on the x-axis corresponds to the analyte concentration in sample. The relation of two methods related to standard curve and standard addition curve is constructed by plotting the different analyte concentrations from standard curve on a x-axis and the different analyte concentrations from standard addition curve on a y-axis. If there is an identical result for both methods, the slope and intercept value of the regression line will be closed to 1 and 0, respectively. This implies that there is no matrix effect and the calibration curve can be used for the analysis of analyte concentration in sample (Miller and Miller, 2005).

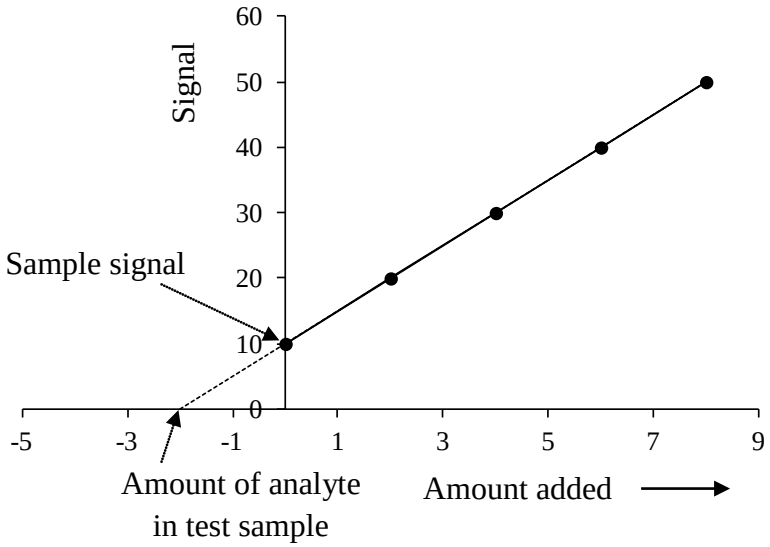


Figure 1.9 The method of standard additions (derived from Miller and Miller, 2005)

1.6.4 Accuracy and precision

Accuracy is the closeness of a single (x_i) or of a mean ($\bar{x}$) of a set of results to the true value (μ). The true value is usually obtained from the certified reference material (CRM). Accuracy is expressed as either an absolute error,

$E = x_i - \mu$

$E = \bar{x} - \mu$

or a percent relative error (E_r),

$$E_r = \frac{\bar{x} - \mu}{\mu} \times 100$$

However, the matrix of CRM is not suitable for real sample matrix. Therefore, the recovery is used to evaluate the accuracy and studied by spiking known standard into the real sample. The percentage of recovery is calculated as described in Equation 1.24 (EURACHEM, 1998).

$$\text{Recovery}(\%) = \frac{C_1 - C_2}{C_3} \times 100 \qquad (1.24)$$

Where C_1 is the concentration of added standard determined in spiked sample

C_2 is concentration of analyte determined in unspiked sample

C_3 is concentration of standard added in sample

Precision describes the reproducibility of results obtained from two or more replicate measurement, or measurements that have been made in exactly the same way. The terms used to describe the precision of a set of replicate data are standard deviation, variance or coefficient of variation. The standard deviation (SD) and the coefficient of variation (CV) are given by the Equation 1.25 and 1.26, respectively (Skoog *et al*., 1996).

$$SD = \sqrt{\frac{\sum_{i=1}^{n} \left(x_i - \bar{x}\right)^2}{n-1}} \qquad (1.25)$$

$$CV = \frac{SD}{\bar{x}} \times 100 \qquad (1.26)$$

Where n is the number of replicate data

x_i is a measurement

$\bar{x}$ is a mean of measurement

1.7 Objectives

1.7.1 To develop method of the extraction of CoQ_{10} from *Artemia*

1.7.2 To study the purification method for CoQ_{10} from crude extract of *Artemia* by column chromatography

1.7.3 To study the preservation method for purified CoQ_{10}

1.8 Expected benefits

The developed method of the extraction of CoQ_{10} in *Artemia* focused on the cell lysis by the organic acid treatment coupled with the solvent extraction. The advantages of developed extraction method are low cost consuming, simple and fast to release CoQ_{10} from *Artemia*. Moreover, the analysis of CoQ_{10} by HPLC-DAD is the selective, accurate and precise method. The extraction method of CoQ_{10} from *Artemia* could be applied to industry. The proposed method for the purification yielded high CoQ_{10} and the preservation of CoQ_{10} with β-cyclodextrin are low cost and can stabilize CoQ_{10} for a long period.

CHAPTER 2
EXPERIMENTAL

2.1 Overall scope

This work was divided into two parts. The first part related to the development of sample preparation method including cell lysis and CoQ_{10} extraction from *Artemia*. The second part related to the purification of extract with silica gel column chromatography and the preservation of purified CoQ_{10} by using *β*-cyclodextrin. The scope of this work was summarized in Figure 2.1.

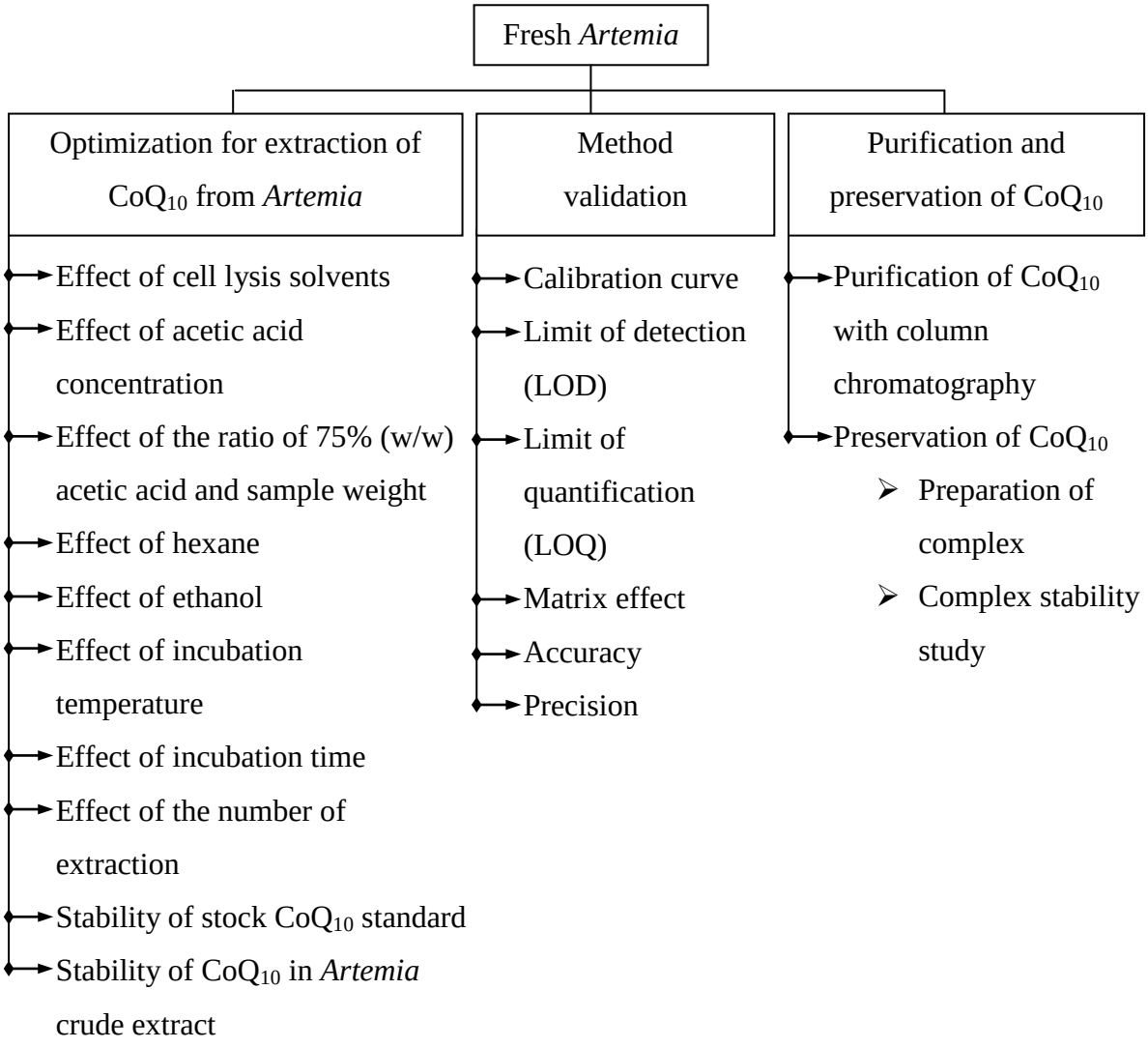


Figure 2.1 Scope of the experiment for CoQ_{10} from *Artemia*.

2.2 Chemicals

2.2.1 Standard chemical

Coenzyme Q_{10} (greater than 98% purity) was purchased from Sigma-Aldrich (USA).

2.2.2 General chemicals

General chemicals used throughout experiment are listed in Table 2.1. Deionized (DI) water was obtained from a Maxima ultrapure water instrument (ELGA, England).

Table 2.1 General chemicals used throughout experiment

Chemicals	**Grade**	**Brand**	**Country**
Absolute ethanol	HPLC	RCI Labscan	Thailand
Absolute ethanol	AR	RCI Labscan	Thailand
Acetic acid	AR	Merck	Germany
Acetone	AR	RCI Labscan	Thailand
Aluminum sheets of silica gel 60 GF_{254} (20 cm × 20 cm, 0.2 mm layer thickness)	TLC	Merck	Germany
Chloroform	AR	RCI Labscan	Thailand
d_1-Chloroform	NMR spectroscopy	Merck	Germany
β-Cyclodextrin 100%	R&D	Sigma	Singapore
Ethyl acetate	AR	RCI Labscan	Thailand
Hexane	AR	J. T. Baker	USA
Hydrochloric acid	AR	RCI Labscan	Thailand
Methanol	HPLC	RCI Labscan	Thailand
2-Propanol	HPLC	RCI Labscan	Thailand
Silica gel 100 (70-230 Mesh ASTM)	AR	Merck	Germany

2.3 Laboratory glasswares

2.3.1 Glasswares used for CoQ_{10} extraction

1) Amber vial with silicone septum, 1.5 mL
2) Beaker, 100 mL
3) Dropper
4) Glass bottle
5) Glass rod
6) Glass vacuum filter holder
7) Graduated cylinder, 10 mL
8) Graduated cylinder, 100 mL
9) Hypodermic syringe, 10 mL
10) Measuring pipette, 10 mL
11) Round bottom flask, 100 mL
12) Test tube, 60 mL
13) Volumetric flask, 50 mL
14) Volumetric flask, 100 mL
15) Watch glass

2.3.2 Glasswares used for CoQ_{10} purification

1) Amber vial with silicone septum, 1.5 mL
2) Beaker, 150 mL
3) Capillary tube, 1 mm i.d.
4) Clear vial, 10 mL
5) Dropper
6) Glass column, 30.0 cm × 2.0 cm i.d.
7) Glass rod
8) Graduated cylinder, 100 mL

2.3.3 Glasswares used for CoQ_{10} preservation

1) Amber vial with silicone septum, 1.5 mL
2) Beaker, 20 mL
3) Glass rod

4) Watch glass

5) Graduated cylinder, 10 mL

6) Petri dish, 15 mm × 60 mm

7) Volumetric flask, 5 mL

2.3.4 Cleaning of glasswares

Glasswares were cleaned with a detergent solution (Tepol Pure, Thailand) and tap water. After that, they were rinsed with distilled water and soaked in 10 % (v/v) nitric acid for at least 12 hours. After washing with distilled water, they were dried in the oven at 100°C, but test tubes and vials were furnaced at 200-300°C at least 7 hours. Volumetric flask, cylinder and pipette, after soaking in 10% (v/v) nitric acid, were washed with distilled water and air dried.

2.4 Apparatus and materials

All apparatus and materials used are listed in Table 2.2.

Table 2.2 Apparatus and materials used throughout experiment

Apparatus	**Brand**	**Country**
Aluminum foil	Diamond	USA
Analytical balance, 0.001 g	Mettler Toledo, PB 303	Switzerland
Analytical balance, 0.0001 g	Mettler Toledo, AB204-S	Switzerland
Aspirator	JEIO TECH, VE-11	Korea
Block heater	Techne, DB-3D	England
Freezer	Umac scientific, UM-Fd200	China
Holder syringe, 13 mm	Millipore	USA
Hot air oven	Memmert, UM 500	Germany
Hot plate & stirrer	Heidolph, MR 3001	Germany
Micropipette, 10-100 µL	Brand	Germany
Micropipette, 100-1000 µL	Brand	Germany
Nylon membrane filters, 0.2 µm	Whatman	England
Nylon membrane filters, 0.45 µm	Whatman	England

Table 2.2 Apparatus and materials used throughout experiment (continued)

Apparatus	Brand	Country
Rotary evaporator	Eyela, N-1001	China
Homemade stability test chamber (30 cm × 60 cm × 30 cm)	-	-
Ultrasonic cleaner	Auto Science, AS10200BDT	China
UVA lamp 40W	Phillips	Netherlands
Vortex mixer	Uzusio, VTX-3000L	Japan
Water bath	Memmert, W 350	Germany

2.5 Instruments

2.5.1 High-performance liquid chromatograph (HPLC)

The HPLC analyses were performed on a Agilent 1200 series and controlled by a computer using Agilent Chemstation software (Agilent Technologies, USA). The Agilent ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm i.d., 5 µm thickness) with guard column (12.5 mm × 4.6 mm i.d., 5 µm thickness) was used. A 20 µL sample was injected into the column with the column temperature set at 27°C. The mobile phase consisted of methanol and ethanol (5:95, v/v) with the isocratic elution at the flow rate of 1.0 mL min^{-1}. The diode array detection was set at 275 nm.

2.5.2 UV-VIS spectrophotometer

The spectrum of CoQ_{10} was recorded on Lambda 45 UV-VIS spectrophotometer (PerkinElmer, USA) with a quartz cell of 1 cm light path.

2.5.3 ^{1}H-Nuclear magnetic resonance spectrometer (^{1}H NMR)

The ^{1}H NMR spectrum of purified CoQ_{10} was recorded on a FTNMR, Bruker Avance (300 MHz). d_1-Chloroform ($CDCl_3$) was used as a solvent.

2.5.4 Mass spectrometer

The purified CoQ_{10} was identified by direct injecting to the mass spectrometer (Micromass, U.K.) in electrospray ionization positive mode. The time of flight mass analyzer was monitored at *m/z* 885.

2.6 Preparation of samples

Fresh *Artemia* samples were collected from the Ecological Aquaculture Research Unit at Department of Technology and Industry, Faculty of Science and Technology, Prince of Songkla University, Pattani campus during 2011-2012. Samples were washed with DI water about 10 mL g^{-1} sample and kept in a plastic bottle at 4°C before analysis. Moisture content of sample was analyzed by AOAC 930.15 method for the calculation of the CoQ_{10} yield based on *Artemia* dry weight.

2.7 Preparation of solutions

2.7.1 A stock standard solution of CoQ_{10} at 100 μg mL^{-1}

A 5.0 mg of CoQ_{10} was dissolved in absolute ethanol, followed by sonication for 10 min. A solution was adjusted to 50.00 mL with ethanol, and aliquots were taken and kept in a 10-mL glass bottle at -20°C when not in use. The solutions were used within 3 months and protected from the light by using amber bottles or wrapping with aluminum foil.

2.7.2 A working standard solution of CoQ_{10} at 10 μg mL^{-1}

Working standard solution was freshly prepared by pipetting 1000 μL of stock standard solutions into a 10-mL volumetric flask and volume was adjusted to the mark with ethanol.

2.7.3 Calibration curve of CoQ_{10}

The calibration curve of CoQ_{10} was obtained by diluting of the stock standard solution of 10.0 and 100.0 μg mL^{-1}. The concentrations of CoQ_{10} standard were prepared at 1.0, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 μg mL^{-1}, as shown in the Table 2.3. Then the standard solution was injected to HPLC.

Table 2.3 The preparation of calibration curve of CoQ_{10}

Stock concentration of CoQ_{10} ($\mu g\ mL^{-1}$)	**Volume of CoQ_{10} from stock standard solution (μL)**	**Volume of ethanol (μL)**	**Final concentration of CoQ_{10} ($\mu g\ mL^{-1}$)**
10.0	100.0	900.0	1.0
10.0	250.0	750.0	2.5
10.0	500.0	500.0	5.0
10.0	1000.0	0.0	10.0
100.0	200.0	800.0	20.0
100.0	300.0	700.0	30.0
100.0	400.0	600.0	40.0
100.0	500.0	500.0	50.0

2.8 Preparation of solution for extraction of CoQ_{10}

2.8.1) 70% (v/v) Ethanol

A 70.0 mL of absolute ethanol was added into a 100-mL volumetric flask and adjusted to the mark with DI water. A solution was transferred to a glass bottle.

2.8.2) 9.2% (w/w) Hydrochloric acid

A 25.0 mL of 37% (w/w) hydrochloric acid was added into a 100-mL volumetric flask containing about 50 mL of DI water in fume hood. The volume of solution was adjusted to the mark with DI water and transferred to a glass bottle.

2.8.3) 0.1% (w/w) Hydrochloric acid

A 1.00 mL of 9.2% (w/w) hydrochloric acid was pipetted into a 100-mL volumetric flask containing about 50 mL of DI water. The volume of solution was adjusted to the mark with DI water and transferred to a glass bottle.

2.8.4) 5% (w/w) Acetic acid

A 5.0 mL of glacial acetic acid was added into a 100-mL volumetric flask containing about 50 mL of DI water in fume hood. The volume of solution was adjusted to the mark with DI water and transferred to a glass bottle.

2.8.5) 25% (w/w) Acetic acid

A 25.0 mL of glacial acetic acid was added into a 100-mL volumetric flask containing about 50 mL of DI water in fume hood. The volume of solution was adjusted to the mark with DI water and transferred to a glass bottle.

2.8.6) 50% (w/w) Acetic acid

A 50.0 mL of glacial acetic acid was added into a 100-mL volumetric flask containing about 40 mL of DI water in fume hood. The volume of solution was adjusted to the mark with DI water and transferred to a glass bottle.

2.8.7) 75% (w/w) Acetic acid

A 75.0 mL of glacial acetic acid was added into a 100-mL volumetric flask containing about 10 mL of DI water in fume hood. The volume of solution was adjusted to the mark with DI water and transferred to a glass bottle.

2.9 Methods

2.9.1 Selection of the HPLC conditions

The important HPLC conditions for the separation of CoQ_{10} such as mobile phase, column and detector were considered to obtain the rapid separation of CoQ_{10} from *Artemia*. The mobile phase system was evaluated with three systems: methanol:ethanol (5:95, v/v) (Cao *et al*., 2006), 2-propanol:ethanol:methanol (35:24:41, v/v/v) (Souchet and Laplante, 2007) and methanol:hexane (83:17, v/v) (Zhong *et al*., 2009). The CoQ_{10} extract from *Artemia* was used for the comparison of three mobile phase systems with the HPLC condition mentioned in section 2.5.1.

2.9.2 Optimization for extraction of CoQ_{10} from *Artemia*

The solid-liquid extraction coupled with cell lysis was used for the extraction of CoQ_{10} from *Artemia*. Parameters affecting the cell lysis and extraction, *i.e.*, cell lysis solvents, concentration of acetic acid, the ratio of 75% (w/w) acetic acid and sample weight, volume of hexane used as an extractant, volume of ethanol, incubation temperature, incubation time and the number of extraction were studied. The highest yield of CoQ_{10} was chosen for the optimization of each parameter except the recovery which was used for optimizing the number of extraction.

2.9.2.1 The effect of cell lysis solvent on CoQ_{10} extraction

The extraction method was modified from Tian *et al*. (2010b) who found that the highest content of CoQ_{10} from *Agrobacterium tumefaciens* cell was obtained when using 3 M hydrochloric acid under the incubation in water bath at 84°C for 35 min before the solvent extraction. Therefore, cell lysis solvents were firstly optimized before the extraction of CoQ_{10} from *Artemia*. The cell lysis solvents, *i.e.*, DI water, 70% (v/v) ethanol, absolute ethanol, 0.1% (w/w) HCl, 9.2% (w/w) HCl and glacial acetic acid were studied.

The experiment was carried out as follows:

1) Weigh fresh *Artemia* 1.0 g to the nearest 0.001 g into a 60-mL test tube wrapped with aluminum foil for the protection from light.

2) Add 5.00 mL of cell lysis solvents as mentioned above into the sample.

3) Shake the sample gently and incubate it in water bath at 84°C for 35 min.

4) Allow the solution to cool at room temperature and vortex mix vigorously for 1 min.

5) Add 5.00 mL of ethanol and 10.00 mL of hexane as an extractant and vortex mix vigorously for 1 min.

6) Leave to equilibrate at room temperature. Transfer by pipette the upper phase into a 25-mL round bottom flask.

7) Repeat the extraction procedure given in step 5 to 6. Combine and evaporate extract to dryness by a rotary evaporator.

8) Re-dissolve the residue with 2.00 mL of ethanol. Filter the solution through 0.2 μm Nylon membrane before HPLC analysis.

2.9.2.2 The effect of acetic acid concentration on CoQ_{10} extraction

Although glacial acetic acid provided the best results, it would not be appropriate for the CoQ_{10} production when using a lot of raw material of *Artemia* due to its smell. Therefore, the concentrations of acetic acid were further studied.

The experiment was carried out as follows:

1) Weigh fresh *Artemia* 1.0 g to the nearest 0.001 g into a 60-mL test tube wrapped with aluminum foil.

2) Add 5.00 mL of concentrations of acetic acid at 5%, 25%, 50%, 75% and 100% (w/w) into the sample.

3) Shake the sample gently and incubate it in water bath at 84°C for 35 min.

4) Allow the solution to cool at room temperature and vortex mix vigorously for 1 min.

5) Add 10.00 mL of hexane and vortex mix vigorously for 1 min.

6) Add 5.00 mL of ethanol and shake the sample gently.

7) Leave to equilibrate at room temperature. Transfer by pipette the upper phase into a 25-mL round bottom flask.

8) Repeat the extraction procedure given in step 5 to 7. Combine and evaporate extract to dryness by a rotary evaporator.

9) Re-dissolve the residue with 2.00 mL of ethanol. Filter the solution through 0.2 μm Nylon membrane before HPLC analysis.

2.9.2.3 The effect of the ratio of 75% (w/w) acetic acid and sample weight on CoQ_{10} extraction

The acidity of the sample solution was crucial to achieve the effective cell lysis (Tian *et al*., 2010b). Therefore, the ratio of 75% (w/w) acetic acid and sample weight was further studied.

The experiment was carried out as same as topic 2.9.2.2 except step 2 which various volumes of 75% (w/w) acetic acid at 2.50, 5.00, 10.00 and 15.00 mL were added.

2.9.2.4 The effect of hexane volume on CoQ_{10} extraction

Hexane was commonly used for CoQ_{10} extraction from several tissues such as pelagic fish tissues (Souchet and Laplante, 2007), litchi pericarp (Rao *et al*., 2011) or palm oil (Han *et al*., 2006). Therefore, hexane was chosen for extracting CoQ_{10} from *Artemia* and the volume of hexane was further studied to increase the partition of CoQ_{10} to organic phase (Moldoveanu and David, 2002).

The experiment was carried out as same as topic 2.9.2.3 except step 2 which 5.00 mL of 75% (w/w) acetic acid was added and step 5 which various volumes of hexane at 2.50, 5.00, 10.00 and 15.00 mL were added.

2.9.2.5 The effect of ethanol volume on CoQ_{10} extraction

The most effective extraction was obtained when using ethanol coupled with hexane for CoQ_{10} extraction from pelagic fish, litchi pericarp, food materials (Souchet and Laplante, 2007; Rao *et al*., 2011 and Mattila *et al*., 2000) Therefore, volume of ethanol was further investigated.

The experiment was carried out as same as topic 2.9.2.4 except step 5 which 5.00 mL of hexane was added and step 6 which various volumes of ethanol at 0.00, 2.50, 5.00 and 10.00 mL were added.

2.9.2.6 The effect of incubation temperature on CoQ_{10} extraction

Tian *et al*. (2010b) reported that the highest CoQ_{10} yield from *Agrobacterium tumefaciens* cell was obtained by the extraction of sample at 84°C. However, high energy at 84°C was consumed. Therefore, the suitable incubation temperature for cell lysis of CoQ_{10} extraction from *Artemia* was further optimized.

The experiment was carried out as same as topic 2.9.2.5 except step 3 which the sample was incubated at various temperatures, *i.e.*, 30 (room temperature), 55, 75, 85 and 100°C in water bath for 35 min and step 6 which 5.00 mL of ethanol was added.

2.9.2.7 The effect of incubation time on CoQ_{10} extraction

The incubation time was studied to obtain the complete cell disruption. The experiment was carried out as same as topic 2.9.2.6 except step 3 which the sample was incubated at room temperature for several days, *i.e.*, 1, 3, 5 and 7 days.

2.9.2.8 The number of extraction on CoQ_{10} extraction

The efficiency of extraction can be increased when extracting repeatedly (Harvey, 2000). Therefore, the numbers of extractions, 2 and 3 times, were optimized to obtain the highest extraction efficiency. The experiment was carried out as same as topic 2.9.2.7 except step 3 which the sample was incubated at room temperature for 24 hours.

2.9.3 The stability of stock CoQ_{10} standard

The stability of stock CoQ_{10} standard was studied to check the shelf life of stock standard solution by observing the sensitivity of calibration curve in the range from 1.0 to 50.0 μg mL^{-1} of CoQ_{10}. The calibration curve was freshly prepared from a stock CoQ_{10} standard as described in section 2.7.1. Then each concentration was injected in duplicates into HPLC. All slopes of independent calibration curve were compared by ANOVA at 95% confidence level.

2.9.4 The stability of CoQ_{10} in *Artemia* crude extract

Each of three replicates of the CoQ_{10} extract was stored in a 2-mL amber vial at -20°C. The stability of CoQ_{10} extract was performed by injecting extract into HPLC at the interval of 7 days. The amount of CoQ_{10} was compared by ANOVA at 95% confidence level.

2.10 Method validation of CoQ_{10} extraction

2.10.1 Calibration curve

The concentrations of CoQ_{10} standard at 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 20.0, 30.0, 40.0 and 50.0 μg mL^{-1} were prepared for the linearity. Ten replicates were prepared for each concentration. The linear regression was plotted between known concentration of CoQ_{10} (x-axis) and peak area of CoQ_{10} obtained from HPLC

(y-axis). The assessment of linearity was expressed by the linear equation and the coefficient of determination (R^2) should be closed to 1.

2.10.2 Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) are the analyte concentration giving a signal equal to the blank or background signal plus three and ten standard deviations of blank, respectively. The LOD and LOQ were calculated as described in Equation 1.22 and 1.23 (Chapter 1), respectively.

2.10.3 Matrix effect

Matrix effect was studied by using a standard addition method (Miller and Miller, 2005). It involved the addition of CoQ_{10} standard in the concentration range of 1.0-50.0 µg mL^{-1} into the 500.0 µL of sample solution and ethanol was added to obtain 1000.0 µL of final solution volume. Three replicates of samples were performed. The evaluation of matrix effect was performed by using the regression line. The regression line was plotted between the concentration of CoQ_{10} standard from calibration curve on x-axis and the concentration of CoQ_{10} from standard addition curve on y-axis. If there is no matrix effect, the slope and intercept of regression line of both concentrations will cover 1 and zero, respectively at 95% confidential interval (CI).

2.10.4 Accuracy and precision

The accuracy of CoQ_{10} extraction was carried out by recovery method (EURACHEM, 1998). The recovery of CoQ_{10} was assessed by two methods. The first method was performed by spiking 450.0 µL of 20.0 µg mL^{-1} and 630.0 µL of 100 µg mL^{-1} CoQ_{10} standard into samples and making volume to 6.00 mL of sample solutions to obtain the final concentrations of CoQ_{10} at 1.5 and 10.5 µg mL^{-1}, respectively. The second method involved with the addition of CoQ_{10} standard in the concentration range of 1.0-50.0 µg mL^{-1} in sample solutions. The extraction procedure was performed as described in section 2.11.

The precision is expressed in the term of the percent relative standard deviation or coefficient of variation (CV) (Skoog *et al.*, 1996). The precision of the

CoQ_{10} analysis was studied by intra- and inter-day precisions. The intra-day precisions were obtained from the measurement of two concentration levels of CoQ_{10} at the same day and the inter-day precisions of two concentration levels of CoQ_{10} were determined for 5 days.

2.11 Determination of CoQ_{10} in *Artemia*

Fresh *Artemia* (1.0 g) was weighed to the nearest 0.001 g into a 60-mL test tube wrapped with aluminum foil for the protection from light. Then 5.00 mL of 75% (w/w) acetic acid was added. Sample was gently mixed and then incubated at room temperature for 24 hours. After that, sample was vigorously mixed by a vortex mixer for 1 min. The extraction was performed by adding 5.00 mL of hexane, followed by vigorous vortex mixing for 1 min and 5.00 mL of ethanol was added, followed by gentle shaking. After allowance to settle for equilibrium at room temperature, the upper phase was pipetted into a round bottom flask. The extraction of aqueous phase was repeated twice, and each extracted solution was combined and evaporated to dryness by a rotary evaporator. The residue was re-dissolved with 2.00 mL ethanol and filtered through 0.2 μm Nylon membrane before HPLC analysis.

2.12 Purification of CoQ_{10} extract

Aparted from the use of HPLC technique to determine CoQ_{10} in a crude sample, the matrix in crude extract will interfere the CoQ_{10} analysis due to the low concentration of CoQ_{10} in extract and it would not be appropriate to use for commercial products or industrial application. Therefore, the purification of CoQ_{10} in *Artemia* crude extract obtained from the optimal condition (section 2.11) was proposed by using an adsorption chromatography with silica gel used as an adsorbent. The silica gel column was prepared by transferring slurry of silica gel 100 in the mixture of 5% acetone and hexane into the glass column (30.0 cm × 2.0 cm i.d.). About 700 mg of dried crude extract was re-dissolved with hexane and the solution was passed through the column. The column was eluted with the gradient elution of acetone and hexane until the separation of CoQ_{10} was achieved. Each fraction was identified by thin-layer chromatography (TLC) which was the aluminum sheets of silica gel 60 GF_{254} and visualized under ultraviolet light. The yellow fraction

containing CoQ_{10} was combined and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was dissolved with dropwise addition of a little volume of hexane and methanol until the turbid solution was obtained. The solution was stood at room temperature until the crystal was obtained. Then the re-crystallization of CoQ_{10} was performed by re-dissolving the residue with ethanol at 40°C. The solution was cooled at 4°C until a crystal was formed. Purified CoQ_{10} was characterized by HPLC, ^{1}H NMR and mass spectroscopy. The separation efficiency of CoQ_{10} was expressed by %purity of CoQ_{10}, %HPLC purity and %recovery which were calculated as described in Equation 2.1, 2.2 and 2.3, respectively.

$$\text{Purity of } CoQ_{10} = \frac{C \times V \times 100}{wt \times 10^{6}} \qquad (2.1)$$

Where Purity of CoQ_{10} is % (w/w) purity of CoQ_{10} in crude extract or purified CoQ_{10}

C is concentration of CoQ_{10} ($\mu g\ mL^{-1}$) from a calibration curve

V is the final volume of a sample solution (mL)

wt is a weight of crude extract or purified CoQ_{10} (g)

$$\%\text{HPLC purity} = \frac{A}{B} \times 100 \qquad (2.2)$$

Where A is the peak area of CoQ_{10}

B is the sum of peak area of all components in HPLC chromatogram

$$\%\,\text{Recovery} = \frac{P}{E} \times 100 \qquad (2.3)$$

Where P is the amount of CoQ_{10} in purified CoQ_{10}

E is the amount of CoQ_{10} in crude extract

2.13 Preservation of CoQ_{10}

The preservation of CoQ_{10} has been performed since CoQ_{10} is sensitive to light. The use of cyclodextrin seems to be the promising method to preserve CoQ_{10} due to its simple and ease to operation. Moreover, the purification by using α- and γ-cyclodextrins increases considerably the cost of production (Astray *et al*., 2009). Thus, the *β*-cyclodextrin was chosen to preserve CoQ_{10} for long time storage (Fir *et al*., 2009). The preservation of CoQ_{10} with *β*-cyclodextrin was assessed by using the accelerated condition with UV light.

2.13.1 Preparation of inclusion complex between CoQ_{10} and *β*-cyclodextrin

A preparation of inclusion complex was modified from Fir *et al*. (2009). A whole experiment should be protected from light by wrapping with aluminum foil. A complex in the ratio of 1:1 molar ratio of CoQ_{10} and *β*-cyclodextrin was prepared. *β*-Cyclodextrin (1.5 g) was weighed into a 50-mL beaker, and 15.0 mL of DI water was added. The sample was stirred at 80°C to obtain a clear solution. A 1.122 g of CoQ_{10} was dissolved with 20.0 mL of hexane. The *β*-cyclodextrin solution and CoQ_{10} solution were mixed by stirring at 80°C until the formation of the yellow homogeneous paste was obtained. The outer CoQ_{10} in complex was washed with hexane at 4°C. The complex was kept in a refrigerator until the requirement for the stability test.

2.13.2 Determination of CoQ_{10} in complex

About 20 mg of complex was dissolved with 10.0 mL ethanol. The sample solution was sonicated to obtain a clear solution. The solution was transferred to a 25-mL volumetric flask and adjusted to the mark with ethanol. The sample was filtered through 0.2 µm Nylon membrane and diluted by pipetting 250.0 µL sample solution into a 1.5-mL amber vial and 250.0 µL of ethanol was added. The CoQ_{10} content was analyzed by HPLC condition as mentioned in section 2.5.1. The CoQ_{10} content in complex was calculated as described in Equation 2.4.

$$\text{Conc.}(\%, \text{w/w}) = \frac{\text{conc.}\left(\mu\text{g mL}^{-1}\right) \times \text{V} \times \text{dilution factor} \times 100}{1 \times 10^{6} \times \text{wt}} \qquad (2.4)$$

Where Conc. (%, w/w) is the concentration of CoQ_{10} in complex

Conc. (µg mL^{-1}) is the concentration of CoQ_{10} from a calibration curve

V is the final volume of a complex solution (mL)

wt is a weight of complex (g)

Dilution factor is $\frac{0.50}{0.25} = 2$

2.13.3 Photostability test

The stability of CoQ_{10} was undertaken under low-pressure mercury vapour fluorescent lamps emitting long wave UV radiation between 350-400 nm in a homemade stability test chamber. The distance between the sample and light source was 10-15 cm (Fir *et al*., 2009). Figure 2.2 shows the set up of photostability test for CoQ_{10}.

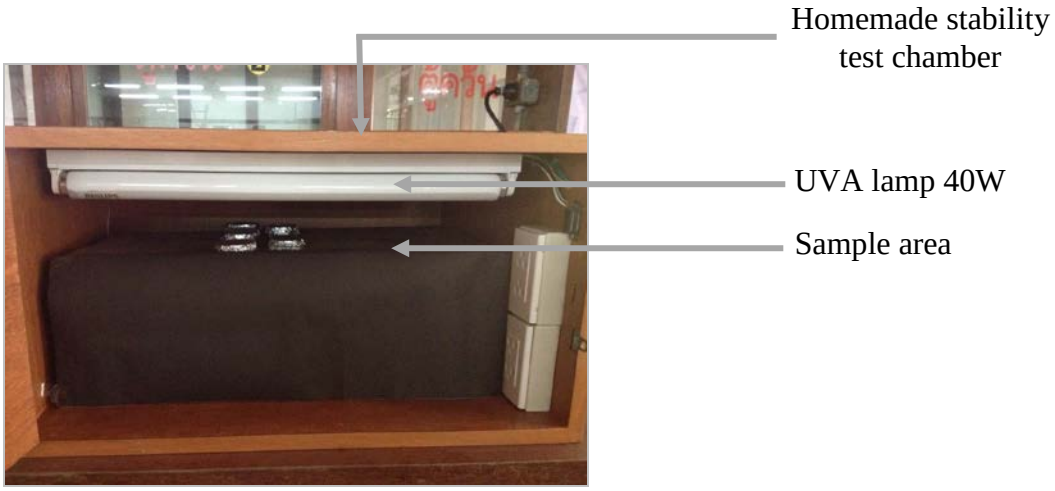


Figure 2.2 Photostability test set up.

2.13.3.1 Preparation of free CoQ_{10} as control

a) Non-UV exposure of free CoQ_{10}

There were 2 sets of CoQ_{10} investigated in this experiment. Fifteen petri dishes were set up. The first set of three petri dishes was added with 300.0 µL of

3.3 mg mL^{-1} of free CoQ_{10} in hexane, air dried and wrapped with aluminum foil for the protection from light. Each of the first set of free CoQ_{10} was exposed under UVA light for 30 min. The distance between the free CoQ_{10} and light source was about 10 cm. The second, third, fourth and fifth set of replicates were treated in the same way as the first set except that each set was exposed for 1, 2, 4 and 8 hours, respectively. After exposure of the various periods, all petri dishes were removed and the remaining CoQ_{10} was analyzed as described in section 2.13.3.3.

b) UV exposure of free CoQ_{10}

The experiment was performed as the same way as the method described in section 2.13.3.1 (a) but no petri dishes were wrapped with aluminum foil.

2.13.3.2 Preparation of complex

a) Non-UV exposure of complex

Fifteen petri dishes were set up. The first set of three petri dishes was spread with 0.0115 g of complex and wrapped with aluminum foil for the protection from light. Each of the first set of complex was exposed under UVA light for 30 min. The distance between the complex and light source was about 10 cm. The second, third, fourth and fifth set of replicates were treated in the same way as the first set except that each set was exposed for 1, 2, 4 and 8 hours, respectively. After exposure of the various periods, all petri dishes were removed and the remaining CoQ_{10} was analyzed as described in section 2.13.3.3

b) UV exposure of complex

The experiment was performed as the same way as the method described in section 2.13.3.2 (a) but no petri dishes were wrapped with aluminum foil.

2.13.3.3 Determination of remaining CoQ_{10}

After the end of certain exposure, the remaining CoQ_{10} was examined. Either CoQ_{10} in standard or complex was rinsed by adding about 2 mL of ethanol from the petri dish twice. The solution was transferred to a 25-mL volumetric flask, sonicated for 10 min to obtain a clear solution and adjusted to the mark with ethanol.

The solution was filtered through 0.2 µm Nylon membrane. The remaining CoQ_{10} in solution was analyzed by HPLC condition described in section 2.5.1. The remaining of CoQ_{10} after UVA exposure was calculated as described in Equation 2.5.

$$\text{Remaining } CoQ_{10} = \frac{C_t}{C_0} \qquad (2.5)$$

Where C_t is the concentration of CoQ_{10} (%, w/w) after UVA exposed for t hours

C_0 is the initial of the concentration of CoQ_{10} (%, w/w)

2.13.3.4 The rate of photodegradation of CoQ_{10}

The relationship between the remaining CoQ_{10} and exposed time were plotted. The rate constant and the half life of CoQ_{10} ($t_{1/2}$) can be calculated as mentioned in section 1.5.4 (Chapter 1).

CHAPTER 3
RESULTS AND DISCUSSION

The determination of CoQ_{10} from *Artemia* has not been found. Therefore, the aim of this thesis was to study of the CoQ_{10} extraction from *Artemia* by using acid-treatment followed by solvent extraction. The content of CoQ_{10} was analyzed by means of HPLC-DAD. Consequently, the purification of CoQ_{10} was studied to obtain the purified form before applying for industry and the purified CoQ_{10} was preserved with *β*-cyclodextrin for long time storage before manufacturing.

3.1 Selection of the HPLC conditions

The reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection has been commonly used for determination of CoQ_{10} due to high selectivity and sensitivity (Karpińska *et al*., 2006). The important HPLC conditions for the separation of CoQ_{10} such as detector, column and mobile phase were considered. The structure of CoQ_{10} is a hydrophobic compound containing benzoquinone ring and 10 units of isoprenoid chain and can absorb UV light at 275 nm (Figure 3.1). Mattila *et al*. (2000) compared in-line connected diode array (DAD) and electrochemical (ECD) detectors for HPLC analysis of coenzymes Q_9 and Q_{10} in food materials. They found that the LOD from ECD was 20-fold more sensitive than DAD. However, ECD was less selective than DAD since it required the purification step. Therefore, DAD was chosen as the HPLC detector in this work and set at 275 nm for the analysis of CoQ_{10}. Figure 3.1 shows the spectrum of CoQ_{10} standard obtained by UV-VIS spectrophotometry.

Carbon 18 (C18) stationary phase has been used for RP-HPLC analysis of CoQ_{10} due to the hydrophobicity of CoQ_{10} (Cao *et al*., 2006 and Xue *et al*., 2012). In this work, the Agilent ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm i.d., 5 µm thickness) was available and used for the separation of CoQ_{10}. The mobile phase systems obtained from literature reviews were evaluated to obtain a rapid separation of CoQ_{10}. The three mobile phase systems including methanol:ethanol

(5:95 v/v), 2-propanol:ethanol:methanol (35:24:41 v/v/v) and methanol:hexane (83:17 v/v) were compared for the optimization of the separation of CoQ_{10} extract (Cao *et al*., 2006; Souchet and Laplante, 2007 and Zhong *et al*., 2009), and chromatographic term obtained from each system was considered. The chromatographic term and the typical HPLC chromatogram of CoQ_{10} in *Artemia* extract under three mobile phases were shown in Table 3.1 and Figure 3.2, respectively. The chromatographic term of all mobile phase systems provided complete separation with symmetric value and resolution being closed to reference value of 1 and more than 1, respectively (Skoog *et al*., 1996 and Weiss, 2004). The results showed that all mobile phase systems provided good separation with no signals caused by other substances in the matrix interfered at the signal peak of CoQ_{10}. The mobile phase of methanol:ethanol (5:95 v/v) was suitable for the separation because of its short analysis time. In addition, ethanol is less toxic when compared with hexane and 2-propanol. Therefore, the mixture of methanol and ethanol at 5:95 (v/v) was chosen as the suitable mobile phase in this work.

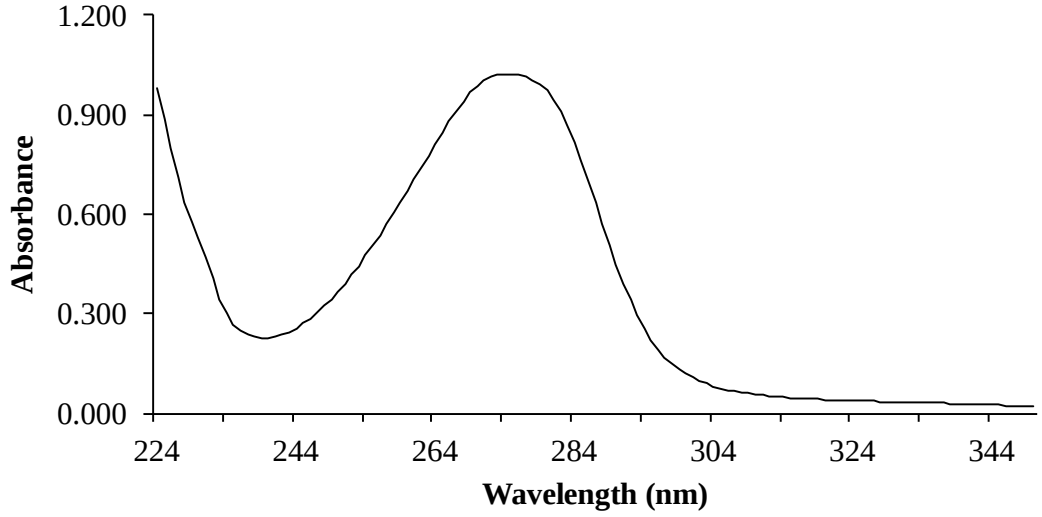


Figure 3.1 The spectrum of CoQ_{10} standard at 50 µg mL^{-1} in absolute ethanol

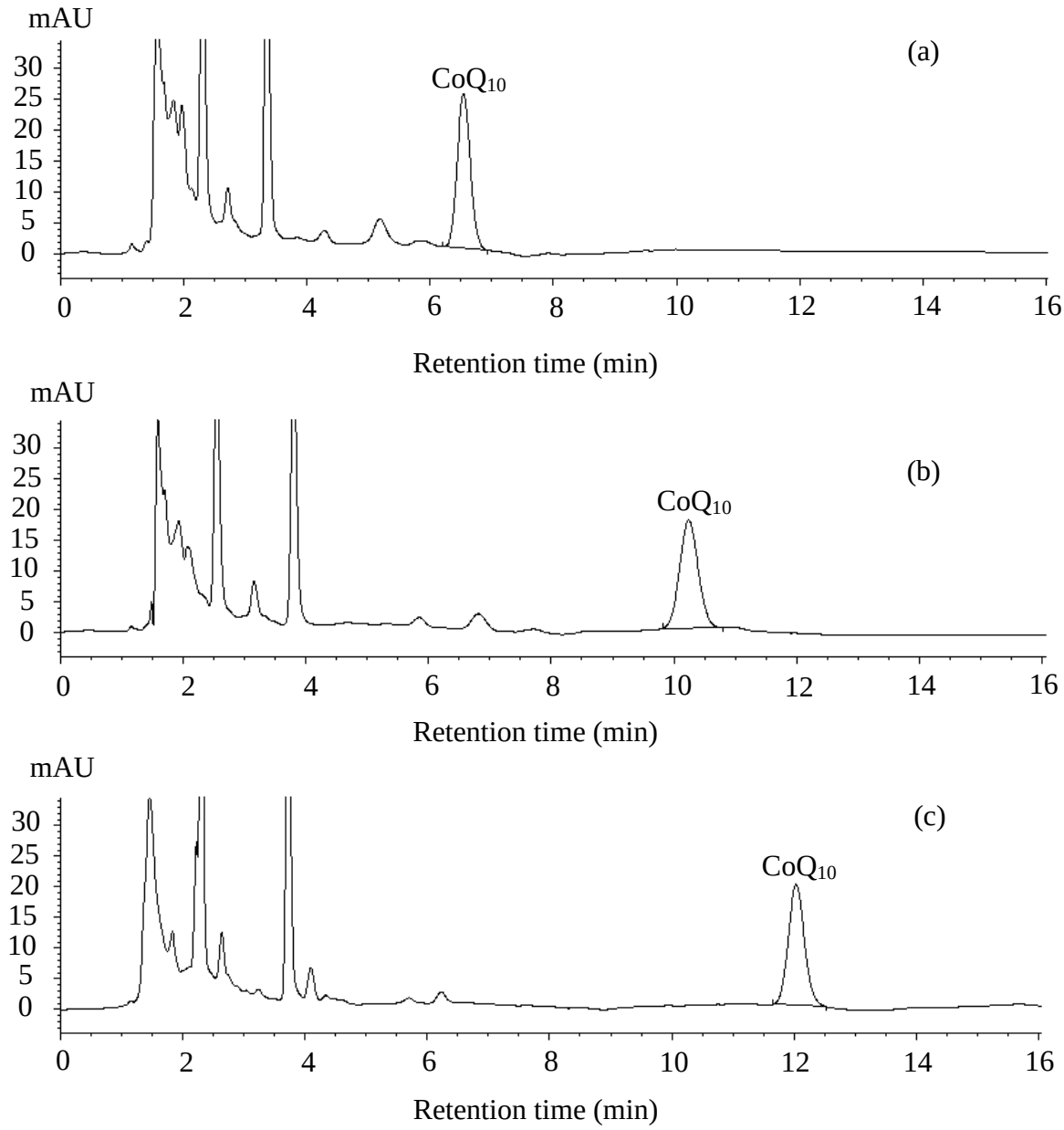


Figure 3.2 The typical HPLC chromatogram of CoQ_{10} at 20.0 µg mL^{-1} in *Artemia* extracted under the three mobile phase systems: (a) methanol:ethanol (5:95 v/v), (b) 2-propanol:ethanol:methanol (35:24:41 v/v/v) and (c) methanol:hexane (83:17 v/v).

Table 3.1 Chromatographic term of CoQ_{10} under three mobile phase systems

Mobile phase	Retention time (min)	Symmetry[a]	Resolution[b]
Methanol:ethanol (5:95, v/v)	6.531±0.002	0.89±0.01	1.57±0.04
2-Propanol:ethanol:methanol (35:24:41, v/v/v)	10.239±0.019	0.93±0.01	6.99±0.04
Methanol:hexane (83:17, v/v)	12.056±0.063	0.91±0.01	15.60±0.10

[a] and [b] calculated from equation 1.5 and 1.4, respectively (Chapter 1, page 12 and 13)

3.2 Optimization for extraction of CoQ_{10} from *Artemia*

The solid-liquid extraction was used for CoQ_{10} extraction from *Artemia*. The good extraction efficiency depends on the partition between sample and solvent. Since CoQ_{10} was found in intracellular part, therefore, the cell lysing step in order to release CoQ_{10} from cell membrane is necessary (Tian *et al*., 2010b). The studied parameters for extraction efficiency were the type of cell lysis solvent, the acetic acid concentration, the ratio of 75% (w/w) acetic acid and sample weight, the volume of hexane, the volume of ethanol, the incubation temperature, the incubation time and the number of extraction. The yield of CoQ_{10} in μg g^{-1} based on dry weight of *Artemia* was used for considering the performance of extraction. The recovery of CoQ_{10} was considered for the number of extraction. The *Artemia* sample for optimization of extraction method was obtained from various *Artemia* lots due to the pilot study of the cultured *Artemia*.

3.2.1 The effect of cell lysis solvent on CoQ_{10} extraction

Tian *et al.* (2010b) studied the cell lysis of *Agrobacterium tumefaciens* and found that the highest content of CoQ_{10} was obtained when 3 M hydrochloric acid (HCl) was used under the incubation temperature at 84°C for 35 min before solvent extraction. *Artemia* cell is not complicated, therefore, the cell lysis method for *Artemia* was also studied by using other solvents including HCl as mentioned by Tian *et al*. (2010b). To increase the yield of CoQ_{10} from *Artemia*, various types of solvents, *i.e.*, DI water, 70% and 100% (v/v) ethanol, 0.1% and 9.2% (w/w) HCl and glacial

acetic acid were studied for cell lysis. As shown in Figure 3.3, the highest of CoQ_{10} content was obtained by glacial acetic acid, followed by 9.2% (w/w) HCl and absolute ethanol since they could gently lyse cell membrane of *Artemia* to release CoQ_{10}. Low amount of CoQ_{10} was observed when using 0.1% (w/w) HCl and DI water. This result was probably caused by polarity and acid strength. Glacial acetic acid is more polar than ethanol, resulting in high efficient disruption of *Artemia* cell. Although hydrochloric acid is more acidic than glacial acetic acid, the highest yield of CoQ_{10} was obtained by glacial acetic acid because non-polar part of glacial acetic acid can contact and penetrate cell membrane easily (Ni *et al*., 2008). Based on the mentioned fact, glacial acetic acid was chosen as a cell lyser of *Artemia* cell membrane.

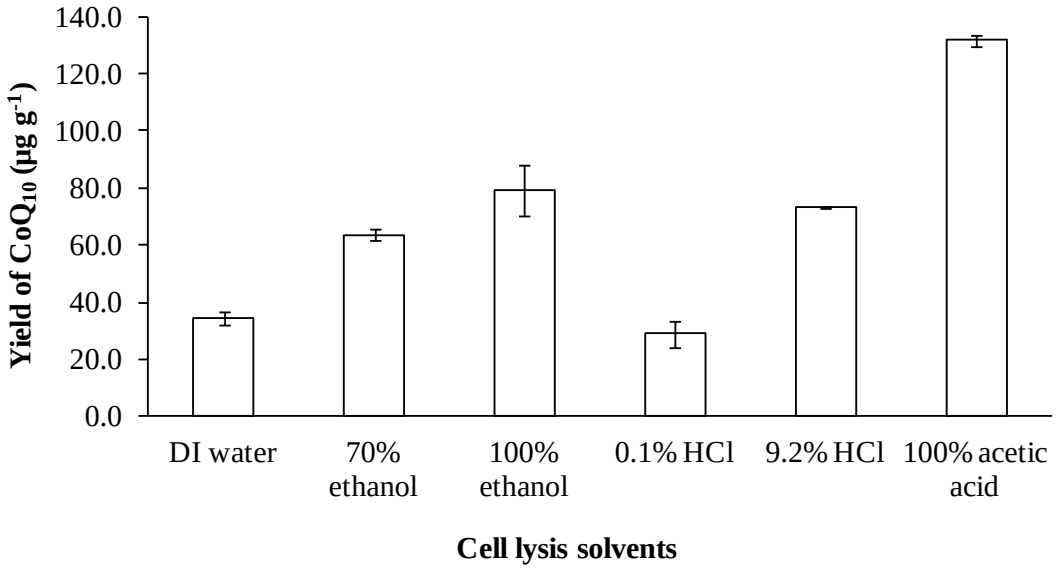


Figure 3.3 The effect of cell lysis solvents on the CoQ_{10} extraction (n=4)

3.2.2 The effect of acetic acid concentrations on CoQ_{10} extraction

Although glacial acetic acid provided the best result, it would not be appropriate for the CoQ_{10} production when working with big scale of raw material of *Artemia* due to glacial acetic acid smell. Therefore, 5.00 mL of the concentrations of acetic acid were studied in the range between 5-100% (w/w). As shown in Figure 3.4, the concentration of acetic acid lower than 75% (w/w) provided the incomplete separation between organic phase and aqueous phase, resulting in low extraction efficiency. The problem was solved by adding 5 mL of DI water as mentioned by

Hagerman *et al.* (2001) or ethanol with gentle shaking to increase the separation of two phases. The separation was clearly obtained when ethanol was used because ethanol can dissolve lipid of *Artemia*. The yield of CoQ_{10} increased and reached the highest when 75% (w/w) acetic acid was used. After that, its yield was found to be constant. Therefore, 75% (w/w) acetic acid was chosen in this work.

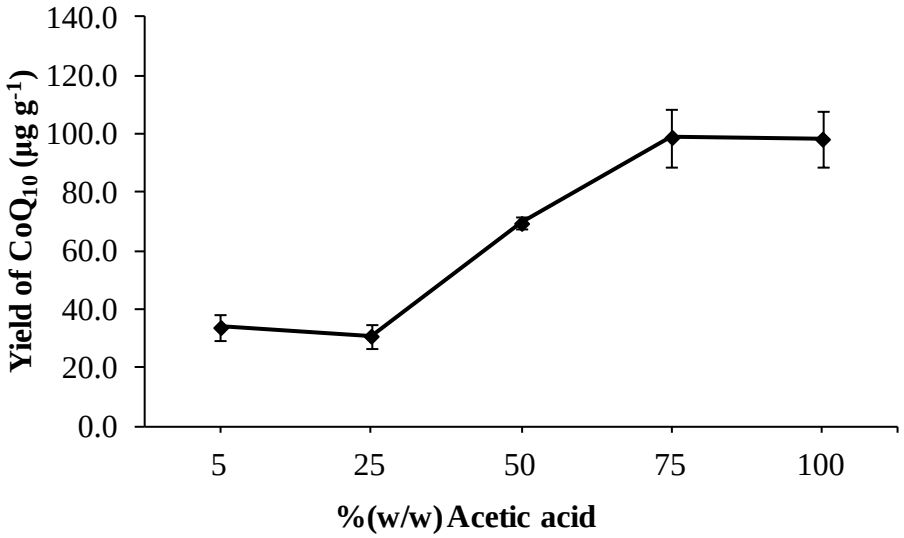


Figure 3.4 The effect of acetic acid concentrations on the CoQ_{10} extraction (n=4)

3.2.3 The effect of the ratio of 75% (w/w) acetic acid and sample weight on CoQ_{10} extraction

The extraction efficiency depends on the partition of CoQ_{10} into solvent and acid sample cell lysis. Tian *et al.* (2010b) mentioned that the acidity of the sample was most critical in achieving the effective cell lysis. Therefore, the ratios of 75% (w/w) acetic acid from 2.50 to 15.00 mL and one gram of fresh *Artemia* were investigated. As shown in Figure 3.5, the yield of CoQ_{10} was significantly increased when increasing the ratio from 2.50 to 5.00 mL g^{-1} due to complete contact between cell lysis solvent and sample and was constant at the ratio of 10.00 mL g^{-1}. The ratio of acid to fresh *Artemia* at 15.00 mL g^{-1} provided the poor separation between organic solvent and aqueous phase, resulting in low yield of CoQ_{10} and high variation of analysis. Therefore, the ratios of 75% (w/w) acetic acid and sample weight at 5.00 and 10.00 mL g^{-1} were considered. Tian *et al.* (2010b) reported that the highest yield of CoQ_{10} from *Agrobacterium tumefaciens* cell was obtained with the ratio of acid

solution volume and dried cells of bacteria at 10.00 mL g^{-1}. However, the ratio of 75% (w/w) acetic acid and sample weight at 5.00 mL g^{-1} was chosen for cell lysis of wet *Artemia* in order to reduce the use of solvent.

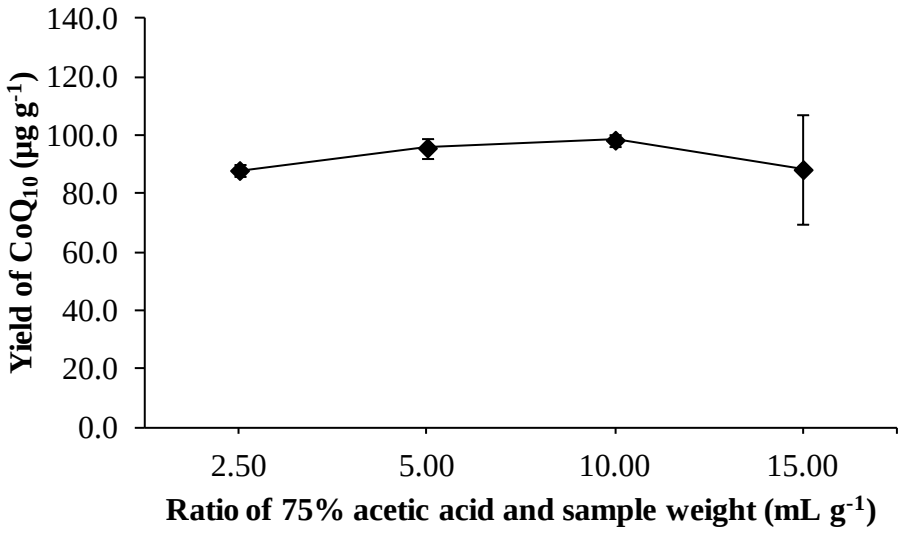


Figure 3.5 The effect of the ratios of 75% (w/w) acetic acid and sample weight (mL g^{-1}) on the CoQ_{10} extraction (n=4)

3.2.4 The effect of hexane volume on CoQ_{10} extraction

Hexane has been used as an extractant because it can dissolve CoQ_{10} (Ondarroa *et al*., 1986) and highly evaporate when concentrating the extract. It has also been commonly used for CoQ_{10} extraction of pelagic fish tissues (Souchet and Laplante, 2007), litchi pericarp (Rao *et al*., 2011), palm oil (Han *et al*., 2006) or tobacco leaves (Zu *et al*., 2006). The volumes of hexane ranged between 2.50 to 15.00 mL were studied for extracting CoQ_{10} from *Artemia*. The result is shown in Figure 3.6. The lowest yield of CoQ_{10} was obtained when using 2.50 mL of hexane because the ratio of organic solvent and aqueous phase was not suitable. The yield of CoQ_{10} increased with the volume of hexane up to 5.00 mL and was constant until 15.00 mL. Five milliliters of hexane has been reported by Souchet and Laplante (2007) for the extraction of CoQ_{10} from pelagic fish. Therefore, 5.00 mL of hexane was chosen in this work for extracting CoQ_{10} from *Artemia.*

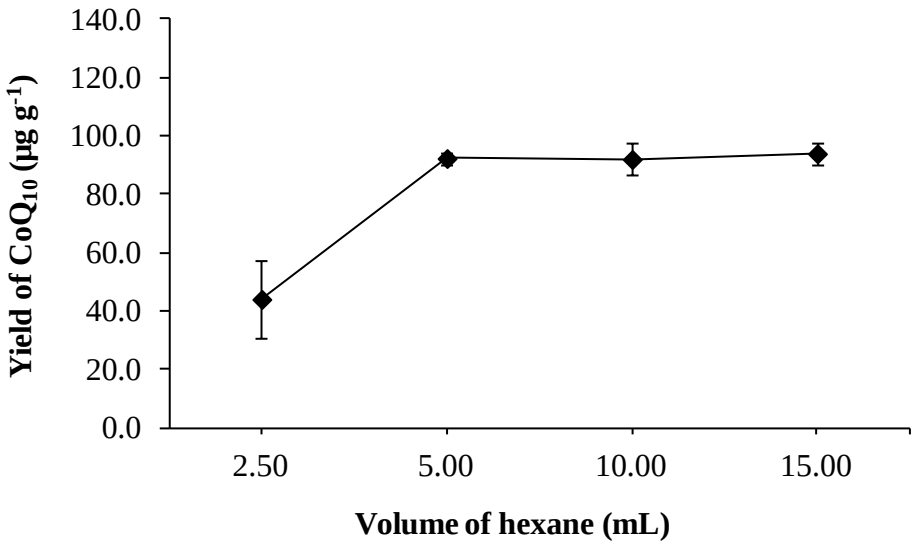


Figure 3.6 The effect of hexane volume on the CoQ_{10} extraction (n=4)

3.2.5 The effect of ethanol volume on CoQ_{10} extraction

Ethanol was used for the protein precipitation in animal tissues (Souchet and Laplante, 2007) and as the co-solvent for increasing the solubility of CoQ_{10}, resulting in high extraction efficiency (Ondarroa *et al*., 1986). Additionally, ethanol can penetrate through the wet cell membrane (Ni *et al*., 2008). Mattila *et al*. (2000) and Souchet and Laplante (2007) used ethanol and hexane for CoQ_{10} extraction from food and animal tissue. Moreover, Zu *et al*. (2006) used methanol or absolute ethanol for extraction of CoQ_{10} from fresh tobacco leave and found that absolute ethanol provided more extraction efficiency than methanol. Thus, ethanol was used in this work. As shown in Figure 3.7, the yield of CoQ_{10} was slightly increased with increasing volume of ethanol up to 5.00 mL. Morover, it was found that when ethanol was used in the extraction of CoQ_{10} from *Artemia*, the HPLC signal of other components at the retention time ranged between 1.0-4.0 min decreased (Figure 3.8). This showed that ethanol could promote the solubility of CoQ_{10} in hexane phase. The volume of ethanol up to 10.00 mL provided the low extraction efficiency with poor separation because the ratio of organic solvent and aqueous phase was not suitable. Therefore, 5.00 mL of ethanol was chosen for the optimal extraction.

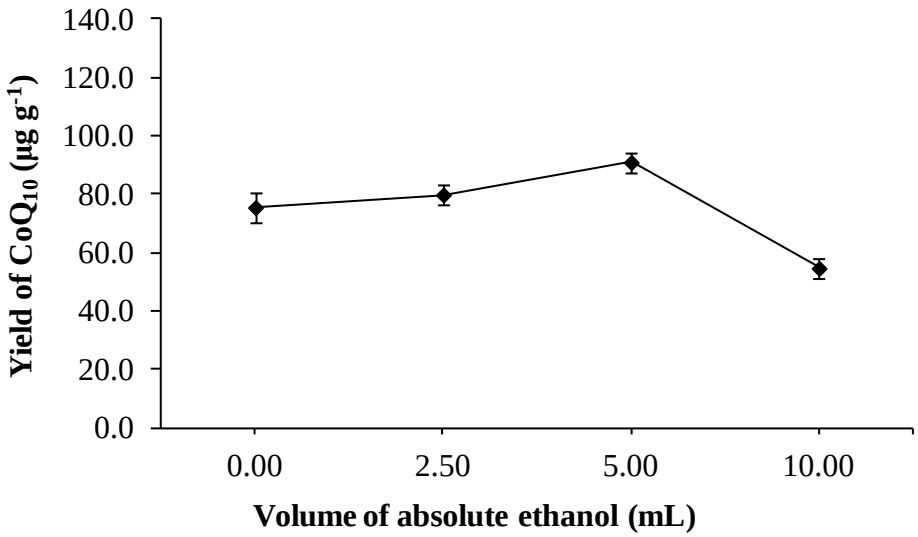


Figure 3.7 The effect of ethanol volume on the CoQ_{10} extraction (n=4)

3.2.6 The effect of incubation temperature on CoQ_{10} extraction

Tian *et al.* (2010b) studied the cell lysis of *Agrobacterium tumefaciens* and found that the highest content of CoQ_{10} was obtained when using 3 M hydrochloric acid (HCl) under the incubation at 84°C for 35 min before the solvent extraction. The heat treatment can lyse cell structure to release of intracellular components. The incubation temperatures at room temperature to 100°C were studied for extraction of CoQ_{10} from *Artemia*. As shown in Figure 3.9, the yield of CoQ_{10} obtained from the extraction at room temperature was not significantly different from that obtained from the extraction using increased incubation temperature. Therefore, the incubation temperature at room temperature was chosen for this study with low energy consumption and the reduction of extraction step.

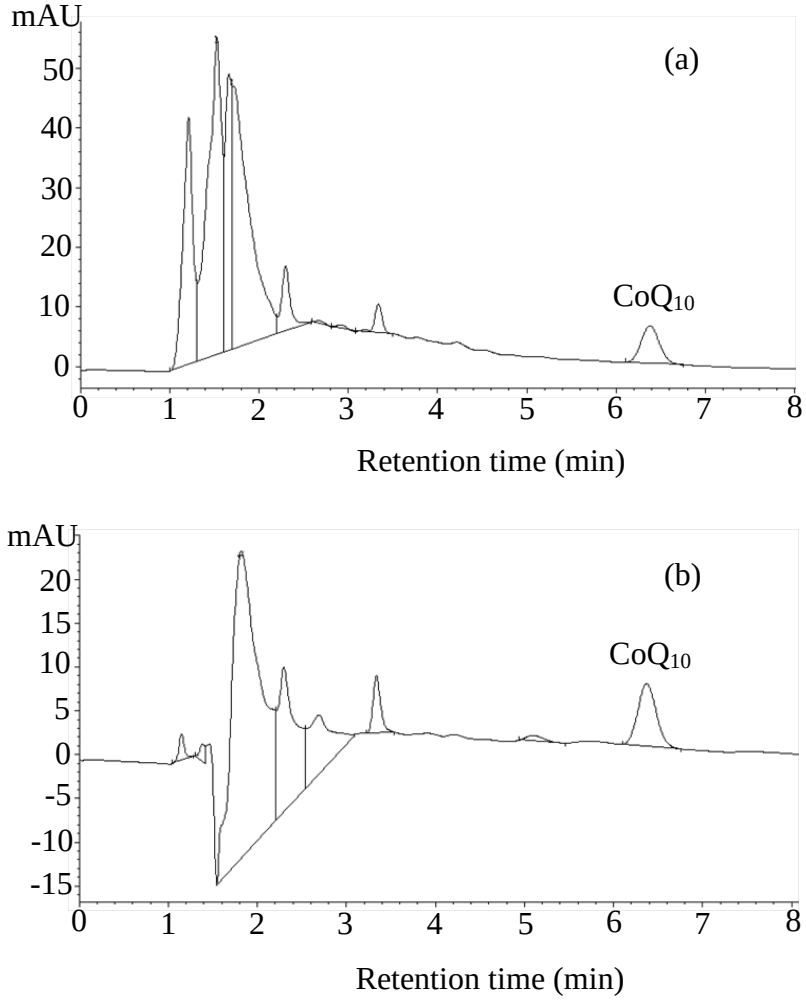


Figure 3.8 The HPLC chromatogram of CoQ_{10} in *Artemia* extract obtained by (a) without adding ethanol and (b) adding 5.00 mL of ethanol

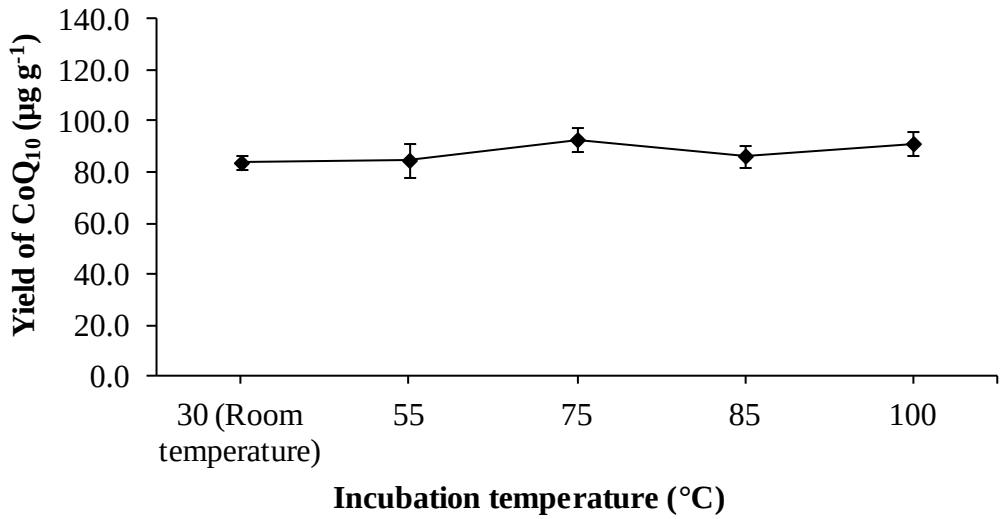


Figure 3.9 The effect of incubation temperatures on the CoQ_{10} extraction (n=4)

3.2.7 The effect of incubation time on CoQ_{10} extraction

Preliminary test of the incubation at room temperature for 35 min and overnight was performed. The CoQ_{10} content from *Artemia* was significantly increased after incubated for overnight (100.7±5.0 µg g^{-1} dry weight) when compared to room temperature for 35 min (83.8±2.9 µg g^{-1} dry weight). Therefore, the incubation time for cell lysis was further studied in the range of 1 to 7 days to obtain the highest content of CoQ_{10} from *Artemia*. The result showed that the yields of CoQ_{10} were not significantly different when using different incubation times (Figure 3.10). However, the trend of yield of CoQ_{10} decreased when time increased. Therefore, the further study should involve with the incubation time in the range of 35 min to overnight. In this study, the incubation time of 24 hours was chosen for the optimal condition.

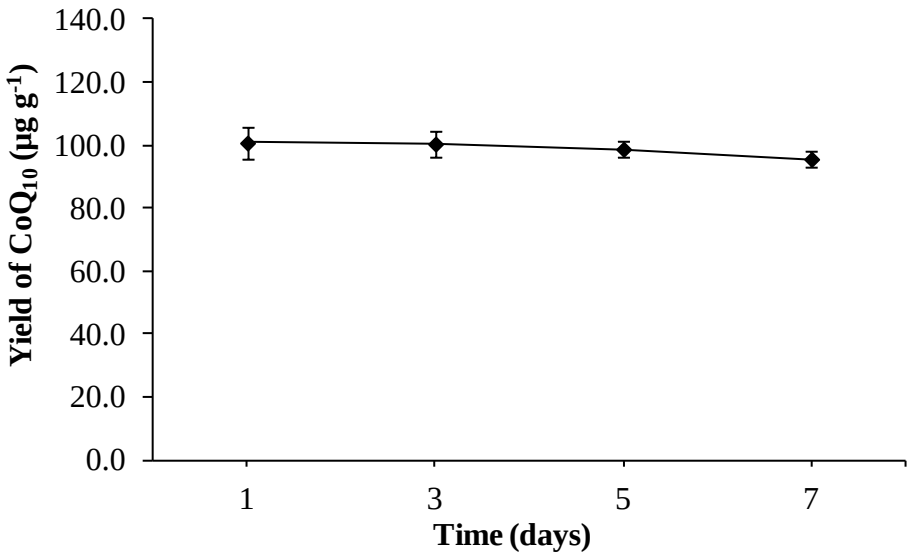


Figure 3.10 The effect of incubation time on the CoQ_{10} extraction (n=4)

3.2.8 The number of extraction on CoQ_{10} extraction

The number of extraction was studied with the recovery of CoQ_{10} by spiking 10.5 µg mL^{-1} of CoQ_{10} standard into a sample. The good recovery at 95.2±2.7% was obtained with extraction number of 3 times (Figure 3.11). This result was similar to results obtained for the extraction of CoQ_{10} from other biological samples including tobacco leaves (Zu *et al*., 2006) and food sample (Pyo and Oh, 2011). Therefore, the number of extraction of 3 times was chosen for the optimum condition of CoQ_{10} extraction from *Artemia*.

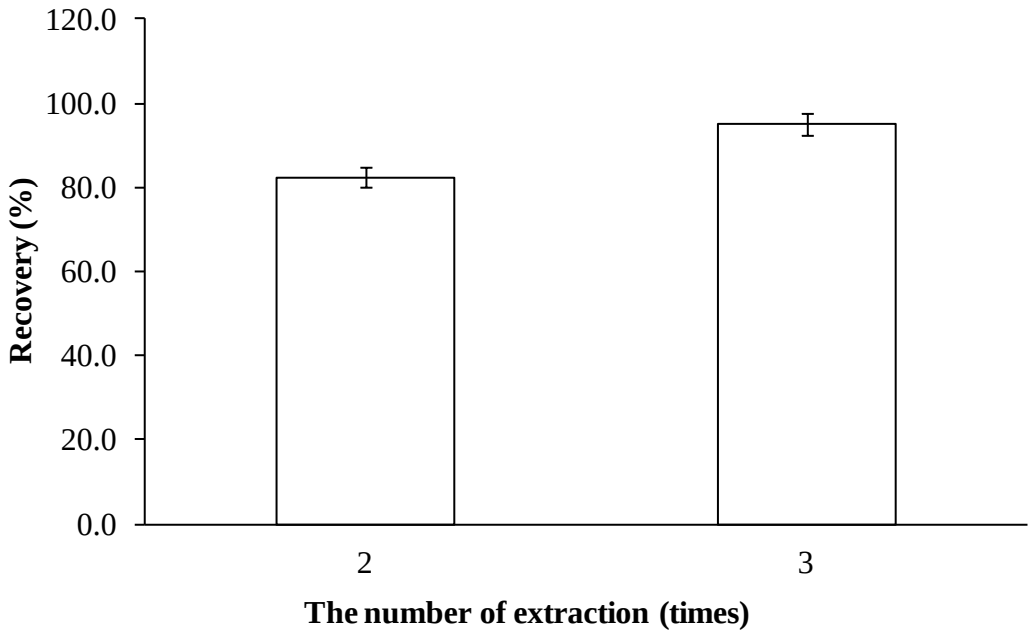


Figure 3.11 The numbers of extraction on the recovery of CoQ_{10} (n=6)

3.3 The stability of stock CoQ_{10} standard

The stock of CoQ_{10} standard in ethanol was stored at -80°C (Kubo *et al*., 2008 and Pyo and Oh, 2011) or -40°C (Souchet and Laplante, 2007) before analysis. The stability of stock CoQ_{10} standard at -20°C was assessed by using the slope values of independent calibration curve of CoQ_{10} in the range of 1.0 to 50.0 µg mL^{-1} for 3 months. As shown in Figure 3.12, the slope values of 12 calibration curves were in the range from 19.6205 and 20.2916 which were not significantly different at 95% confidence level. Therefore, the stock of CoQ_{10} standard can be kept for at least 3 months at -20°C.

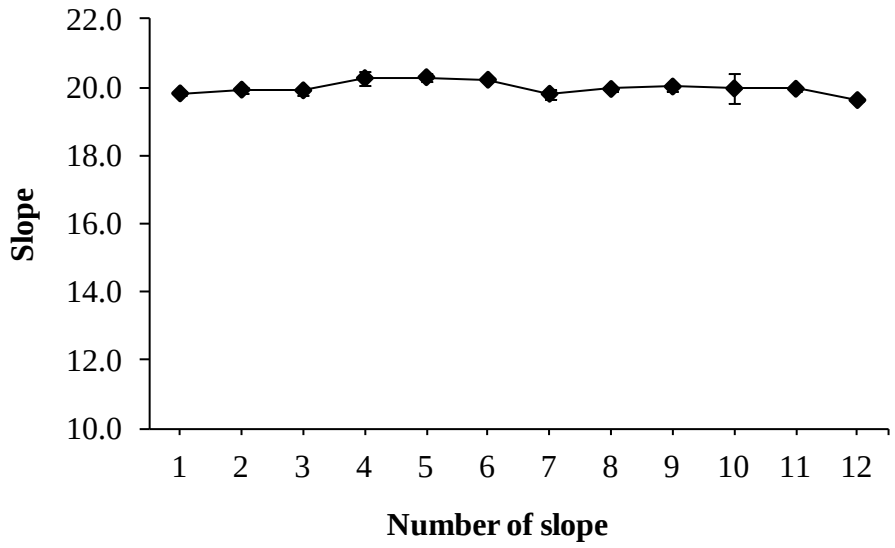


Figure 3.12 Slope of calibration curve of CoQ_{10} observed for 3 month

3.4 The stability of CoQ_{10} in *Artemia* crude extract

Artemia samples were extracted as mentioned in section 2.11 and the extracts were stored at -20°C before HPLC analysis. The stability of CoQ_{10} in crude extract was investigated for 15 weeks and the peak area of the remaining CoQ_{10} with time was used for data analysis. As shown in Figure 3.13, the percentage of peak area of remained CoQ_{10} was stable within 4 months when stored at -20°C. Therefore, it is suggested that crude extract could be stored at -20°C for at least 4 months to prolong the shelf life of CoQ_{10}.

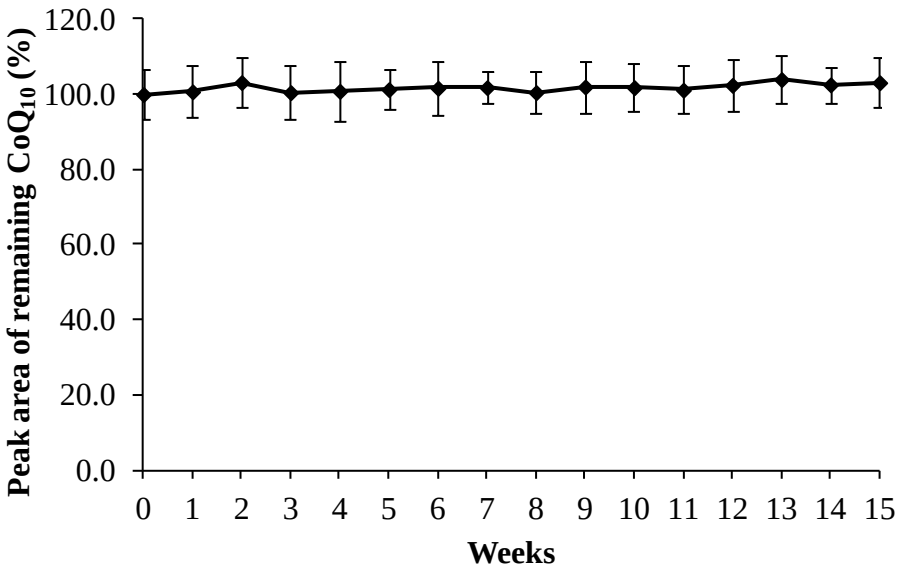


Figure 3.13 Stability of CoQ_{10} in *Artemia* crude extract

3.5 Method validation of CoQ_{10} extraction

The performance of method including the calibration curve, LOD, LOQ, matrix effect, accuracy and precision were validated for the determination of CoQ_{10} in *Artemia* extract.

3.5.1 Calibration curve

The calibration curve was the relationship between peak area of CoQ_{10} obtained from HPLC and concentration of CoQ_{10} which covered the concentration of CoQ_{10} in the sample solution. As shown in Figure 3.14, the calibration curve of CoQ_{10} was found to be linear in the concentration range of 1.0-50.0 μg mL^{-1} with a good correlation coefficient of 1.000.

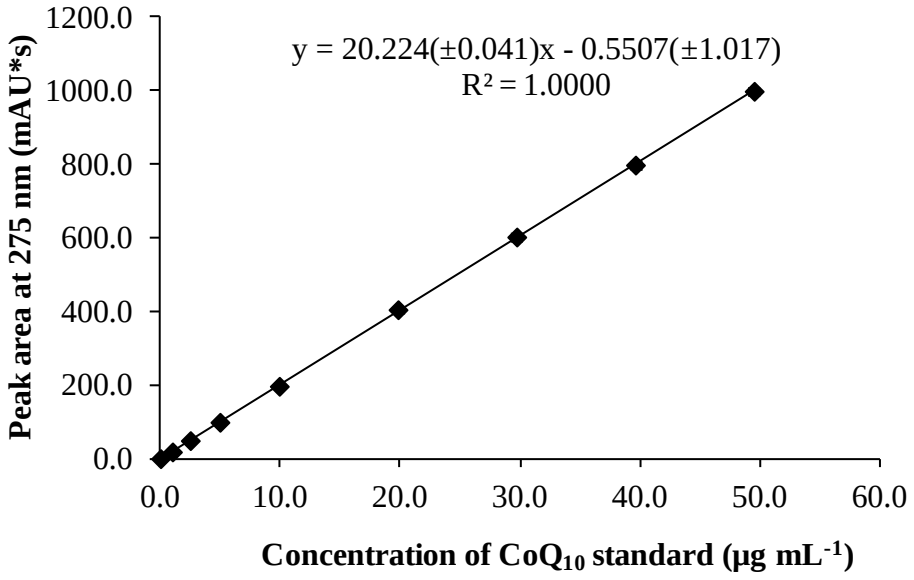


Figure 3.14 Calibration curve of CoQ_{10} in the range of 1.0-50.0 µg mL^{-1}

3.5.2 Limit of detection (LOD) and limit of quantification (LOQ)

The calculation of limit of detection (LOD) and limit of quantification (LOQ) using the linear equation of y = 20.229x - 0.726 were calculated as presented in Table A1. Therefore, the LOD was 0.3 µg mL^{-1} at the signal-to-noise ratio (S/N) of 3 while LOQ was 1.1 µg mL^{-1} at S/N of 10.

3.5.3 Matrix effect

The matrix effect was achieved by using the method of standard addition (Miller and Miller, 2005). The concentration of CoQ_{10} ranged from 1.0-50.0 µg mL^{-1} was determined by HPLC analysis. The relationship between the concentration of CoQ_{10} from standard addition assigned to the y-axis and the concentration of CoQ_{10} from calibration curve assigned to the x-axis was shown in Figure 3.15. The linear equation of this relationship was y = 0.9996(±0.0068)x - 0.0038(±0.1725) with the coefficient of determination (R^2) of 0.9997±0.3640. The use of *t* value for the degree of freedoms (*t*=2.36) gives the 95% confidence levels for the slope and intercept as follows:

$$b = 0.9996 \pm (2.36 \times 0.0068) = 0.9836 - 1.0156$$

$$a = (-0.0038) \pm (2.36 \times 0.1725) = (-0.4109) - 0.4033$$

From these values, it is shown that the calculated slope and intercept did not different significantly from ideal values of 1 and 0, respectively (Miller and Miller, 2005). Therefore, the matrix did not affect the analysis of CoQ_{10} in real sample and the calibration curve was used to quantify CoQ_{10} in *Artemia* in this work.

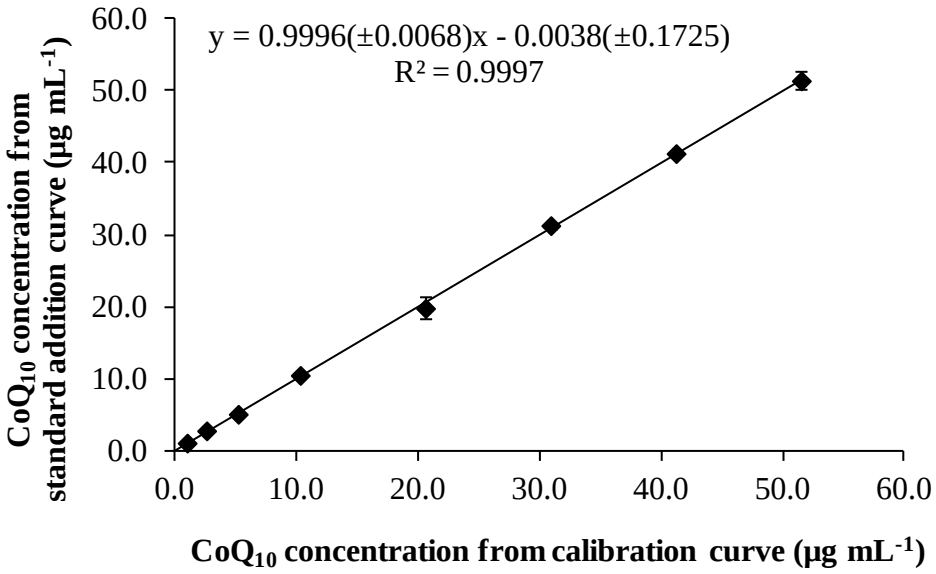


Figure 3.15 The relationship between CoQ_{10} concentration (µg mL^{-1}) from calibration curve (x-axis) and CoQ_{10} concentration (µg mL^{-1}) from standard addition curve (y-axis)

3.5.4 Accuracy and precision

The reliability of the development of CoQ_{10} extraction from *Artemia* was obtained by using accuracy and precision. The recovery of CoQ_{10} was determined by spiking CoQ_{10} standard at 1.5 and 10.5 µg mL^{-1} to the *Artemia* sample. Mean recoveries of CoQ_{10} ranged from 96% to 100% for intra-day accuracy (Table 3.2) and 94% for inter-day accuracy (Table 3.3). Moreover, the recovery of CoQ_{10} in the concentration range of 1.0-50.0 µg mL^{-1} was found to be in the range from 97-115% (Table 3.4).

The precision of the analysis of CoQ_{10} at 1.5 and 10.5 µg mL^{-1} was studied by intra- and inter-day precisions and expressed in term of CV. The CV values ranged from 0.5-9.1% for intra-day precision and 4.3-8.5% for inter-day precision (Table 3.2 and 3.3, respectively). These CV values obtained are acceptable within the range mentioned by AOAC (2002).

Table 3.2 Recovery and intra-day precision of the extraction of CoQ_{10} at 1.5 and 10.5 µg mL^{-1} spiked into *Artemia* sample (n=6)

Concentration of CoQ_{10} spiked (µg mL^{-1})	**% Recovery (mean±SD)**	**% CV**
1.5	96.1±8.7	9.1
10.5	99.6±0.5	0.5

Table 3.3 Recovery and inter-day precision of CoQ_{10} at 1.5 and 10.5 µg mL^{-1} spiked into *Artemia* sample within 5 days (n=30)

Concentration of CoQ_{10} spiked (µg mL^{-1})	**% Recovery (mean±SD)**	**% CV**
1.5	93.8±8.0	8.5
10.5	94.3±4.0	4.3

Table 3.4 Recovery and intra-day precision of CoQ_{10} in the concentration range of 1.0-50.0 µg mL^{-1} spiked into *Artemia* sample solution (n=10)

Concentration of CoQ_{10} spiked (µg mL^{-1})	**% Recovery (mean±SD)**	**% CV**
1.0	115.1±12.3	10.7
2.6	108.7±2.4	2.2
5.2	96.7±2.0	2.0
10.3	97.8±0.9	1.0
20.6	97.5±7.5	7.7
30.9	100.9±1.3	1.2
41.2	99.8±0.2	0.2
51.5	99.2±2.5	2.5

3.6 Determination of CoQ_{10} in *Artemia*

The quantitative analysis of CoQ_{10} in this thesis initially focused on samples obtained from the Ecological Aquaculture Research Unit at Department of Technology and Industry, Faculty of Science and Technology, Prince of Songkla University, Pattani campus. The moisture, protein and fat contents of this sample were found in the range of 89-92%, 52-62% and 6-7%, respectively. These values indicated that *Artemia* is as high source of protein and fat as that reported by Léger *et al*. (1987). The developed extraction method for the determination of CoQ_{10} provided the mean concentration of CoQ_{10} in *Artemia* at 236±29 μg g^{-1} on a dry weight basis which was comparable to the CoQ_{10} content in the fermented food such as fish and shrimp reported by Pyo and Oh (2011) (Table 3.5). This means that *Artemia* can be the good source of CoQ_{10}. Other reports of concentration of CoQ_{10} detected in many kinds of samples were reported in Table 3.5. The differences on concentration may be influenced by sample types or the fermentation process. Heart tissues of pelagic fish contained the high content of CoQ_{10} due to its high abundance in mitochondria (Mattila *et al*., 2000 and Souchet and Laplante, 2007). Moreover, the fermentation process by diverse microorganisms during manufacture might affect the production of CoQ_{10} in food (Pyo and Oh, 2011).

The amounts of CoQ_{10} from *Artemia* in this study are found to be highly variable probably because of many factors such as the types of food for culture of *Artemia* and age of *Artemia*.

Table 3.5 CoQ_{10} content in various samples detected by other studies

Sample	**CoQ_{10} content (μg g^{-1} dry weight)**	**Reference**
Artemia	177-303	This work
Pork heart	63.4[a]	Mattila *et al*., 2000
Tobacco leaves	9.9-15.1	Zu *et al*., 2006
Heart tissues of pelagic fish	105.5-148.4[a]	Souchet and Laplante, 2007

Table 3.5 CoQ_{10} content in various samples detected by other studies (continued)

Sample	CoQ_{10} content ($\mu g\ g^{-1}$ dry weight)	Reference
Soybean oils	97.6	Rodríguez-Acuna *et al*., 2008
Japanese food		Kubo *et al*., 2008
- Meat	3.24-107[a]	
- Fish and shellfish	0.29-106[a]	
- Vegetables	0.08-6.34[a]	
Fermented food based on		Pyo and Oh, 2011
- soybean	13.1-122.4	
- fish and shrimp	293.8	
- Vegetables	98.4	
Litchi pericarp tissues	90-134	Rao *et al*., 2011
Bee pollen	ND-192.8	Xue *et al*., 2012

[a] wet weight

ND is not detectable

3.7 Purification of CoQ_{10} extract

The separation of CoQ_{10} in *Artemia* from matrices was successful by reversed phase HPLC, however, the CoQ_{10} purity from HPLC was quite low about 28% and the use of instrument to purify CoQ_{10} would increase the cost of analysis. The purification of CoQ_{10} by silica gel column chromatography followed by recrystallization has been proposed before the CoQ_{10} production in the industry. Commonly, CoQ_{10} was purified by using normal phase adsorbents such as silica gel (Hagerman *et al*., 2001 and Cao *et al*., 2006) or reverse phase adsorbents such as amino-propyl type (Rodríguez-Acuna *et al*., 2008). However, silica gel is 13 or 20 times cheaper than the amino-propyl type or C18-RP type, respectively (http://www.sigmaaldrich.com/singapore.html, accessed January 22, 2012). Moreover, silica gel has advantages such as simple operation and ability to separate chemicals with similar characters and structures (Zhang *et al*., 2012). Therefore, the purification of the CoQ_{10} in crude extract was performed by using the silica gel

column chromatography. Preliminary TLC assays were performed to obtain a CoQ_{10} fraction free of other constituents. The intense yellow color was checked and it was found that the best elution system for silica gel chromatography was a mixture of acetone and hexane (5:95, v/v). The yellow fraction containing CoQ_{10} was collected, evaporated to dryness under a gentle stream of nitrogen at 40°C and re-crystallized by ethanol. A 4.6 mg of a yellow crystal as the purified CoQ_{10} was obtained from the crude extract of 100 g wet *Artemia* (10 g dry weight) and the purified CoQ_{10} was characterized by spectrophotometry (Figure 3.16), HPLC (Figure 3.17), mass spectrometry (Figure 3.18) and ^{1}H NMR (Figure 3.19). The separation efficiency of CoQ_{10} was expressed by % purity of CoQ_{10}, % HPLC purity and % recovery, as shown in Table 3.6. The amount of purified CoQ_{10} obtained in this study was comparable to the study by Ikeda and Kagei (1979). Ikeda and Kagei (1979) reported the purification of CoQ_{10} by silica gel chromatography of plant tissue cultures, in which the yellow crystals of CoQ_{10} were 6.7, 3.2, 3.3 and 3.1 mg obtained from 100 g dry rice cells, soybean cells, peanut cells and Ruta cells, respectively. Therefore, silica gel chromatography can be practically used for purification of CoQ_{10} from *Artemia*.

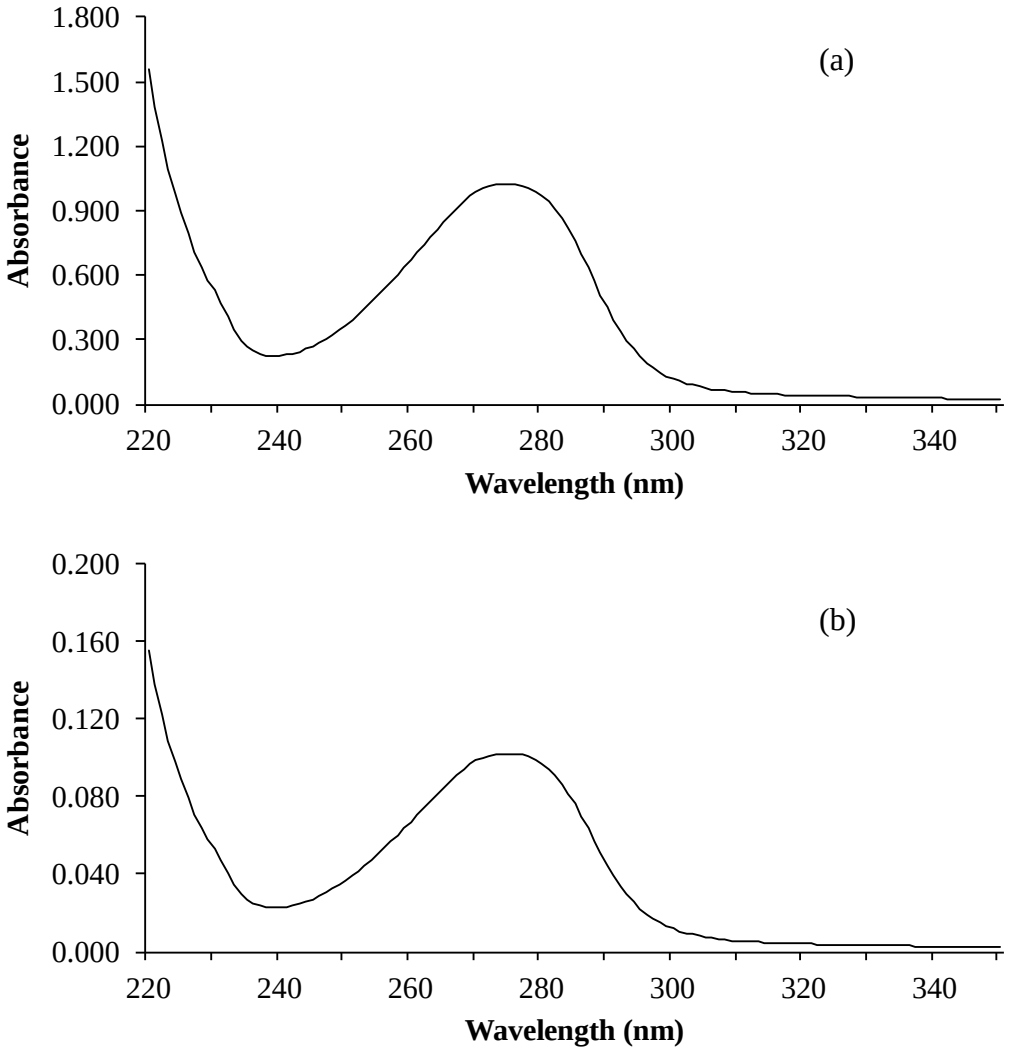


Figure 3.16 The spectra of (a) CoQ_{10} standard and (b) purified CoQ_{10} in ethanol

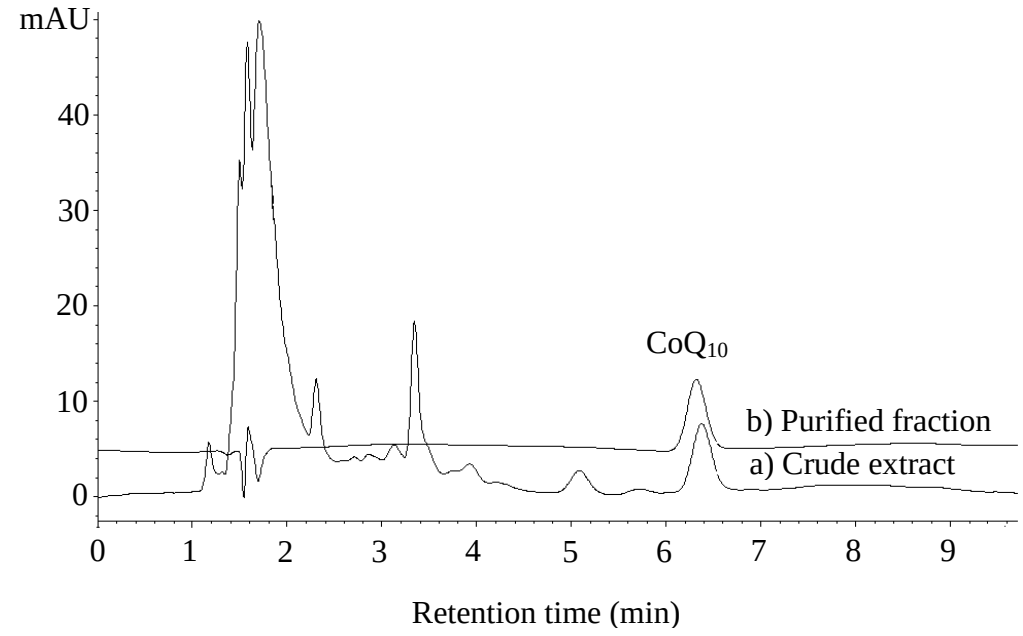


Figure 3.17 HPLC chromatogram of CoQ_{10} in (a) crude extract and (b) purified fraction by DAD detection at 275 nm

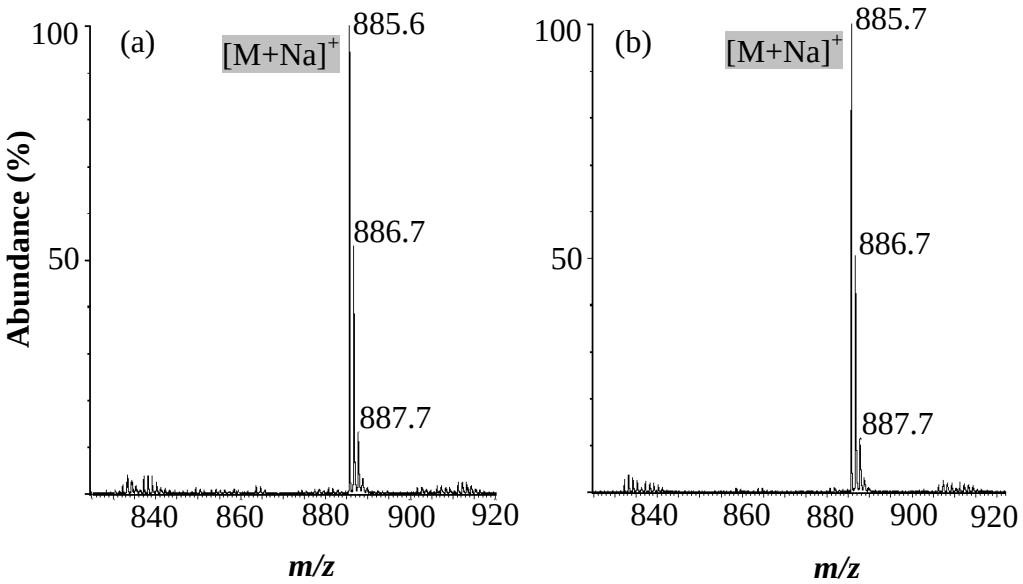
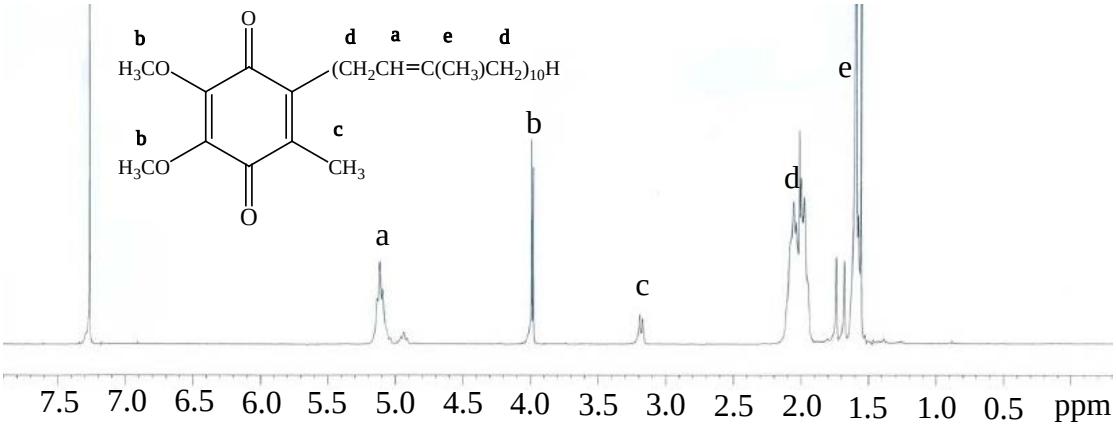
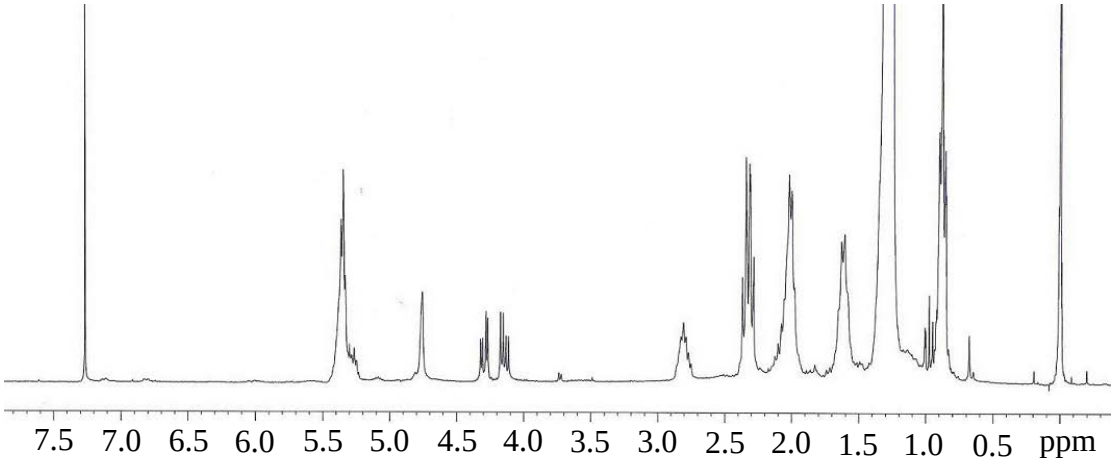


Figure 3.18 ESI^+ mass spectra of CoQ_{10} identified in (a) CoQ_{10} standard and (b) purified CoQ_{10}. Molecular weight (M) of CoQ_{10} is 863.34 g mol^{-1}.

(a) CoQ_{10} standard



(b) Crude extract



(c) Purified CoQ_{10}

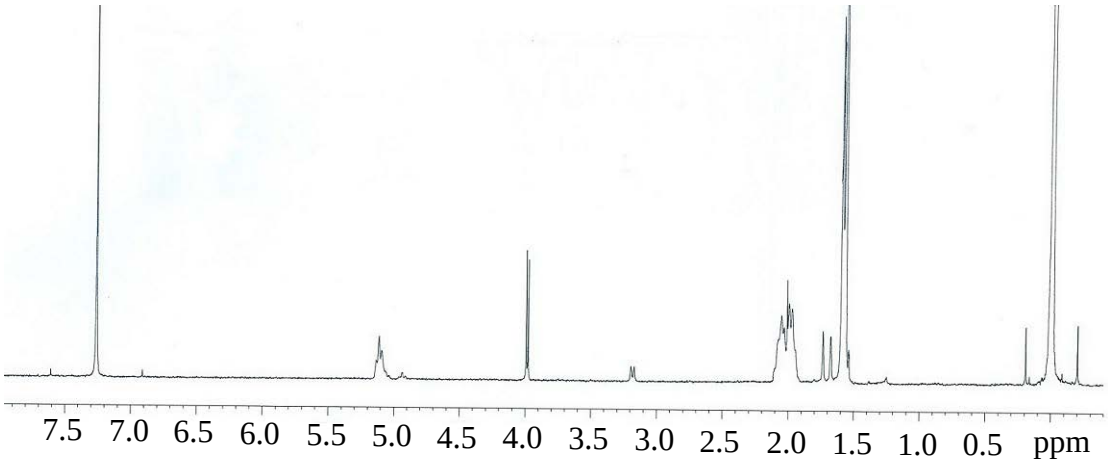


Figure 3.19 ^{1}H NMR spectra of (a) CoQ_{10} standard, (b) *Artemia* crude extract and (c) purified CoQ_{10} in crude extract by column chromatography

Table 3.6 The separation efficiency of CoQ_{10} by silica gel chromatography with subsequent crystallization

Parameter	Crude extract	Purified CoQ_{10}
Purity (%)	0.07	65.1
HPLC purity (%)	28.4	100.0
Recovery (%)	-	55.0

3.8 Preservation of CoQ_{10}

CoQ_{10} is sensitive to light (EP 4, 2002) and may degrade during shipping and storage processes. The encapsulation of CoQ_{10} in *β*-cyclodextrin can improve photostability of CoQ_{10} (Yang and Song, 2006 and Fir *et al*., 2009). Therefore, the preservation of CoQ_{10} with *β*-cyclodextrin before manufacturing will be further studied. A *β*-cyclodextrin is commonly used for the stabilization of flavours in food industry (Astray *et al*., 2010), the improvement of photostability of drug in pharmaceutical industry (El-Kemary *et al*., 2011 and Loftsson and Brewster, 1996). A *β*-cyclodextrin is a truncated-cone polysaccharide made up of seven D-glucose monomers linked by α-1,4-glucose bonds which exhibits a hydrophobic central cavity and a hydrophilic outer surface (Miyamoto *et al*., 2009 and Gu *et al*., 2011). Yang and Song (2006) reported that an inclusion complex technique for CoQ_{10} with *β*-cyclodextrin can improve the photostability and sensitivity for CoQ_{10} in samples. Consequently, *β*-cyclodextrin was chosen for a study of the preservation of CoQ_{10} in *Artemia* crude extract with the inclusion complex technique. Water is the most common solvent used. However, in case of lipophilic guest that making complexation slow, either ethanol or hexane was used to dissolve CoQ_{10} for the inclusion complex (Del Valle, 2004 and Astray *et al*., 2009). The CoQ_{10} content in complex was determined by HPLC when using ethanol and hexane as described in section 2.13.2. It revealed that CoQ_{10} content in the complex was 5.5% and 8.6% (w/w) for ethanol and hexane, respectively. Therefore, hexane was chosen since it was able to dissolve CoQ_{10} more than ethanol.

The photostability study of CoQ_{10} encapsulated within *β*-cyclodextrin was accelerated by the application of UVA light. The degradation profile of CoQ_{10}

between remaining CoQ_{10} and exposure time was shown in Figure 3.20. Non-UV exposed complex and non-UV exposed free CoQ_{10} were used as control samples to ensure the degradation depending on a UVA light. Both of control samples were found to be stable while the signals from both UV exposed complex and UV exposed free CoQ_{10} were found to be decreasing significantly during 8 hours of exposure. During 0.5-2 hours of exposure time, UV exposed free CoQ_{10} was sharply decreased while UV exposed complex gradually decomposed. After 8 hours, the percentage of remaining CoQ_{10} of complex is 15% higher than that of free CoQ_{10}. Figure 3.21 and Figure 3.22 show the first and second kinetics pattern of CoQ_{10} in complex and free CoQ_{10}, respectively. As shown in Figure 3.21, the photodegradation related between $\ln(C_o/C_t)$ and exposure time for complex fitted a pseudo first order kinetics. The half life of CoQ_{10} in complex was 2.29 hours. For the degradation of free CoQ_{10}, it presented the second order kinetics with the regression line shown in Figure 3.22 and the half life of free CoQ_{10} of 0.85 hours.

The half life of CoQ_{10} in complex was 3 times more than free CoQ_{10}. From these results, the inclusion complex formation between CoQ_{10} and *β*-cyclodextrin was suitable for the improvement of photostability of CoQ_{10} in *Artemia*.

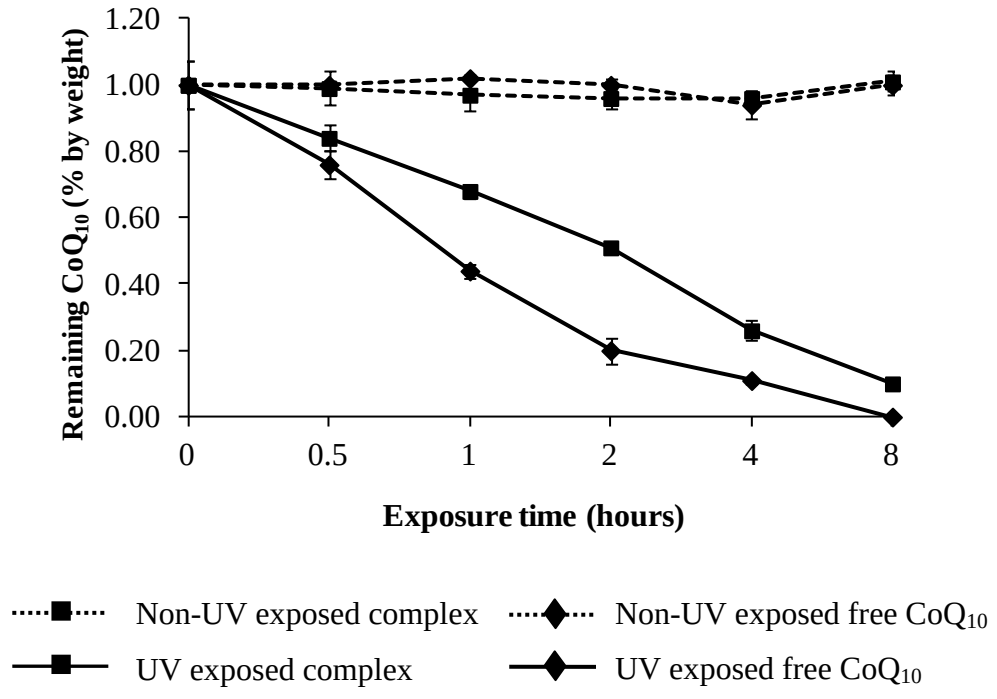


Figure 3.20 Effect of *β*-cyclodextrin on the photostability of CoQ_{10}

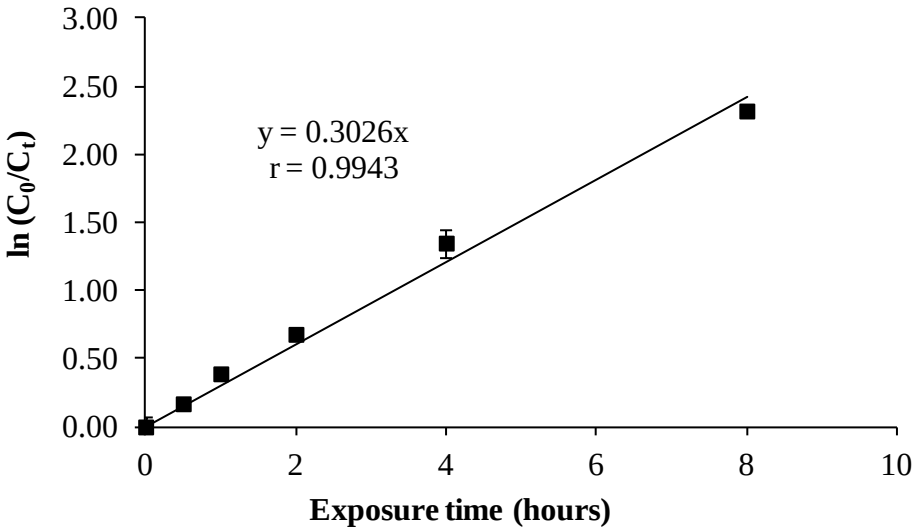


Figure 3.21 The relationship between $\ln(C_0/C_t)$ and exposure time of complex

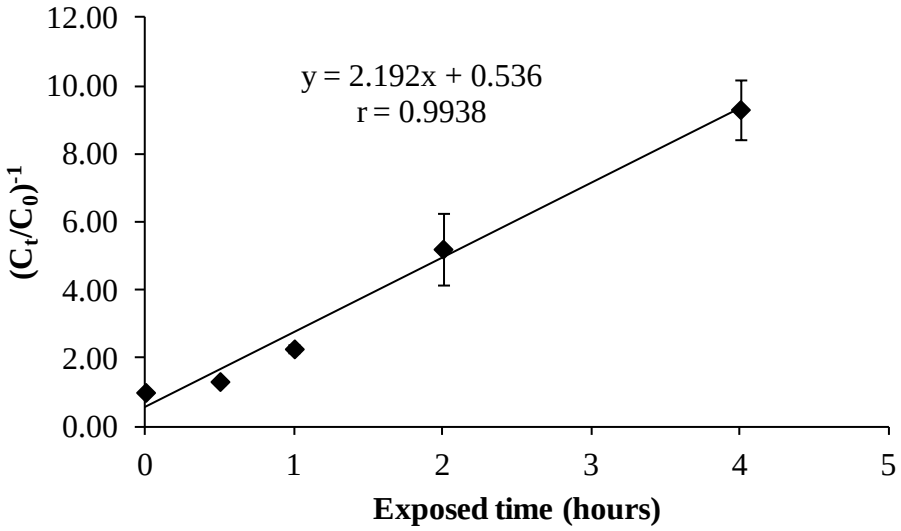


Figure 3.22 The relation of (remained CoQ_{10})$^{-1}$ and exposure time of free CoQ_{10}

CHAPTER 4
CONCLUSIONS

4.1 Overall conclusions

The main objectives of this research were to investigate the separation of CoQ_{10} from *Artemia* and preservation of CoQ_{10}. The extraction of CoQ_{10} was studied on the yield of CoQ_{10} with the several effects including the cell lysis solvents, the concentrations of acid, the ratio of cell lysis solvent and sample weight, volume of hexane as an extractant, volume of ethanol, the incubation temperature, the incubation time and the number of extraction. The optimal condition of CoQ_{10} extraction was obtained by incubating one gram of sample with 5.00 mL of 75% (w/w) acetic acid at room temperature for overnight, followed by extraction three times with 5.00 mL of hexane and 5.00 mL of ethanol to increase the separation of aqueous phase and organic phase. The determination of CoQ_{10} by high-performance liquid chromatography with diode array detection (HPLC-DAD) was carried out by XDB C18 column for separation of CoQ_{10} which was eluted by the mixture of methanol and ethanol (5:95, v/v). The detection was set at 275 nm.

This method was found to be reliable with the linear calibration curve at the concentration ranged of 1.0-50.0 μg mL^{-1}. The limit of detection and limit of quantification were achieved at 0.3 and 1.1 μg mL^{-1}, respectively. The concentrations obtained from the calibration curve and these obtained from standard additions were identical. Thus, it was no matrix effect and the calibration curve was used for the quantification of CoQ_{10} in *Artemia*. The mean recovery was achieved from 94 to 100% with the intra- and inter-day precisions of 0.5-9.1%. Under these optimal conditions, CoQ_{10} could be extracted in the range from 177 to 303 μg g^{-1} dry weight of *Artemia*. Compared to the study by Tian *et al*. (2010b), the developed method was found to be simple, low toxic solvent use, low energy consuming, no special requirement of instruments and efficient extraction procedure for CoQ_{10} extraction from *Artemia*. This extraction method could be a practical method for industrial practice.

The purity of CoQ_{10} in crude extract was low, resulting in the inappropriate form for commercial products or industrial application. Therefore, the purification of CoQ_{10} was performed by silica gel column chromatography and the elution with the mixture of acetone and hexane (5:95, v/v). Subsequently, the re-crystallization of CoQ_{10} was performed by ethanol. This method can increase the purity of CoQ_{10} from 0.07 to 65% in purified CoQ_{10}. The purification of CoQ_{10} method provided low cost consuming and simple operation.

During shipping and storage process, CoQ_{10} might be degraded under light. The preservation of CoQ_{10} was investigated by inclusion complex of CoQ_{10} with β-cyclodextrin with the photostability test. The complex and free CoQ_{10} were tested under UVA light for 8 hours. The photostability of CoQ_{10} with β-cyclodextrin was improved three times against free CoQ_{10}. This preservation method of CoQ_{10} was found to be easy and could be used for food industry and pharmaceutical manufacturing.

4.2 Recommendations

1) The incubation time in the range from 35 min to overnight should be further investigated.
2) CoQ_{10} is stable at high temperature as reported by Fir *et al*. (2009), in which the stability of CoQ_{10} obtained from differential scanning calorimetry was up to 250°C, but it is sensitive to light. Therefore, CoQ_{10} should be protected from light if the production of *Artemia* is performed by heat at high temperature.
3) Since CoQ_{10} can absorb UV light at 275 nm, thus, purified CoQ_{10} can be confirmed by spectrophotometer which is the available instrument in routine laboratory.
4) Preparation of a complex between CoQ_{10} and β-cyclodextrin should be optimized such as the ratio of substrate and stirring time.
5) After extraction, the residue of *Artemia* sample can be used for animal feed due to high nutrient of *Artemia*.

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Appendix A
Calculation of LOD and LOQ

The calculation of limit of detection (LOD) and limit of quantification (LOQ) using the linear equation of y = 20.229x - 0.726 were calculated as presented in Table A1.

Table A1. The calculation of LOD and LOQ of CoQ_{10}

x_i	y_i	$\hat{y}_i$	$y_i - \hat{y}_i$	$(y_i - \hat{y}_i)^2$
0.00	0.00	-0.73	0.73	0.53
0.25	3.90	4.33	-0.43	0.18
0.50	9.24	9.39	-0.15	0.02
0.99	18.34	19.30	-0.96	0.92
2.48	49.09	49.44	-0.35	0.12
4.95	98.96	99.41	-0.44	0.20
7.43	149.31	149.57	-0.26	0.07
9.90	196.86	199.54	-2.68	7.17
19.80	405.11	399.80	5.31	28.15
29.70	602.78	600.07	2.71	7.35
39.60	798.42	800.33	-1.91	3.65
49.50	999.03	1000.60	-1.57	2.45

$$\sum_i (y_i - \hat{y}_i)^2 = 50.81$$

$$S_{y/x} = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-2}} = \sqrt{\frac{50.81}{10}} = 2.25$$

$$x_{LOD} = \frac{3S_{y/x}}{b} = \frac{3 \times 2.25}{20.229} = 0.33$$

$$x_{LOQ} = \frac{10S_{y/x}}{b} = \frac{10 \times 2.25}{20.229} = 1.11$$

Therefore, the LOD was 0.3 µg mL^{-1} at the signal-to-noise ratio (S/N) of 3 while LOQ was 1.1 µg mL^{-1} at S/N of 10.

Appendix B

The summary of methods

B1. The optimal method of extraction, purification and preservation of CoQ_{10} from *Artemia*

B1.1. The extraction method of CoQ_{10} from *Artemia*

1) Weigh fresh *Artemia* 1.0 g to the nearest 0.001 g into a 60-mL test tube wrapped with aluminum foil.
2) Add 5.00 mL of 75% (w/w) acetic acid.
3) Shake the sample gently and incubate at room temperature for 24 hours.
4) Vortex mixture vigorously for 1 min.
5) Add 5.00 mL of hexane and vortex mixture vigorously for 1 min.
6) Add 5.00 mL of ethanol and shake the sample gently.
7) Leave to equilibrate at room temperature. Transfer by pipette the upper phase into a 25-mL round bottom flask.
8) Repeat of two times of the extraction procedure given in step 5 to 7. Combine and evaporate extract to dryness by a rotary evaporator.
9) Re-dissolve the residue with 2.00 mL of ethanol. Filter the solution through 0.2 µm Nylon membrane before HPLC analysis.

B1.2. HPLC condition for determination of CoQ_{10}

Column	: Agilent ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm i.d., 5 µm thickness) with guard column (12.5 mm × 4.6 mm i.d., 5 µm thickness)
Column temperature	: 27°C
Mobile phase	: Ethanol:methanol (95:5 v/v)
Flow rate	: 1.0 mL min^{-1}
Injection volume	: 20 µL
DAD detection	: 275 nm

The HPLC chromatograms of CoQ_{10} standard and CoQ_{10} in crude extract are shown in Figure B1.

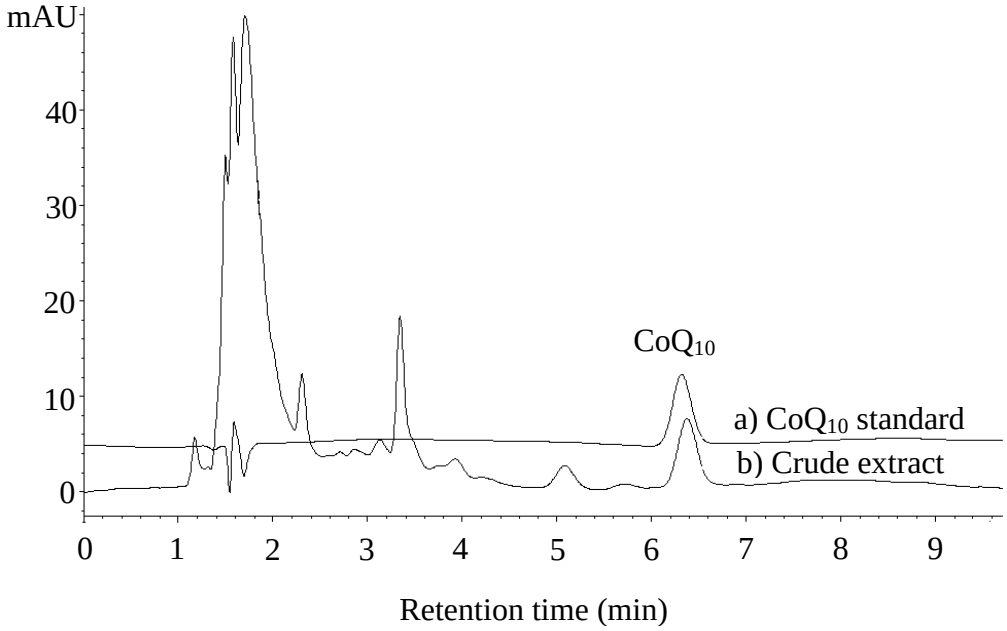

Figure B1. The typical HPLC chromatogram of CoQ_{10} in (a) CoQ_{10} standard and (b) *Artemia* crude extract

B1.3. Purification of CoQ_{10}

1) Mix silica gel 100 with 5% acetone in hexane.
2) Pour this slurry into the glass column (30.0 cm × 2.0 cm i.d.).
3) Re-dissolve of about 700 mg of crude extract with hexane.
4) Load a sample solution to the top of the column.
5) Elute with 5% of acetone in hexane.
6) Collect the yellow fraction.
7) Evaporate the yellow fraction under a gentle stream of nitrogen at 40°C.
8) Identify the CoQ_{10} fraction by TLC.
9) Dissolve the residue with a little volume of hexane, then drop by drop of methanol until a turbid solution was obtained. Stand at room temperature until the crystal was formed.
10) Re-crystallize of CoQ_{10} with ethanol and leave to cool at 4°C until the crystal was again formed.

11) Elucidate the structure of CoQ_{10} by spectrophotometry, HPLC, ^{1}H NMR and mass spectroscopy.

B1.4. Preservation of CoQ_{10}

B1.4.1. Preparation of inclusion complex between CoQ_{10} and *β*-cyclodextrin

1) Dissolve *β*-Cyclodextrin (1.5 g) with 15.0 mL of DI water in a 50-mL beaker.
2) Stir at 80°C to obtain a clear solution.
3) Dissolve 1.122 g of CoQ_{10} with 20.0 mL of hexane.
4) Mix *β*-cyclodextrin solution with CoQ_{10} solution. Stir at 80°C until the formation of the yellow homogeneous paste was obtained.
5) Wash the outer CoQ_{10} in complex with hexane at 4°C.
6) Keep the complex in a refrigerator until the requirement of the stability test.

B1.4.2. Determination of CoQ_{10} in complex

1) Dissolve 20 mg of complex with 10.0 mL ethanol.
2) Sonicate a sample solution to obtain a clear solution.
3) Transfer the solution to a 25-mL volumetric flask and adjust to the mark with ethanol.
4) Filter the sample solution through 0.2 µm Nylon membrane. Dilute by pipetting 250.0 µL sample solution into a 1.5-mL amber vial and add 250.0 µL of ethanol.
5) Analyze CoQ_{10} content by HPLC condition as mentioned in section 4.2.2.

B1.4.3. Photostability test

1) Add 300.0 µL of 3.3 mg mL^{-1} of free CoQ_{10} in hexane into petri dish or spread about 10 mg of complex in petri dish.
2) Air dry at room temperature.
3) Wrap with aluminum foil for either of non-UV exposure complex or free CoQ_{10} for control samples.

4) Expose complex and free CoQ_{10} under UVA light for 0.5, 1, 2, 4 and 8 hours.

5) Remove each petri dish after the certain exposure and analyze the remaining CoQ_{10} by HPLC.