METHOD VALIDATION FOR DETERMINATION OF CYANURIC ACID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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MAY 2014

Method Validation for Determination of Cyanuric acid by High Performance Liquid Chromatography (HPLC)

By

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A project report submitted to the Department of Chemical Science,

Faculty of Science,

Universiti Tunku Abdul Rahman

in partial fulfillment of the requirement for the degree of

Bachelor of Science (Hons) Chemistry

MAY 2014

ABSTRACT

METHOD VALIDATION FOR DETERMINATION OF CYANURIC ACID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Ooi Yen Han

Cyanuric acid has been adulterated in food to boost the apparent nitrogen level and caused various accidents, such as renal lithiasis in infants and young children in China, December 2008. It is highly toxic when administered with melamine as an insoluble complex, melamine-cyanurate crystal precipitated in kidney and lead to renal failure. Two different determination methods of cyanuric acid have been utilised in this work by using High Performance Liquid Chromatography coupled with ultraviolet detector (HPLC-UV). Water has been used as mobile phase for determination method I whereas sodium 1-heptanesulphonate-acetonitrile (83:17, v/v) has been implemented as mobile phase for method II. Detection was being carried out at 210 nm for method I and 213 nm for method II. The retention time observed for cyanuric acid in method I and II were 2.875 min and 2.806 min respectively. Both of the determination methods were validated according to International Conference on Harmonization ICH Q2 (R1) guidelines. The LOD and LOQ of cyanuric acid detected in method I which used water as mobile phase were 0.02 and

0.06 µg/mL respectively. On the other hand, 0.08 and 0.25 µg/mL were calculated to be the respective LOD and LOQ of cyanuric acid analysed in method II. Robustness of both analytical methods was tested using Plackett–Burman design and evaluated by "Design Expert" software. 12 experiments were conducted to assess 11 factors. Types of water used, mobile phase pH, column temperature, flow rate, detection wavelength, equilibrium time and detector response constant were the seven factors utilised for robustness test of method I (four dummy factors). As for method II, the factor: type of water used was replaced by percentage of sodium 1-heptanesulphonate and the rest of the factors were similar with method I. It was found that: (1) type of water used, column temperature and flow rate affected the responses significantly in method I, (2) column temperature and flow rate affected significantly the responses determined in method II, and (3) method I that utilised water as mobile phase is more robust than method II that used sodium 1-heptanesulphonate-acetonitrile as mobile phase.

ABSTRAK

Asid sinurik telah dicemari dalam makanan untuk meningkatkan tahap nitrogen dan menyebabkan pelbagai kemalangan, seperti lithiasis buah pinggang di kalangan bayi dan kanak-kanak di China, Disember 2008. Ia adalah sangat toksik apabila dicemari dengan melamin kerana kompleks larut, melamin - cyanurate kristal dicetuskan di dalam buah pinggang dan membawa kepada kegagalan buah pinggang. Dua kaedah penentuan yang berlainan telah digunakan dengan menggunakan Kromatografi Cecair Prestasi tinggi beserta dengan pengesan ultraungu (HPLC-UV). Air telah digunakan sebagai fasa bergerak bagi kaedah penentuan I manakala natrium 1- heptanesulphonate - asetonitril (83:17 , v/v) telah dilaksanakan sebagai fasa bergerak bagi kaedah II. Pengesanan telahdijalankan di 210 nm bagi kaedah I dan 213 nm bagi kaedah II. Masa tahanan diperhatikan untuk asid sinurik dalam kaedah I dan II adalah masing-masing 2.875 min dan 2,806 min . Kedua-dua kaedah penentuan telah disahkan menurut Persidangan Antarabangsa mengenai harmonisasi ICH Q2 garis panduan (R1). LOD dan LOQ bagi asid sinurik dikesan dalam kaedah I yang menggunakan air sebagai fasa bergerak adalah 0.02 dan 0.06 μg / mL. Selain daripada itu, 0.08 dan 0.25 μg / mL telah dikira dan dipastikan sebagai LOD dan LOQ yang dianalisis oleh kaedah II. Keteguhan kedua-dua kaedah analisis telah diuji dengan menggunakan reka bentuk Plackett - Burma dan dinilai oleh " Design Pakar" perisian. 12 percubaan telah dijalankan untuk menilai 11 faktor. Jenis-jenis air yang digunakan , pH fasa bergerak , suhu ruangan , kadar , pengesanan panjang gelombang , masa keseimbangan dan sambutan pengesan berterusan mengalir

adalah tujuh faktor yang digunakan untuk ujian keteguhan kaedah I (empat faktor dummy). Bagi kaedah II , faktor iaitu jenis air yang digunakan telah digantikan dengan peratusan natrium 1- heptanesulphonate dan seluruh faktor-faktor adalah sama dengan kaedah I. Ia telah didapati bahawa: (1) jenis air yang digunakan , suhu turus dan aliran kadar terjejas balas dengan ketara dalam kaedah I, (2) suhu turus dan kaedah kadar telah didapati mempengaruhi jawapan ditentukan dalam kaedah II , dan (3) Kaedah I yang menggunakan air sebagai fasa bergerak adalah lebih mantap daripada kaedah II yang menggunakan natrium 1- heptanesulphonate - asetonitril sebagai fasa bergerak .

ACKNOWLEDGEMENT

Primarily, I would like to express my gratitude to my project supervisor, Assoc. Prof. Dr Hnin Pwint Aung who has provided me guidance and advice throughout this final year project. I would like to express my deepest appreciation for the care, encouragement, valuable information and the invaluable time that had been shared by Dr. Hnin Pwint Aung that eventually helped me to complete this project.

I would also like to thank to Universiti Tunku Abdul Rahman (UTAR), Faculty of Science for providing equipment and instrumental support to complete my project. Besides, I also want to express my sincere thanks to master student, Elaine Fong who had spent her precious time to guide and help me solving the HPLC technical problems that I faced along the project. Appreciation goes to all laboratory staff in Faculty of Science who had always provided guidance and assistance when I faced problem.

On top of that, I would like to thank to my beloved family members and friends for their support, tolerance, faith and advice that motivated me to complete my project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institution.

_____

(OOI YEN HAN)

Date:

APPROVAL SHEET

This project report entitled **— "Method Validation for Determination of Cyanuric acid by High Performance Liquid Chromatography (HPLC)"** was prepared by OOI YEN HAN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Chemistry at Universiti Tunku Abdul Rahman.

Approved by:

_____ Date: _____

(Assoc. Prof. Dr. HNIN PWINT AUNG)

Supervisor

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FACULTY OF SCIENCE

UNIVERSITY OF TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that OOI YEN HAN (ID No: 10ADB04337) has completed this final year project entitled **— "Method Validation for Determination of Cyanuric acid by High Performance Liquid Chromatography (HPLC)"** supervised by Assoc. Prof. Dr. HNIN PWINT AUNG from the Department of Chemical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my thesis in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(OOI YEN HAN)

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LIST OF ABBREVIATIONS

% RSD	Percentage of relative standard deviation
ANOVA	Analysis of Variance
BSTFA	Bis(trimethyl)silyltrifluoroacetamide
C_7H_{14}-SO_3^-	Heptanesulphonate ion
CYA	Cyanuric acid
DAD	Diode array detection
DI water	Deionised water
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Admistration
GC	Gas chromatography
HPLC	High performance liquid chromatography
ICH	International Conference on Harmonisation
LOD	Limit of Detection
LOQ	Limit of Quantitation/Quantification
m/z	Mass-to-charge ratio
MEL	Melamine
MRL	Maximum residue limit
MRPL	Minimum Required Performance Limit
MS	Single-stage mass spectroscopy
MS/MS	Tandem mass spectrometry
Na^+	Sodium ion
NaDCC	Sodium dichloroisocyanurate

NDC	Non-diary creamer
RP-HPLC	Reversed phase high performance liquid chromatography
RSM	Response Surface Method
SPE	Solid-phase extraction
TMS	Trimethylchlorosilane
USP	The United States Pharmacopeia
UV	Ultraviolet radiation
v/v	Ratio of volume

CHAPTER 1

INTRODUCTION

1.1 Cyanuric acid (CYA)

In March 2007, hundreds of pet foods were recollected once renal failure was diagnosed in the pets consumed the pet foods which have been adulterated with cyanuric acid (CYA) along with the melamine (MEL). It has been revealed that cyanuric acid (and also melamine) was added in pet food ingredients to boost the apparent protein content (Karbiwnyk, et al., 2009). Melamine and cyanuric acid have gathered more worldwide attention as accidents of renal lithiasis in infants and young children occurred in China, December 2008. More than 294,000 cases of urinary tract stone with the hospitalization of 50,000 infants and six deaths were reported from the incident of consumption of the adulterated infant formula (Miao, et al., 2009).

Cyanuric acid is well-known as 1, 3, 5-triazine-2, 4, 6-triol (CAS No. 108-80-5). It has $(CNOH)_3$ as the molecular formula and abbreviated as CYA. It is a structural analogue of melamine as shown in Figure 1.1 (Ono, et al., 1998). Cyanuric acid appeared as white crystalline powder at room temperature, having density as 2.5 g/cm^3. It is highly polar and hydrophilic due to the presence of hydroxide group in the structure, and fairly stable under many environmental conditions (Stipičević, et al., 2013).

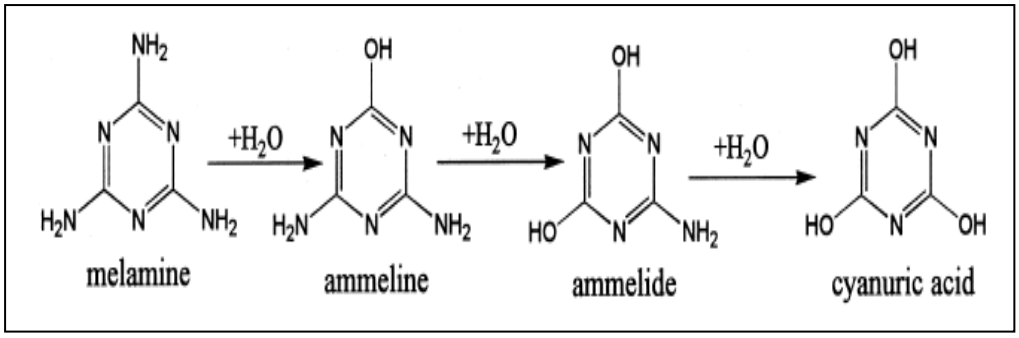


Figure 1.1: Formation of cyanuric acid as melamine's analogue by alkaline hydrolysis (Ono, et al., 1998)

In aqueous solution, cyanuric acid exits as a mixture of keto and enol tautomers. Keto form is known as isocyanuric acid, whereas the enol form is stable under alkaline conditions (pH more than 7.2). The structures of keto and enol form of cyanuric acid are shown in Figure 1.2.

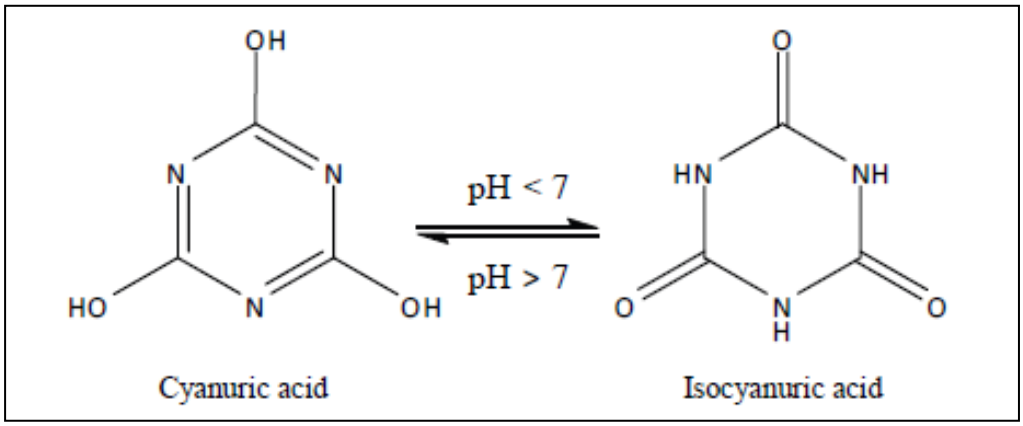


Figure 1.2: The structures of enol (cyanuric acid) and keto (isocyanuric acid) tautomers (Stipičević, et al., 2013)

Apart from being added to animal feed and pet food, cyanuric acid has been discovered to be adulterated into numerous food sources, including protein powder, raw milk, and non-dairy creamer (NDC). It is even more essential that these food sources had been used to manufacture infant powder, biscuits, instant drinks, cookies, soup products and so on (Chao, et al., 2011). Cyanuric

acid is a general ingredient, found as formulation impurity in plastics as it is nitrogen-rich and legally approved as a ruminant feed additive by the U.S. Food and Drug Administration (FDA) (Panuwet, et al., 2010). On top of that, cyanuric acid is also a common compound utilised to manufacture the industrial cleansers, fire retardants, scouring powders, household bleaches, and automatic dishwasher components (Miao, et al., 2009).

There are two major environmental sources of cyanuric acid, often existed as the product of microbial degradation, chemical or photo-oxidation of *s*-triazine herbicides. In fact, cyanuric acid has been acted as the marker of symmetric atriazine herbicide's degradation because cyanuric acid is the eventual hydroxylated metabolite in their degradation pathway. Cyanuric acid can also be detected in water, especially in swimming pool. It is widely applied as a microbicide or disinfectant in water treatment to prevent the destruction of chlorine caused by evaporation and ultraviolet radiation (UV) (Yu, et al., 2009).

Cyanuric acid can be formed through the rapid water hydrolysis of sodium dichloroisocyanurate (NaDCC). In this case, chlorine in the form of hypochlorous acid will be released. As a matter of fact, NaDCC is famously used as disinfectant dealing with body fluid spills, unfortunately illustrate a serious potential hazard to patients and staff. Moreover, this hydrolysis process is an equilibrium reaction, where a relatively low concentration will dissociate to a large extent. Hence, it is obvious that cyanuric acid is the primary environmental as well as toxicological interest when investigating the

chlorinated cyanurates in humans. Cyanuric acid is determined to be slightly toxic; nonetheless, a tolerable daily intake of NaDCC from treated drinking water as not more than 2 mg/kg of body weight per day has been set (Patel and Jones, 2007).

Cyanuric acid has low acute toxicities when administered singly. Nevertheless, the toxicity will be enhanced significantly when cyanuric acid is adulterated with MEL. A poor or nearly insoluble complex so called melamine-cyanurate crystals, as shown in Figure 1.3 has been induced by extending the hydrogen bonding network (Sun, et al., 2010). Aside from that, self-association within melamine-cyanurate complexes lead to precipitated crystal and thus block the kidney tubular, urinary system will be damaged and eventually caused death (Panuwet, et al., 2010).

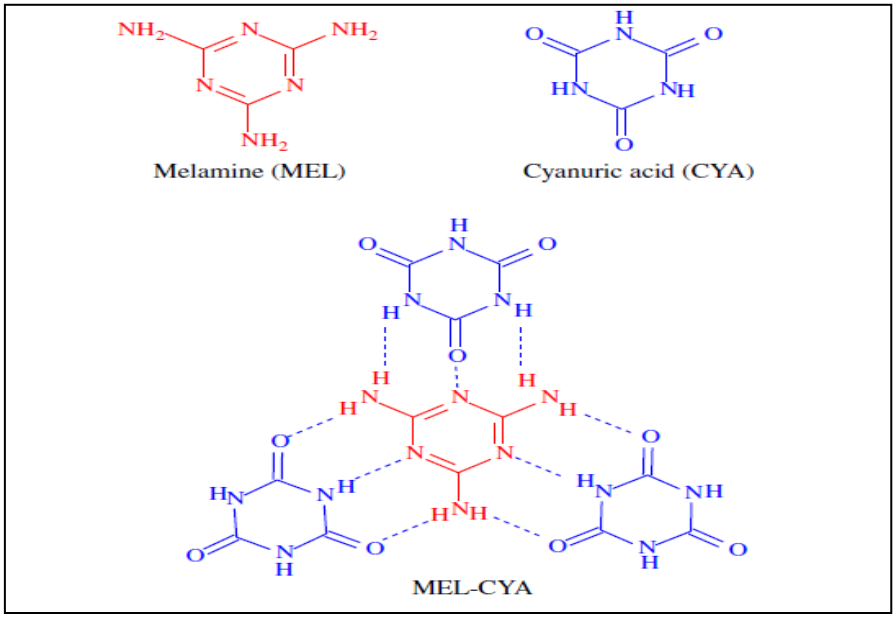


Figure 1.3: The structures of melamine, cyanuric acid and melamine-cyanurate crystal (MEL-CYA) (Sun, et al., 2010)

Evidently, daily intake for both melamine and cyanuric acid as less as 0.63 mg/kg of body weight per day is considered tolerable and has been regulated by FDA for the health and safety purpose (Panuwet, et al., 2010). Maximum residue limit (MRL) was established as 1 and 2.5 mg/kg in milk formula and products, respectively, by Chinese Ministry of Public Health. Furthermore, another provision has also been made by FDA and Chinese Ministry of Agriculture that cyanuric acid and melamine should be detected in food samples simultaneously (Pan, et al., 2013). Minimum Required Performance Limit (MRPL) of MEL and cyanuric acid has been issued within an interim method of LIB4422 by FDA, where the quantitation limit (LOQ) for melamine and cyanuric acid were 25 and 50 μg/kg of tissue and liquid formula respectively and both 200 μg/kg for dry infant formula powder (Deng, et al., 2010).

The determination method of cyanuric acid is indeed very essential in the pharmaceutical aspect, and yet, an intensive method validation is required, in order to support the cyanuric acid determination method as a qualitative and reliable analysis. Generally, a validation study is carried out after a new determination method so to demonstrate that the final method is suitable for the major purpose of that particular method (Enrique, et al., 2008).

1.2 Objectives

Shortly, the aim of this study is to validate the determination methods of cyanuric acid through high performance liquid chromatography (HPLC). To accomplish this aim, several objectives have been established:

I. To determine cyanuric acid by applying two different HPLC conditions through HPLC-UV technique.
II. To validate the cyanuric acid determination method on linearity, detection limit, detection quantification and precision.
III. To study the robustness of determination method of cyanuric acid by Plackett-Burman design and evaluate through “Design Expert” software.

CHAPTER 2

LITERATURE REVIEW

2.1 Melamine (MEL)

As cyanuric acid has been proved to be the hydrolysed analogue of melamine, it is also important to investigate the characteristics of melamine. Melamine, also been called as 2,4,6-triamino-1,3,5-triazine and abbreviated as MEL. It is an organic base and a cyanamide trimer with the structure's skeleton of 1,3,5-triazine, having $C_3H_6N_6$ as the chemical formula. The structure of melamine is shown in Figure 2.1.

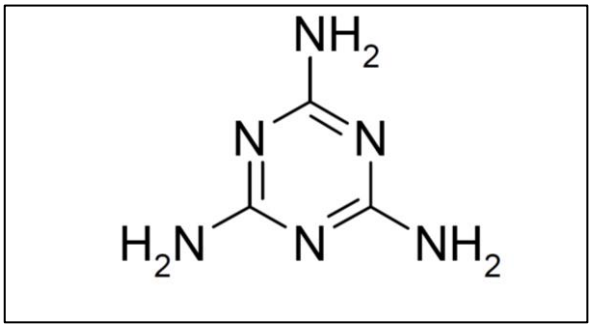


Figure 2.1: Structure of melamine (MEL) (Furusawa, 2012)

Melamine is a synthetic chemical component popularly used as a raw material for fabrication of multipurpose melamine-formaldehyde polymer resins for laminates, coatings, plastics, commercial filters, fabrics, glues or adhesives, dishware and kitchenware, and flame-retardants. Due to the 66.7% of nitrogen percentage, melamine is even illegally added by fraudulent producers to food products to raise the apparent food protein level. Occasionally, melamine

manages to migrate from the contact materials into the food content. Although the melamine's acute toxicity is considered to be low, it will be significantly increased as administered together with cyanuric acid, as insoluble melamine-cyanurate complex is formed (Vaclavik, et al., 2010).

2.2 Determination of Cyanuric Acid

There are evidently many methods developed for the analysis of cyanuric acid in foods, feeds and other samples. The existing analysis methods have been widely reviewed after the adulteration incidents. The methods of analysis are varied, divided into selective quantitative methods, rapid screening methods which are less specific or multi-residue methods. Multi-residue methods can be applied to determine the combination of studied compounds within a single process. The ordinary methods used to detect cyanuric acid include enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC) or gas chromatography (GC) coupled with either tandem mass spectrometry (MS/MS), single-stage mass spectrometry (MS), diode array detection (DAD) or ultraviolet absorption (UV). Characteristic of the methods mentioned above will be summarised in Table 2.1.

Table 2.1: Characteristics of analytical techniques used to determine cyanuric acid (World Health Organisation, 2009)

Analytical technique	Selectivity	Sensitivity	Cost	Purpose	Remarks
ELISA	Low	Low	Low	Screening	Currently for detecting melamine only
HPLC-UV/DAD	Low	Low	Medium	Screening and confirmation	Preferred for screening of melamine and analogues; however, validation is required for confirmation
GC-MS	Medium	Medium	High	Screening and confirmation	Confirmatory method for melamine and analogues
GC-MS/MS	High	High	Very high	Screening and confirmation at trace levels	Confirmatory method for melamine and analogues
LC-MS/MS	High	High	Very high	Screening and confirmation at trace levels	Confirmatory method for melamine and analogues

2.2.1 Sample Preparation

A homogenisation procedure is reviewed to be carried out as preliminary steps, followed by liquid extraction and sometimes by solid-phase extraction (SPE).

Polar solvents are always employed as the extraction solvents as cyanuric acid is highly polar. Polar solvents such as acetic acid (Karbiwnyk, et al., 2009), formic acid (Dobson, et al., 2008) and maleic acid (Patel and Jones, 2007) have been widely used. Acidic solvents are used to increase the solubility of cyanuric acid and denature the proteins in the sample. In addition, acetonitrile-water mixture (Kim, 2009), methanol (Huq, et al., 2009) and phosphate buffer (Sancho et al., 2005) have also been applied as the extraction solvents.

Aside from ultrasonic extraction, centrifugation and filtration, SPE is most often incorporated to further cleanup the sample. Reversed phase or cation

exchange SPE, for example Waters Oasis MCX and Phenomenex Strata X-C SPE are generally used to separate melamine whereas mixed-mode anion exchange sorbents, to quote an example, Waters Oasis MAX is used to isolate cyanuric acid from food and animal feed samples (Tittlemier, 2009).

It has been reviewed that the SPE cartridge has been previously conditioned before extracting the sample, normally by using methanol and water and with the addition of hydrochloric acid (Feng, et al., 2012). Methanol and water are added to wet the surface of the SPE catridge's sorbent and acid is added to activate the sorbent into an active form that manage to retain cyanuric acid (Sigma-Aldrich Corporation, 1998). After that, miscellaneous of methanol solution, or aminated methanol solution with 5% ammonia in methanol (Salman, et al., 2012), 5% ammonium hydroxide in methanol (Wu, et al., 2010) or 25% ammonia in methanol (Sun, et al., 2010) has been added to extract cyanuric acid from the sample mixture. According to Yu (2009), ammonia is added to adjust the pH as cyanuric acid's chromatographic conditions will be stable if the sample is in alkaline form. Nitrogen gas passed through the extract to dry the elute to dryness and residue is then re-dissolved in the solvent, readied to be run.

2.2.2 Separation and Detection of Cyanuric Acid by HPLC

High-performance liquid chromatography (HPLC) can be combined with the usage of UV detection (HPLC-UV), diode array detection (HPLC-DAD) and even with tandem mass spectrometry (HPLC-MS/MS).

Normal phase chromatography normally utilised non-polar mobile phase and polar column. Sakuma, Taylor and Schreiber (2010) has used hydrophilic interaction liquid chromatography (HILIC) column with mobile phase acetonitrile to separate cyanuric acid.

Reversed phase chromatography, principally operated by using a C8 or C18 column to separate the analyst compound. RP-HPLC is highly efficient and manages to distinguish between the chemically similar compounds. The stationary phase of this method is non-polar, employing resin, carbon or silica particle which bonded with alkyl chains or phenyl rings. In contrast, mobile phase used is usually polar solvents such as methanol, acetonitrile and water. (Thermo Electron Corporation, 2004). The biggest disadvantage of RP-HPLC is that ion pairing agent is needed compared with normal phase chromatography. Ion-pair reagent manages to modulate the ionic samples' retention. The hydrophobic part of ion-pair reagent will binds to the stationary phase and leaving the polar (hydrophilic) part facing the mobile phase. The stationary phase has now been changed from non-polar to polar, available for the ionic analytes to bond with it. To quote an example, sodium 1-heptanesulphonate is an ion-pair reagent that tends to dissociate into C_7H_{14}-SO_3^- and Na^+ where the heptane alkyl part will bind to the C18 column while the sulphonate ionic part will bind to the analytes. (Sun, et al., 2010)

The major disadvantage of adding ion-pair reagent is that the uptake and releasing process are slow and need for long equilibrating time. The

insufficient or incomplete process of washing or eluting the column may cause blockage of columns and disrupting the column packing structure.

Several reversed phase HPLC-UV methods have been utilised by measuring the amount of light that absorbed by cyanuric acid in different matrixes. For example, cyanuric acid has been separated by C18 column with phosphate buffer as mobile phase in gluten, rice, pet food (Smith, et al., 2007; Yu, et al., 2009) and milk by implementing C18 column and buffer of citric acid with sodium 1-octanesulfonate as mobile phase (Salman, et al., 2012).

Apart from HPLC-UV, there has been another technique called HPLC-DAD employed in the cyanuric acid determination field. Different from UV detector that only analysing the sample with a specific wavelength, DAD method applies the detection of absorption from UV to VIS region by multiple photodiode arrays for the analyte to obtain wide range wavelength information at one time injection. Cyanuric acid has been examined by HPLC-DAD from 200 nm to 250 nm in cereal flours (Ehling, Tefera and Ho, 2007), pet food (Kim, 2009), and rice sample (Muniz-Valencia, et al., 2008).

2.2.3 Separation and Detection of Cyanuric Acid by LC-MS/MS

High performance liquid chromatography-tandem mass spectrometry undergoes the separation analysis by combining the physical separation of liquid chromatography and mass analysis, in which the compound will be separated based on its polarity or size first and then being transferred to be

analysed according to its relative mass-to-charge ratio (m/z). The advantages of LC-MS/MS over HPLC are that LC-MS/MS being able to provide valuable information about the molecular weight, structure, and purity of a sample.

The difference between HPLC and LC-MS/MS columns is LC-MS/MS column can use shorter column for a faster analysis but HPLC column cannot be too short so to obtain a better peak resolution (Thermo Electron Corporation, 2004).

For determination of cyanuric acid by HPLC-MS/MS, several systems have been exercised, including detecting the cyanuric acid in dairy product and pet food (Sakuma, Taylor and Schreiber, 2010; Tran, et al., 2010), food such as egg, honey, milk, sausage (Deng, et al., 2010; Huq, et al., 2009; Varelis and Jeskelis, 2008), food contact materials such as plastic (Li, et al., 2011), infant formula (Han et al., 2011; Tittlemier, et al., 2008), seafood (Karbiwnyk, et al., 2009), soil (Stipičević, et al., 2013), urine (Panuwet, et al., 2010; Zhang, et al., 2010) and even kidney tissue sample (Filigenzi, et al., 2008).

2.2.4 Separation and Detection of Cyanuric Acid by GCMS

Gas-liquid chromatography-mass spectroscopy functions by two major operations, gas chromatography, which separates the chemical mixture into pure chemical pulses based on the different volatility and relative affinity for the stationary phase while the mass spectrometer is used to quantitate the individual compound. The different and unique retention time of each

compound enables the mass spectrometer to ionize the compounds into fragments, accelerate, deflect, and detect the ionized molecules separately. It is noticeable that the stationary phase for GCMS is inert gas, such as helium.

Derivatisation is needed to increase cyanuric acid's volatility as cyanuric acid is not volatile in gas chromatography due to its high polarity and boiling point. According to Bizkarguenaga, et al. (2013), derivatisation can be performed either off-line or in-line. Off-line derivatisation is separated from the GC analysis hardware, in which it is being conducted after the sample extraction procedure (Miao, et al., 2009; Lutter, et al., 2011; Wang, et al., 2010). Thereby, an additional sample processing step is needed. On the contrary, in-line derivatisation is simultaneously carried out with the analysis step in GC analysis. It is done either by "in-port" derivatisation where sample being derivatised in the hot GC inlet (Tzing anf Ding, 2010) or "in-column" derivatisation in which taken place in GC hot column (Miki, et al., 2008). Bis(trimethyl)silyltrifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMS) is being added to act as stimulator to form the tris-TMS-derivatives of cyanuric acid and optimise the chromatographic as well as mass spectrometric performances (Tzing and Ding, 2010). The derivatisation temperature is set to be 70-75 °C and the incubation duration time basically ranged from 30 to 45 min (Pan, et al., 2013).

It has been discovered that GCMS has been employed to detect CYA in dry protein materials such as rice protein and soy protein (U.S. Food and Drug Administration, 2009), food (Huq, et al., 2009), milk and milk products (Miao,

et al., 2009), cow's milk (Lutter, et al., 2011) infant formulas (Restek Corporation, 2009) and even tissue or human fluid (Wang, et al., 2010). The tris-TMS-derivative of cyanuric acid tends to form a fragment $[M+H]^+$ and observed to be the base peak around m/z 346.00 (Pan, et al., 2013; Tzing and Ding, 2010).

2.2.5 Detection of Cyanuric Acid by ELISA

Another screening method, for example, enzyme linked immunosorbent assay or as known as ELISA, is also available for determination of cyanuric acid. ELISA is a bio-analytical method, uses antibodies and the relative colour changes to identify a compound. Sample's antigens will immobilize on a solid surface and then a specific detection antibody which linked to an enzyme will be applied, forming a complex with the antigents. At this stage, a mild detergent will usually be used to wash the plate to remove proteins and antibodies that are unspecifically bonded. Eventually, an enzymatic substance containing the enzyme's substrate is added by washing process and this reaction produces a detectable visible signal, most commonly a colour change to indicate the quantity of antigen in the sample.

Compared to other determination methods, ELISA is obviously low cost, portable and more sensitive to screen large amount samples, and hence, considered as a routine monitoring tool of cyanuric acid. Lutter, et al. (2011) has applied a "Melamine ELISA kit" from Abraxis to detect cyanuric acid in cow's milk and the relative infant formula, obtaining 0.06 mg/kg and 0.1

mg/kg as the LOD and LOQ results respectively. Moreover, Vaclavik, et al. (2010) has also utilised the direct competitive AgraQuant Melamine Sensitive Assay to determine cyanuric acid in milk powder, obtaining 450 μg/kg as LOD. Apart from examining cyanuric acid in milk and infant formula, Dendele, et al. (2013) also employed Luminex bead-based immunoassays to test for CYA in urinary sample, studying the nephrotoxic effects of CYA.

Some of the techniques mentioned before have been summarized and tabulated in Table 2.2 for a clearer and more detail information.

In this project, the technique introduced is based on HPLC-UV by using two different HPLC conditions, mainly differentiated based on the mobile phase. One of the HPLC methods applied the conditions as referred to Sun, et al. (2010) and the other one utilised the usage of water as mobile phase that is easily available (Furusawa, 2012). Comparison studies were conducted for method validation and robustness test between these two HPLC methods.

Table 2.2: Summary of characteristics and techniques employed to determine cyanuric acid in various samples

Method	Matrix	Machine Conditions	Sensitivity	Reference
HPLC-UV	Liquid milk	Column = C18 column Mobile phase = 10 mM sodium n-heptanesulphonate–acetonitrile (83:17, v/v, pH 2.7) Flow rate = 1 mL/min. Detection wavelength = 235 nm Injection volume = 20 µL	18 µg/kg	Sun, et al., 2010
	Non-dairy creamer	Column = Microsorb-MV 100-5 Phenyl column Mobile phase = methanol: 5 mM potassium phosphate buffer [10:90 (v/v)] (pH 6.5). Flow rate = 0.8 mL/min. Detection wavelength = 203 nm Injection volume = 20 µL	0.03 µg/mL	Chao, et al., 2011
	Pet food	Column = C18 column Mobile phase =1 mmol/L phosphate buffer solution (pH 7.5). Flow rate = 0.5 mL/min. Detection wavelength = 214 nm Injection volume = 20 µL	0.002 µg/mL	Yu, et al., 2009

Table 2.2: Continued

Method	Matrix	Machine Conditions	Sensitivity	Reference
HPLC-DAD	Cereal flours	Column = Phenomenex Prodigy ODS columns (250x4.6mm i.d., 5 µm particle size) Mobile phase = 0.02M sodium phosphate monobasic (pH 3.0) with 85% weight phosphoric acid and 100% acetonitrile Flow rate = 0.5 ml/min Detection wavelength = 200 and 220 nm Injection volume = 20 µL	90 µg/g	Ehling, Tefera and Ho, 2007
	Pet food	Column = C8 column Mobile phase = buffer (10 mM citric acid and 10 mM sodium octanesulfonate adjusted to pH 3.0)-tetrahydrofuran-0.1 M ammonium acetate (70:5:25) Flow rate = 0.5 mL/min Detection wavelength = 236 nm Injection volume = 20 µL	0.1 µg/mL	Kim, 2009
HPLC-MS/MS	Dairy product, pet food	Column = Luna 3u Phenyl-Hexyl (150 mm × 2.0 mm, 3 μm) Mobile phase = 95 % water and 5 % acetonitrile (pH10) Flow rate = 0.12 mL/min Ionisation = Negative mode electrospray ionization source	-	Kim, 2009

Table 2.2: Continued

Method	Matrix	Machine Conditions	Sensitivity	Reference
HPLC-MS/MS	Dairy product, pet food	Column = Waters Atlantis HILIC (150 mm ×2.1 mm i.d., 5 µm particle size) Mobile phase = 10% 100 mmolL^{-1} ammonium formate in acetonitrile (pH 3.2) and 0.1% formic acid in acetonitrile Flow rate = 0.4 mL/min Injection volume = 10 µL Ionisation = Positive mode electrospray ionization	99.1 µg/kg	Deng, et al., 2010
		Column = ZIC-HILIC column (150 x2.1 mm, 5 um, 200A) Mobile phase = Ammonium acetate (10 mmol) and acetonotrile Temperature = 500 °C	0.2 µg/mL	Li, et al., 2011
	Food	Column = MERCK ZIC HILIC column (150x2.1 mm i.d., 5 µm) Mobile phase = 20 mM ammonium acetate and acetonitrile. Flow rate = 0.25 mL/min Injection volume = 10 µL Ionisation = Negative mode electrospray ionization Nebulizer = Nitrogen gas Temperature = 500 ℃	0.25 mg/kg	Han et al., 2011

Table 2.2: Continued

Method	Matrix	Machine Conditions	Sensitivity	Reference
	Infant formula	Column = Hypercarb column (5µm, 100 mm× 2.1 mm) Mobile phase = 0.1% formic acid in de-ionized water (v/v) and acetonitrile. Flow rate = 0.2 mL/min Injection volume = 10 µL	3.5µg/kg	Karbiwnyk, et al., 2009
	Seafood	Ionisation = negative ion mode electrospray ionization Voltage = 3500 V Temperature = 600 ◦C	0.0006 µg/mL	Panuwet, et al., 2010
	Urine	Column = HB-5MS capillary column (30 mx 0.25 mm; film thickness, 0.25 mm) Inlet Temperature = 230 ^{o}C Detector Temperature = 250 ^{o}C Injection Volume = 1 µL Carrier Gas = He at 15psi Oven Program = 70 ^{o}C (1 min), 25 ^{o}C/min to 150 ^{o}C, 3 ^{o}C/min to 190 ^{o}C, 50 ^{o}C/min to 280 ^{o}C (10 min) Ionization = Electron ionization	1 mg/kg	Lutter, et al., 2011

Table 2.2: Continued

Method	Matrix	Machine Conditions	Sensitivity	Reference
GCMS	Cow's milk and related infant formula	Column = 30m DB-5MS 5% phenyl 95% dimethyl-polysiloxane (0.25 mm i.d.; film thickness, 0.25 mm) Inlet Temperature = 280 ^{o}C Detector Temperature = 290 ^{o}C Injection Mode = Splitless Injection Volume = 1 µL Carrier Gas Flow = He at 35 cm/sec Oven Program = 75 ^{o}C (hold 1 minute) to 320 ^{o}C at 15 ^{o}C/minute (hold 2.67 min) for a total run time of 20 min. Ionization = Electron ionization Temperature = 230 ℃ (Source); 150 ℃ (Quad)	10 µg/g	U.S. Food and Drug Administration, 2009
	Dry protein materials	Column = DB-5ms column (30.0 m ×250 m ×0.25 m) Inlet Temperature = 300 ^{o}C Detector Temperature = 280 ^{o}C Injection Mode = Splitless Injection Volume = 1 µL Carrier Gas = He at 1.2 mL/min Oven Program = 75 ◦C (1 min) 30 ◦C /min to 300 ◦C (7.5 min) maintained at 300 ◦C (2.0 min) Ionization = Electron ionization	Tissue = 0.01g/g Body fluid = 0.005g/mL	Wang, et al., 2010

2.3 Validation

As this project's major objective is about the validation of the determination method of cyanuric acid, it is essential that the specific details of method validation are briefly discussed for an overall overview of this project.

Validation is often defined as an independent series of procedures that are put into action for evidencing that a product, service, or complex system does achieve certain specifications. According to International Conference on Harmonisation (2005), validation is designed to signify that experiment design fulfils the intended purpose. Validation basically includes all of the analytical techniques and interpretations, so to prove that a particular method in a given matrix is reproducible and reliable for the studied use. On top of that, there may be a necessity adjustment of the operating conditions to meet certain suitability requirements as system suitability is almost hard to accomplish, and this critical condition rearrangement commands for the validation testing.

Recently, most of the determination methods employing HPLC technique have been validated according to guidelines provided by International Conference on Harmonisation (ICH) (Alaani, Alashkar and Karabet, 2013; Belfast, et al., 2006; Fairbanksa, Leveneb, and Bax, 2013; Floriani, et al., 2014; Kumar, Annapurna and Pavani, 2012; Liu, et al.,2014) and The United States Pharmacopeia (USP) (Ariffin, Ghazali and Kavousi, 2014; Potts, et al., 2012; Evaggelopoulou and Samanidou, 2013).

2.3.1 Parameters of Validation

The fundamental parameters for validation include linearity, limit of detection (LOD), and limit of quantitation (LOQ), selectivity, accuracy, precision, robustness, ruggedness and stability. Linearity is analysing on the linear relationship between the instrumental results against the known concentration of analyte. LOD indicates as the lowest analyte concentration at which detection is feasible, determined by the signal-to-noise ratio while LOQ is the lowest concentration at which some predefined goals for bias and imprecision are met for the detection and the value may be equivalent to the LOD or at a much higher concentration. For selectivity, it is the analytical method's capability to differentiate unequivocally the analyte in the presence of other chemical compounds, such as degradants, contaminants or matrix in the sample. Accuracy is defined as the trueness, dominated as the closeness of mean results obtained to that of the true value, often reported as the percent recovery. Precision is the closeness of individual measures to multiple samplings of the similar sample. The stability parameter should account for the stability of analyte during sample collection and handling, after short term and long term storage (International Conference on Harmonisation, 2005; U.S. Department of Health and Human Services, 2001).

2.3.2 Robustness and Ruggedness

As defined by ICH, robustness is stated as the measure of a method's ability to remain constant as small but deliberate parameters are being varied and used

to analyse the crucial factors that would affect the inter-laboratory precision. On the other hand, ruggedness is indicated as the degree of repeatability of the test results collected under a series of normal test conditions, such as different instruments, laboratories, days and so on. There are many different kinds of design applicable for robustness and ruggedness test which manage to be differentiated by the derived model and the purpose of the study. For example, factorial designs, Plackett–Burman designs, central composite designs and Box–Behnken design. In this project, Plackett-Burman designs are employed based on ICH guidelines.

2.3.3 Plackett-Burman Design

Many designs examine the factors independently which eliminate the correlations among the factors but as for Plackett-Burman, it investigates the overall influences of parameters, interested in the main effects instead of focusing on each of the variations. It specifically has become popular for robustness tests in method validation (Hou, et al., 2011; Li, et al., 2005; Novokmet, et al., 2012). This design screens the two base level of each parameter, "+" and "-" level and 4n experiments are officially needed to examine a maximum of 4n-1 factors. By analysing the design of experiment, the main effect is obtained based on the estimation of the response changes as the factor ranging from the "-" to "+" level (Hibbert, 2012).

2.3.4 Interpretation of Result

The interpretation of results is often done by either statistical or graphical methods, or even by a combination of these two methods. Statistical interpretation can be accomplished by t-test or F-test equipped in nested analysis of variance (ANOVA) approaches (Galiano and Kunert, 2006; Sun and Su, 2002; Zeaiter, et al., 2004). As for the graphical interpretation, drawing of half-normal probability plot (Hou, et al., 2011; Zeaiter, et al., 2004) or normal probability plot (Galiano and Kunert, 2006) can be generated and evaluation of data can be carried out by assuming that the insignificant effect will be located on a straight line that passes through zero whereas significant values deviate from it.

In this project, combination methods of graphical and statistical have been utilised for evaluating the robustness results obtained by constructing the half-normal plot and normal plot, along with the statistic support evident of ANOVA.

Chapter 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

Standard of cyanuric acid (98 % purity) and sodium 1-heptanesulphonate were purchased from Nacalai Tesque (Kyoto, Japan). Distilled water and mineral water (Spritzer brand) was purchased from Tesco, Kampar (Perak, Malaysia). De-ionized (DI) water was prepared from a Milli-Q Plus system at 18.2 MΩcm. Acetonitrile and methanol were in high purity, HPLC grade. Sulphuric acid (95-97 %) was in Analytical Reagent grade. Sodium hydroxide was purchased from R&M Chemicals (Essex, U.K.). Sartolon polyamide membrane filter (0.45 µm) was used to filter the mobile phases.

3.2 Equipment

HPLC analysis was performed with an Agilent Technologies G13114 HPLC machine, an Agilent Technologies G1311A pump, equipped with UV detector (Agilent Technologies 1100) and a 20 µL sample loop at room temperature. A Gemini 5u C18 column (150 x 4.60 mm) was purchased from Phenomenex (Torrance, CA). Dynamica HALO SB-10 single beam UV/Vis spectrophotometer has also been used.

3.3 Determination of UV Spectrum of Cyanuric Acid

UV spectrums of two cyanuric acid determination methods have been generated to determine the maximum absorption wavelength of each of the methods.

3.3.1 Method I: Water as Mobile Phase

1 µg/mL cyanuric acid which is dissolved in distilled water solvent has been scanned from 190.00 nm to 500.00 nm to determine the UV spectrum.

3.3.2 Method II: Sodium 1-heptanesulphonate-acetonitrile as Mobile Phase

The UV spectrum of this method has been generated by scanning 200 µg/mL cyanuric acid which dissolved in methanol: water (1:4, v/v) solvent from 190.00 nm to 300.00 nm.

3.4 Chromatographic Conditions

In this project, two different HPLC conditions, mainly differentiated based on the mobile phase have been applied. One condition used water as the mobile phase whereas the other one utilised sodium 1-heptanesulphonate-acetonitrile as mobile phase.

3.4.1 HPLC Operating Conditions

3.4.1.1 Method I: Water as Mobile Phase

Distilled water has been used as mobile phase at a flow rate of 1.0 mL/min at 25 ^{o}C. Distilled water was maintained at its original pH (pH 7.20), detected at 210 nm with the equilibrium time as 10 minutes and the injection volume of 20 µL. The detector response constant of UV detector is set to be >0.1 min (2s).

3.4.1.2 Method II: Sodium 1-heptanesulphonate-acetonitrile as Mobile Phase

Mobile phase of 10 mM sodium 1-heptanesulphonate – acetonitrile (83:17, v/v, pH 7.30) was used at a flow rate of 1.0 mL/min at 30 ^{o}C. An injection volume of 20 µL was applied by setting the equilibrium time as 10 minutes and the detector response constant of UV detector is >0.1 min (2s). Specific wavelength, 235 nm was selected to detect the responses of cyanuric acid.

3.4.2 Standard Preparation

3.4.2.1 Method I: Water as Mobile Phase

Stock solutions of cyanuric acid were prepared by dissolving 10 mg of CYA in 100mL distilled water to a concentration of 100 µg/mL. Dilution was then being carried out by distilled water to 1, 5, 10, 15 and 20 µg/mL.

3.4.2.2 Method II: Sodium 1-heptanesulphonate-acetonitrile as Mobile Phase

100 μg/mL stock solution cyanuric acid was prepared by dissolving 10 mg of cyanuric acid in 100 mL methanol and water (1:4, v/v). Fresh working standard solutions (1, 5, 10, 15 and 20 μg/mL) were prepared daily by diluting the 100 μg/mL stock solution.

3.5 HPLC Method Validation

The determination methods of cyanuric acid were validated based on the following parameters: linearity, sensitivity, robustness, precision (within- and between-day variability) as guided in ICH.

3.5.1 Linearity

Calibration curve was generated by plotting the peak areas against their relative concentrations, ranging from 1 to 20 μg/mL. The linearity was examined from the linear regression with its regression coefficient.

3.5.2 Sensitivity

Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the calibration curve by the LINEST function in EXCEL, according to the formula:

$$\text{LOD} = 3.3(\frac{S.D.}{Slope})$$

$$\text{LOQ} = 10(\frac{S.D.}{Slope})$$

Where S. D. represents the standard deviation of the slope and slope means the gradient of the regression line.

3.5.3 Precision

3.5.3.1 Method I: Water as Mobile Phase

Precision was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision) of 5 and 10 µg/mL cyanuric acid standard solutions. For intra-day precision, each of the concentrations was injected for six replications within a day. For inter-day, each of the concentrations was injected three times per day for continuous three days. Based on retention time, results were integrated as % RSD of the measurements.

3.5.3.2 Method II: Sodium 1-heptanesulphonate-acetonitrile as Mobile Phase

10 and 15 µg/mL cyanuric acid standard solutions have been used to determine the precision. Each of the concentrations was injected for six replications within a day for intra-day whereas for inter-day injections, each of

the cyanuric aicd standard solutions was injected for three times continuously three days. The results were integrated as % RSD based on retention time.

3.5.4 Robustness

3.5.4.1 Plackett-Burman Design for Method I: Water as Mobile Phase

Plackett-Burman design applied twelve experimental numbers (4n) to investigate eleven factors (4n-1), which are types of mobile phase, mobile phase pH, column temperature (^{o}C), flow rate (mL/min), detection wavelength (nm), equilibrium time (min) and detector response constant (min) while the rest factors are represented by dummy. The levels of factors have been tabulated and shown as Table 3.1.

Table 3.1: Levels of factors examined by Plackett-Burman for robustness in method I: water as mobile phase

Factor	Level (-1)	Nominal	Level (+1)
Type of water	Millipore water	Distilled water	Mineral water
Column temperature, ^{o}C	30.00	25.00	35.00
Equilibrium time, min	5	10	15
Flow rate, mL/min	0.9	1	1.1
Detector response constant, min	>0.05	>0.1	>0.2
Detector wavelength, nm	205.00	210.00	215.00
pH	6.70	7.20	7.70

Twelve sets of run conditions have been arranged by Plackett-Burman and arranged in Table 3.2. All runs were conducted by duplicate injections of 10 µg/mL cyanuric acid standard solution. Results such as peak area and retention time were collected and assessed by "Design Expert" software.

Table 3.2: Twelve sets of run conditions designed by Plackett-Burman in method I: water as mobile phase based on the level +1 and -1

Run	Type of water	Column temperature, ^{o}C	Equilibrium time, min	Flow rate, mL/min	Detector response constant, min	Detector wavelength, nm	pH
1	-1	30.00	5.00	1.10	>0.05	215.00	7.70
2	-1	35.00	15.00	0.90	>0.2	215.00	7.70
3	+1	30.00	15.00	1.10	>0.05	215.00	7.70
4	-1	35.00	15.00	1.10	>0.05	205.00	6.70
5	+1	35.00	5.00	1.10	>0.2	215.00	6.70
6	-1	30.00	5.00	0.90	>0.05	205.00	6.70
7	+1	35.00	15.00	0.90	>0.05	205.00	7.70
8	-1	35.00	5.00	1.10	>0.2	205.00	7.70
9	+1	30.00	15.00	1.10	>0.2	205.00	6.70
10	+1	30.00	5.00	0.90	>0.2	205.00	7.70
11	+1	35.00	5.00	0.90	>0.05	215.00	6.70
12	-1	30.00	15.00	0.90	>0.2	215.00	6.70

-1 represent Millipore water while +1 represent mineral water

3.5.4.2 Plackett-Burman Design for Method II: Sodium 1-heptanesulphonate-acetonitrile as Mobile Phase

This determination method utilised 10 mM sodium 1-heptanesulphonate-acetonitrile (v/v, 83:17) as mobile phase. The factors being chosen in this robustness section include the ratio of sodium 1-heptanesulphonate (%), mobile phase pH, column temperature (^{o}C), flow rate (mL/min), detection wavelength (nm), equilibrium time (min) and detector response constant (min) where the rest of the factors represented by dummy. The levels of factors which have been tabulated were shown as Table 3.3.

Table 3.3: Levels of factors examined by Plackett-Burman for robustness in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

Factor	Level (-1)	Nominal	Level (+1)
Ratio of sodium 1-heptanesulphonate buffer, %	82	83	84
Column temperature, ^{o}C	25.00	30.00	35.00
Equilibrium time, min	5	10	15
Flow rate, mL/min	0.9	1	1.1
Detector response constant, min	>0.05	>0.1	>0.2
Detector wavelength, nm	208.00	213.00	218.00
pH	6.80	7.30	7.80

Twelve sets of run conditions have been designed by Plackett-Burman and tabulated in Table 3.4. By using 10 µg/mL cyanuric acid standard solution, twelve run sets are conducted in duplicate injections. Peak area and retention time results were collected and assessed by "Design Expert" software.

Table 3.4: Twelve sets of run conditions designed by Plackett-Burman in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase based on the level +1 and -1

Run	Ratio of Sodium 1-heptanesulphonate buffer, %	Column temperature, ^{o}C	Equilibrium time, min	Flow rate, mL/min	Detector response constant, min	Detector wavelength, nm	pH
1	82.00	35.00	5.00	1.10	>0.05	218.00	7.80
2	84.00	25.00	15.00	0.90	>0.2	208.00	6.80
3	84.00	25.00	15.00	1.10	>0.05	218.00	7.80
4	84.00	35.00	15.00	1.10	>0.05	218.00	6.80
5	82.00	25.00	5.00	1.10	>0.2	218.00	7.80
6	82.00	35.00	5.00	0.90	>0.05	208.00	6.80
7	82.00	35.00	15.00	0.90	>0.05	208.00	7.80
8	82.00	25.00	5.00	1.10	>0.2	208.00	6.80
9	84.00	25.00	15.00	1.10	>0.2	208.00	7.80
10	84.00	35.00	5.00	0.90	>0.2	208.00	7.80
11	84.00	35.00	5.00	0.90	>0.05	218.00	6.80
12	82.00	25.00	15.00	0.90	>0.2	218.00	6.80

Chapter 4

RESULTS AND DISCUSSION

4.1 UV Spectrum of Cyanuric Acid

The maximum absorption wavelength is needed to be estimated from the UV spectrum obtained so to further used as the detection wavelength in HPLC operating conditions. The results of UV spectrums obtained for both determination methods of cyanuric acid have been tabulated as Table 4.1.

Table 4.1: Results of UV spectrum of cyanuric acid

Mobile Phase	**Method I: Water**	**Method II: Sodium 1-heptanesulphonate-acetonitrile**	
Concentration of cyanuric acid used, µg/mL	1	200	
Maximum absorption wavelength, nm	209.77	193.57	213.13
Absorbance, A	0.36	0.53	0.18

4.1.1 Determination of Detection Wavelength in Method I: Water as Mobile Phase

For UV spectrum of cyanuric acid determined in method I: using distilled water as the mobile phase (Figure 4.1), a maximum absorption peak is observed at 209.77 nm. Therefore, 210 nm is selected to be the wavelength for UV detection of HPLC condition to determine cyanuric acid in water as mobile phase.

4.1.2 Determination of Detection Wavelength in Method II: Sodium 1-heptanesulphonate-acetonitrile as Mobile Phase

As shown in Figure 4.2, the absorption peaks for method II were shown at 193.57 nm and 213 nm. However, 213 nm has been selected to be the detection wavelength of HPLC method to determine cyanuric acid. This is because many compounds manage to absorb the light within 200nm (Lutter, et al., 2011), thus 213nm has been selected instead of 193.57nm.

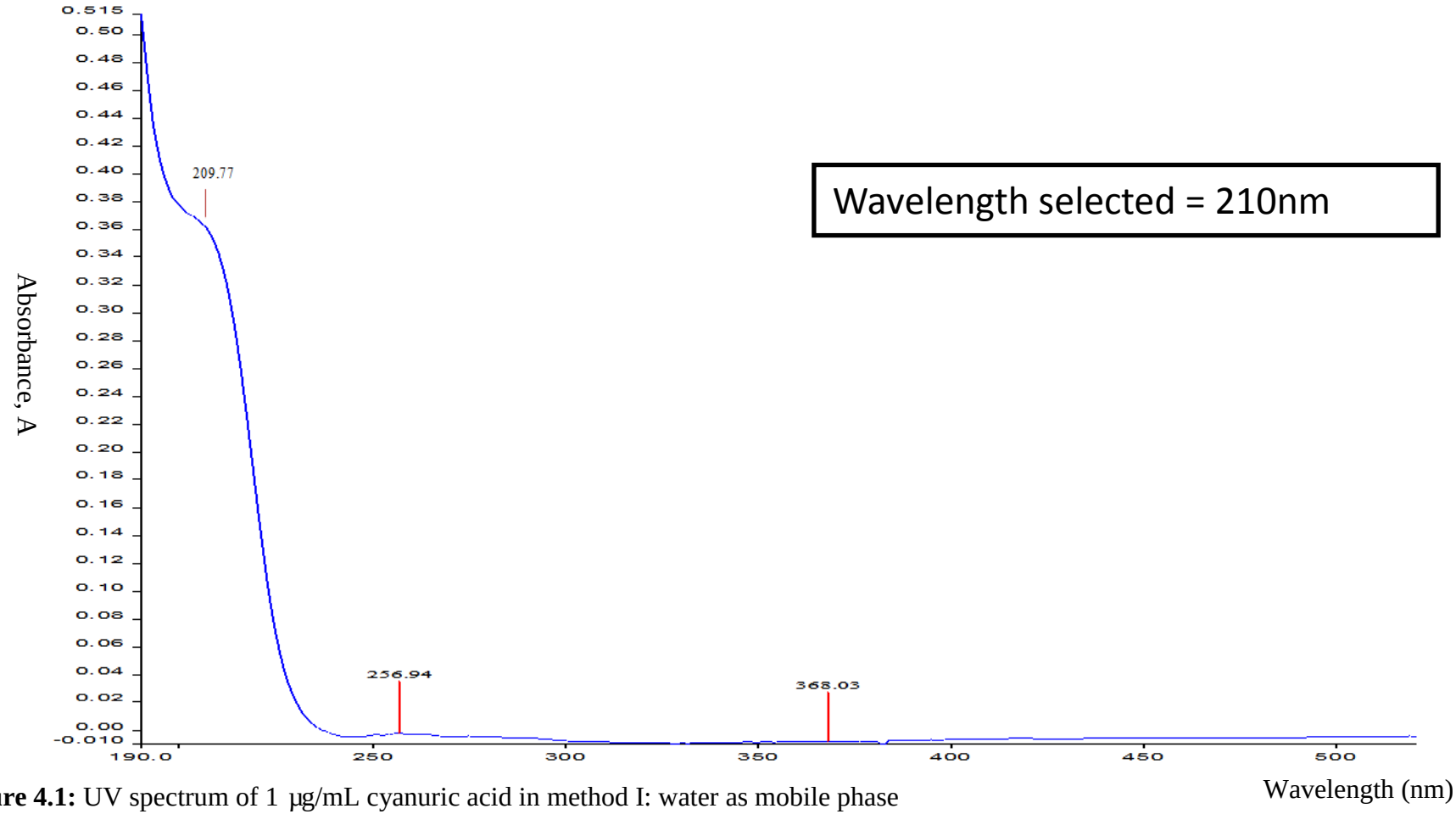


Figure 4.1: UV spectrum of 1 µg/mL cyanuric acid in method I: water as mobile phase

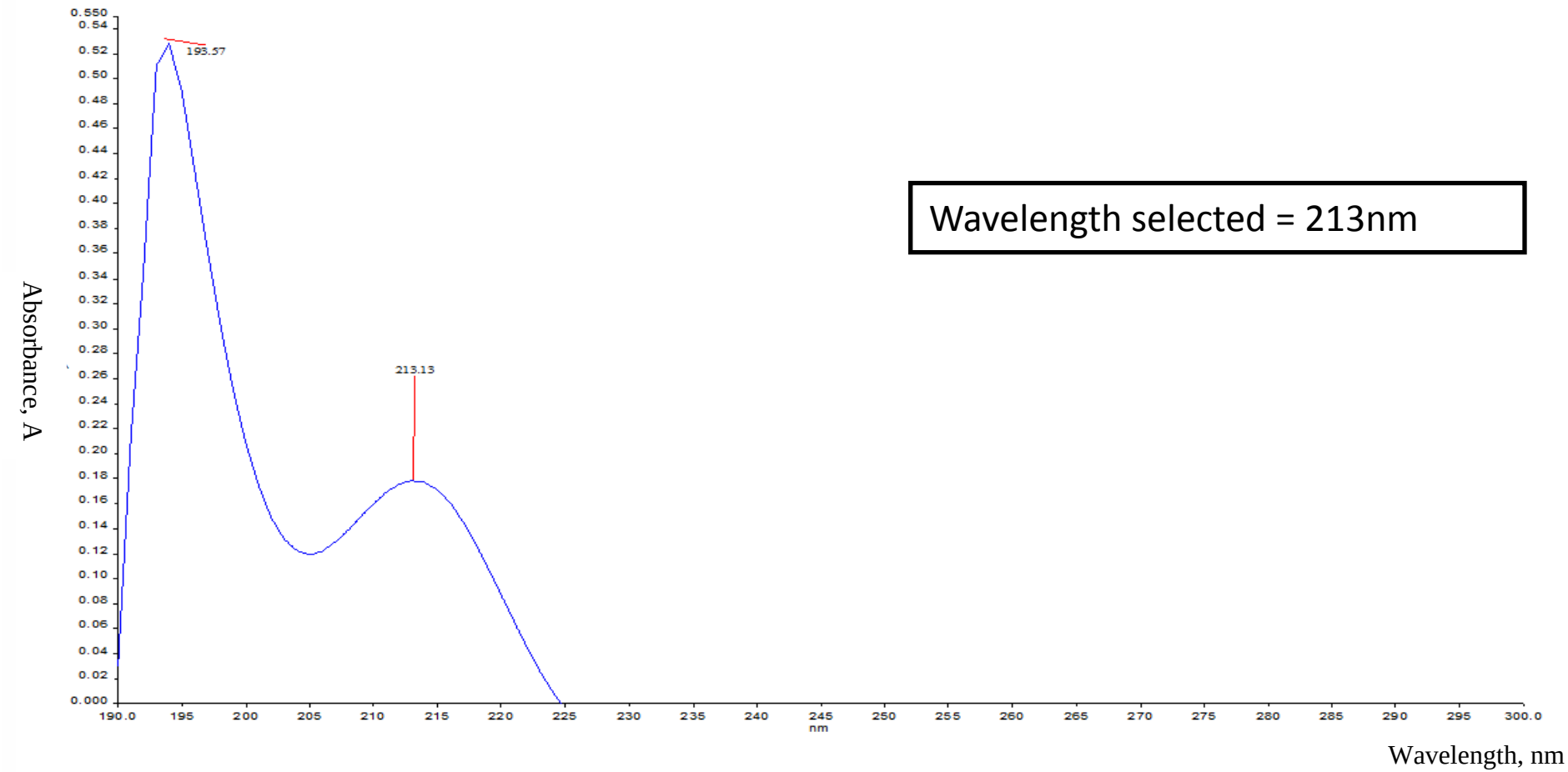


Figure 4.2: UV spectrum of 20 µg/mL cyanuric acid in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

4.2 Chromatogram of Cyanuric Acid

4.2.1 Method I: Water as Mobile Phase

The mobile phase used in this determination method is isocratic elution of 100 % distilled water. In reference to the chromatogram obtained by detecting 20 µg/mL cyanuric acid which attached as Figure 4.3, the retention time of the cyanuric acid is observed to be 2.875 min and the peak area is 1123.62 mAU*min.

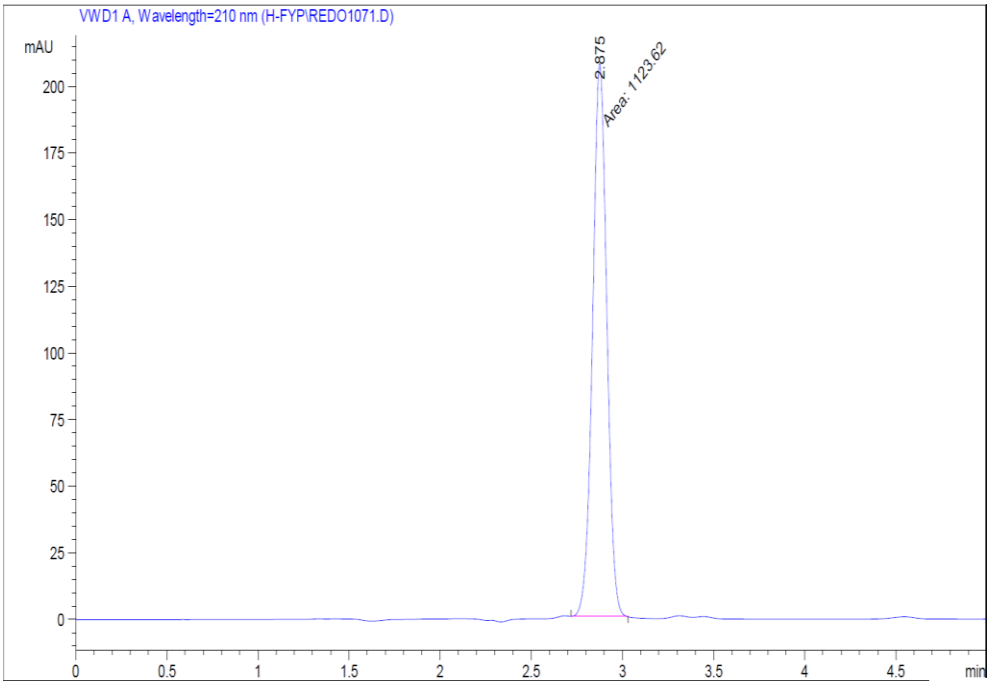
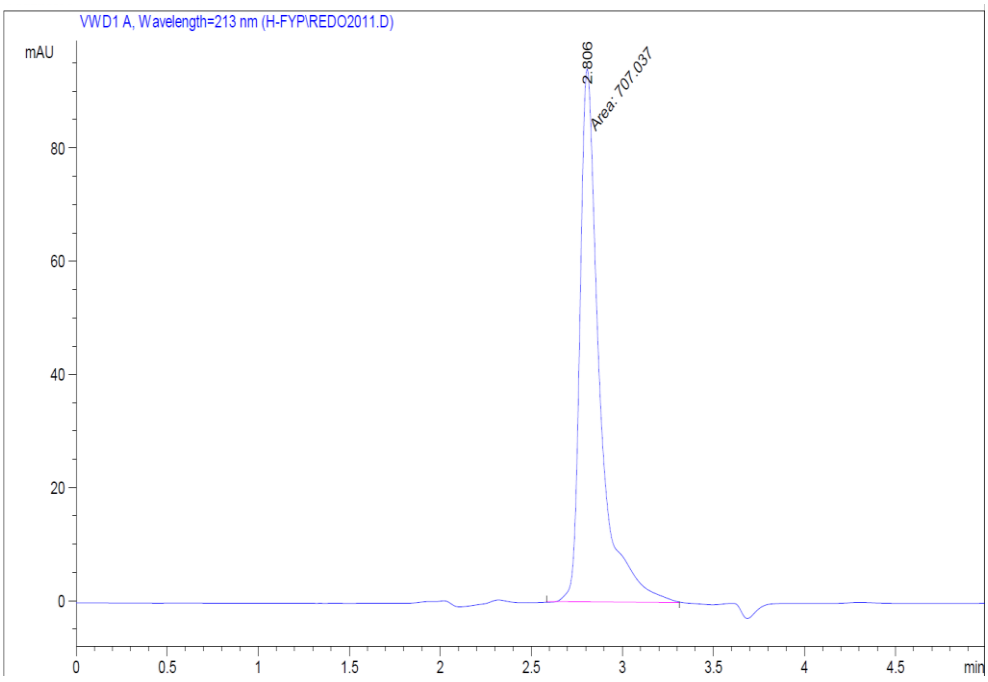


Figure 4.3: Chromatogram of 20 µg/mL cyanuric acid in method I: water as mobile phase, detected at 210nm

4.2.2 Method II: Sodium 1-heptanesulphonate-acetonitrile as Mobile Phase

The mobile phase used for this determination method is set to be an isocratic elution of mixture of sodium 1-heptanesulphonate-acetonitrile (83:17, v/v). The retention time observed for 20 µg/mL cyanuric acid is 2.806 min and the peak area is detected to be 707.037 mAU*min. The chromatogram is attached as Figure 4.4.



Absorbance (mAu)

Time (min)

Figure 4.4: Chromatogram of 20 µg/mL cyanuric acid in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase, detected at 213nm

Tailing effect is spotted in the peak in Figure 4.4. It can be speculated that the interaction between the ion-pair reagent and the packing's active site of C18 is strong that non-homogenous population is formed and thus tailing of peak occurred (Nueu, U.D., 2002).

4.3 Linearity

4.3.1 Method I: Water as Mobile Phase

The linearity was validated using calibration curve (Figure 4.5) of concentration ranging from 1 to 20 µg/mL. The calibration line was all linear with regression coefficient values 0.9999 and the linearity data is tabulated in Table 4.2. The high value of regression coefficient showed that there is a linear relationship between then concentration and peak area results.

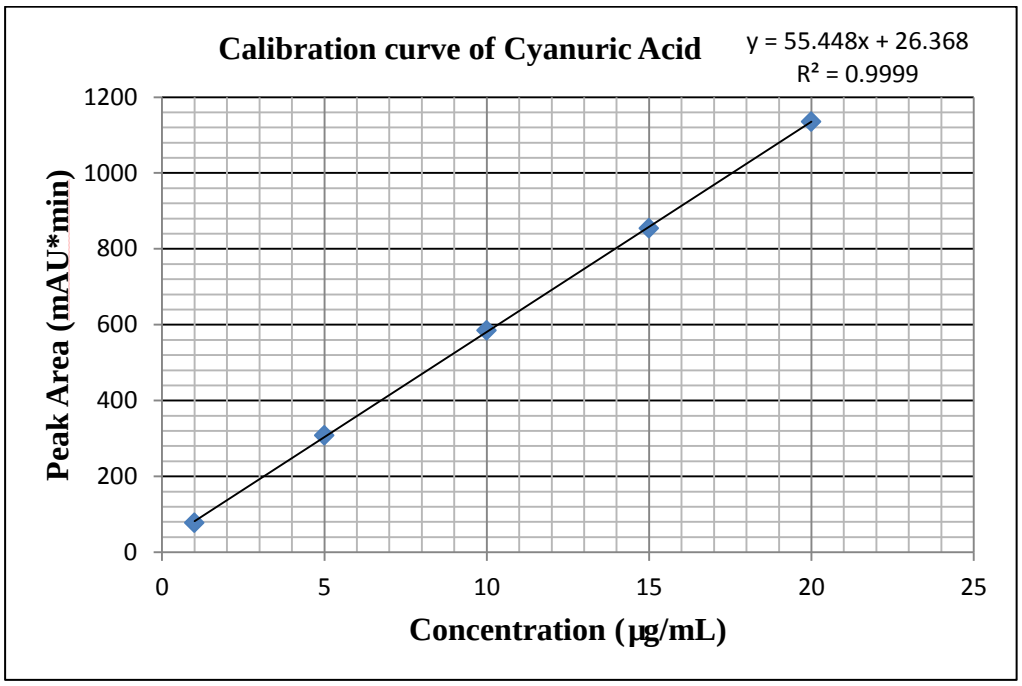


Figure 4.5: Calibration curve of cyanuric acid in method I: water as mobile phase

Table 4.2: Linearity data of cyanuric acid in method I: water as mobile phase

Slope	Intercept	R^2
55.4477 ± 0.3216	26.3681 ± 3.9418	0.9999

4.3.2 Method II: Sodium 1-heptanesulphonate-acetonitrile as Mobile Phase

Calibration curve (Figure 4.6) was assessed based on the linear calibration line ranging from 1 to 20 µg/mL with regression coefficient value as 0.9981. The linearity data is tabulated in Table 4.3. The relationship between concentration and the peak area result is proved to be linear as the regression coefficient is near to 1.

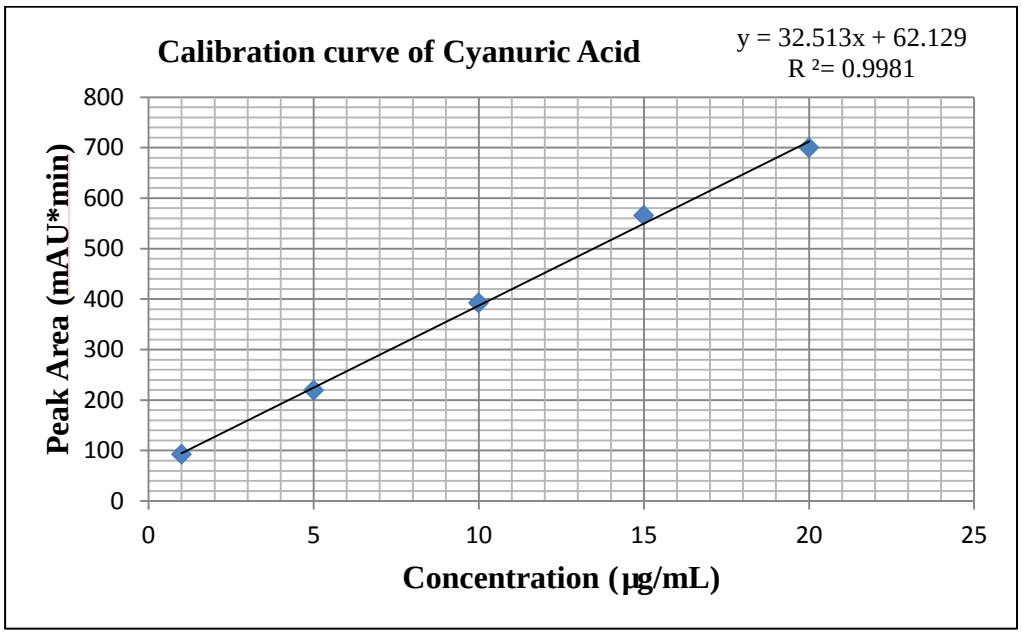


Figure 4.6: Calibration curve of cyanuric acid in method II: sodium 1-heptanesulphonate- acetonitrile as mobile phase

Table 4.3: Linearity data of cyanuric acid in method II: sodium 1-heptanesulphonate- acetonitrile as mobile phase

Slope	Intercept	R^2
32.5135 ± 0.8160	62.1294 ± 10.0002	0.9981

4.4 Sensitivity

There are several methods can be implemented to determine the limit of detection (LOD) and limit of quantification (LOQ). The methods include calculation from the signal-to-noise ratio; calculation from blank's standard deviation and also can be calculated from the calibration line at low concentrations. In this project, the sensitivity is evaluated based on the calibration line through EXCEL's LINEST function. The LOD and LOQ results of the cyanuric acid's determination methods are tabulated in Table 4.4.

Table 4.4: Sensitivity results of cyanuric acid

Mobile Phase	**Method I: Water**	**Method II: Sodium 1-heptanesulphonate-acetonitrile**
Limit of detection (LOD), µg/mL	0.02	0.08
Limit of quantification (LOQ), µg/mL	0.06	0.25

There was a higher sensitivity of cyanuric acid detected in method I that utilised water as mobile phase if compared with method II which used sodium 1-heptanesulphonate-acetonitrile as mobile phase. This is because the detection wavelength chosen in method II is the wavelength that shown the second highest absorption (reason explained in Section 4.1.2). Thus, the lowest concentration of cyanuric acid managed to be determined in method II is higher than method I.

4.5 Precision

The values of % RSD for intra-day and inter-day variation for both of the cyanuric acid determination methods are shown in Table 4.5.

The % RSD values for method I that employed water as mobile phase were all falling below 2% limit, indicating that this determination method is reproducible and repeatable. In addition, low %RSD value of the inter-day precision (intermediate precision) demonstrate a relatively stable and inert chemical properties of cyanuric acid in distilled water even after a period of time (three days).

On the other hand, the intermediate precision (inter-day) results obtained for method II are evidently unsatisfied compared to method I precision as the % RSD values exceed 2% limit. This finding suggested that the cyanuric acid dissolved in methanol: water (1:4, v/v) is most likely to undergo some chemical degradation or variation in its chemical nature. The retention factor of cyanuric acid has been adjusted after storing for continuous three days.

In order to make a comparison between these two methods, determination method I is investigated to have higher precision than method II. Cyanuric acid is discovered to be more stable and chemically inert to be dissolved and stored in water as the inter-day % RSD value of method I found within the limit. Nonetheless, intermediate precision values of cyanuric acid in method II fall out of the limit.

Table 4.5: Intra-day and inter-day precision results of cyanuric acid

Mobile Phase	**Method I: Water**			**Method II: Sodium 1-heptanesilphonate-acetonitrile**		
Standard no.	Concentration	Intra-day precision	Inter-day precision	Concentration	Intra-day precision	Inter-day precision
1	5.0 µg/mL	2.836	2.557	10.0 µg/mL	2.947	2.805
2		2.849	2.544		2.946	2.944
3		2.846	2.557		2.949	2.949
4		2.859	2.551		2.944	2.833
5		2.876	2.553		2.948	2.814
6		2.874	2.545		2.948	2.948
Mean		2.857	2.551		2.947	2.882
Standard Deviation		0.016	0.006		0.002	0.072
% RSD		0.560	0.22		0.061	2.485
1	10.0 µg/mL	2.735	2.546	15.0 µg/mL	2.836	2.823
2		2.719	2.545		2.849	2.818
3		2.797	2.561		2.846	2.818
4		2.72	2.549		2.859	2.822
5		2.769	2.562		2.876	2.959
6		2.711	2.558		2.874	2.974
Mean		2.742	2.554		2.857	2.869
Standard Deviation		0.034	0.0077		0.016	0.076
% RSD		1.240	0.30		0.560	2.639

4.6 Robustness Test

The chromatographic responses of detection of cyanuric acid include peak area and retention time have been collected and analysed. Plackett-Burman design has been applied with twelve sets of run conditions to examine seven factors and four dummy factors.

4.6.1 Method I: Water as Mobile Phase

4.6.1.1 Chromatographic Responses Results of Cyanuric Acid

The chromatographic responses results have been tabulated in Table 4.6. The responses have been analysed individually by "Design Expert" software to determine the significant factors for each responses.

Table 4.6: Responses results of cyanuric acid in method I: water as mobile phase

Run	Peak area, mAU*min	Retention time, min
1	427.819	3.138
2	1144.35	2.318
3	964.014	1.714
4	624.687	1.867
5	943.097	1.831
6	197.576	3.615
7	733.963	2.269
8	618.659	1.906
9	660.353	1.733
10	784.535	2.119
11	1122.78	2.271
12	142.549	3.465

4.6.1.2 Analysis on Peak Area Response of Cyanuric Acid

Half-normal plot of peak area response (Figure 4.7) has been produced by "Design Expert" software to screen the standardise effect of all seven factors on peak area (type of mobile phase, column temperature, equilibrium time, flow rate, detector response constant and detector wavelength). The effect of four dummy factors has been excluded. The Analysis of Variance (ANOVA) results tabulated as Table 4.7.

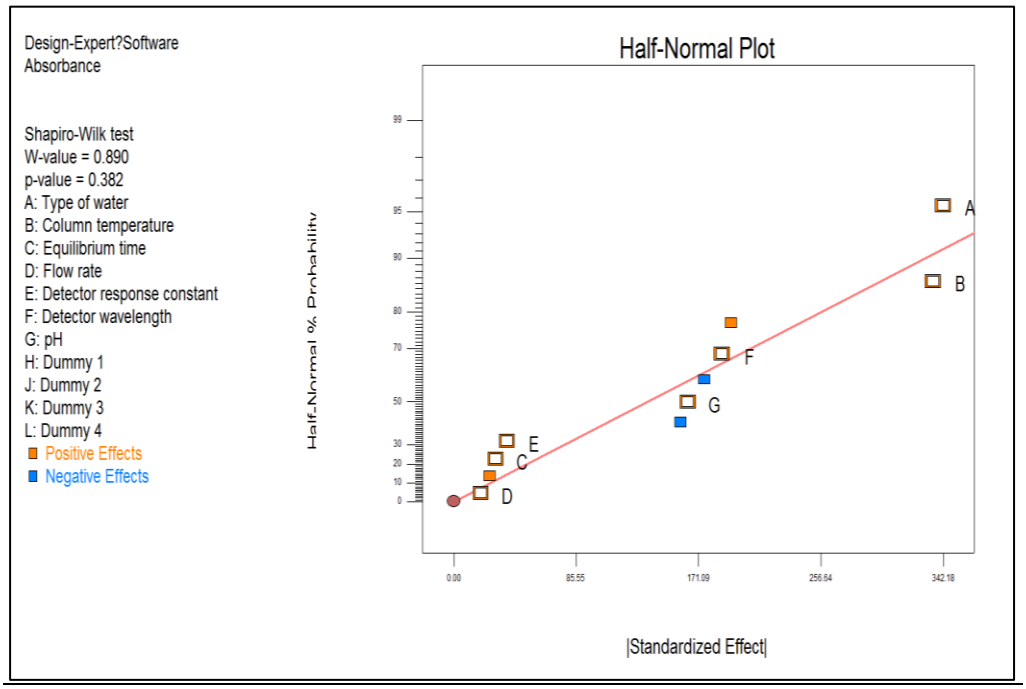


Figure 4.7: Half-normal plot of cyanuric acid peak area in method I: water as mobile phase for seven factors, excluding the dummy

Table 4.7: ANOVA results of peak area for all factorial models of cyanuric acid in method I: water as mobile phase

Source	Sum of Square	df	Mean Square	F value	p-value Prob>F
Model	8.818E+005	7	1.260E+005	1.78	0.3009
A-Type of water	3.513E+005	1	3.513E+005	4.97	0.0896
B-Column temperature	3.369E+005	1	3.369E+005	4.77	0.0943
C-Equilibrium time	2565.09	1	2565.09	0.036	0.8581
D-Flow rate	1061.81	1	1061.81	0.015	0.9083
E-Detector response constant	4132.95	1	4132.95	0.059	0.8208
F-Detector wavelength	1.054E+005	1	1.054E+005	1.49	0.2889
G-pH	80408.17	1	80408.17	1.14	0.3461
Residual	2.825E+005	4	70630.63		
Corrected Total	1.164E+006	11			

0.0500 has been regulated to be the significant level. Model term that has "Prob>F" value that is less than 0.0500 will be indicated as significant. However, the p-value of the overall model and seven factors are all more than 0.0500. This showed that the model and factors are all insignificant. Some factors with large p-values have to be eliminated so to investigate the specific factors those are significant to the model.

Factor A (type of water used as mobile phase) and factor B (column temperature) have been selected. The selected half-normal plot and selected factorial model of ANOVA have been included as Figure 4.8 and Table 4.8 respectively.

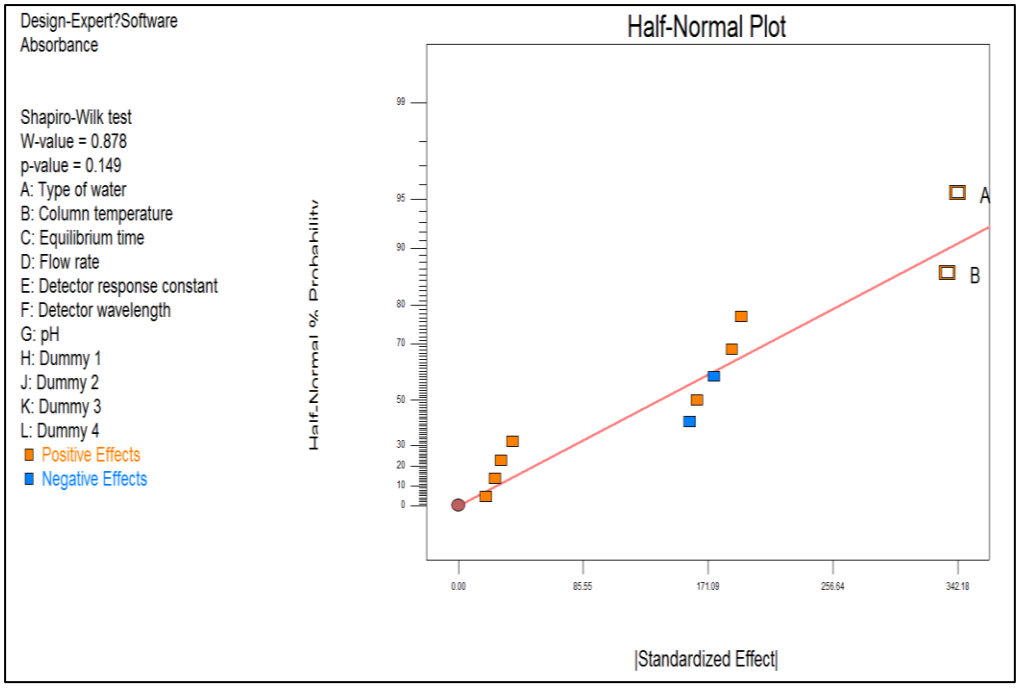


Figure 4.8: Half-normal plot of cyanuric acid peak area in method I: water as mobile phase for two selected factors

Table 4.8: ANOVA results of peak area for selected factorial models of cyanuric acid in method I: water as mobile phase

Source	Sum of Square	df	Mean Square	F value	p-value Prob>F
Model	6.882E+005	2	3.441E+005	6.50	0.0179
A-Type of water	3.513E+005	1	3.513E+005	6.64	0.0299
B-Column temperature	3.369E+005	1	3.369E+005	6.37	0.0326
Residual	4.761E+005	9	52903.06		
Corrected Total	1.164E+006	11			

After eliminating others factors, factors A (type of water) and B (column temperature) is found to be deviated from the linear line. The consequence results of p-value for whole model, type of water and column temperature reduced to 0.0179, 0.0299 and 0.0326 respectively. Therefore, it can be

explained that these two factors are significant factors that affected the peak area response significantly.

Generally, reliability of experimental results obtained can be tested by normal residual plot. If the results gained gathered around a central point with no left or right bias, the normality assumption can be fulfilled. Normal plot of residuals (Figure 4.9) has been evaluated, as the points of factors were distributed along the central line, it explained that the data obtained for cyanuric acid peak area are normally distributed.

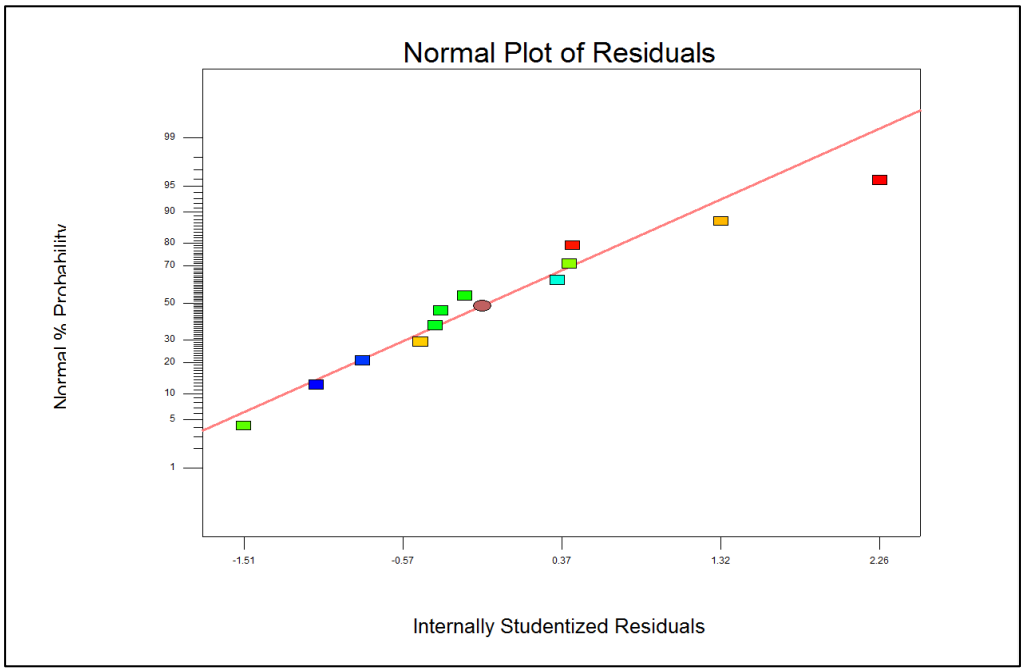


Figure 4.9: Normal plot of residuals of cyanuric acid peak area in method I: water as mobile phase

Equations related to the two found significant factors, type of water and column temperature have been yielded. For future experiment, the peak area results can be predicted by just substituting the known values of water types and column temperature.

For experiments that employed Millipore water as mobile phase, the equation is expressed as:

Peak area = -1652.30342 + 67.02284 * Column temperature

While for experiments that utilized mineral water as the mobile phase, the equation is written as:

Peak area = -1310.11869 + 67.02284 * Column temperature

4.6.1.3 Analysis on Retention Time Response of Cyanuric Acid

As attached as Figure 4.10, half-normal plot has been composed by just including seven HPLC condition factors, screening the standardise effects on the retention time of cyanuric acid. ANOVA results for all seven factors have been evaluated and tabulated as Table 4.9. The effects of dummy factors have been neglected.

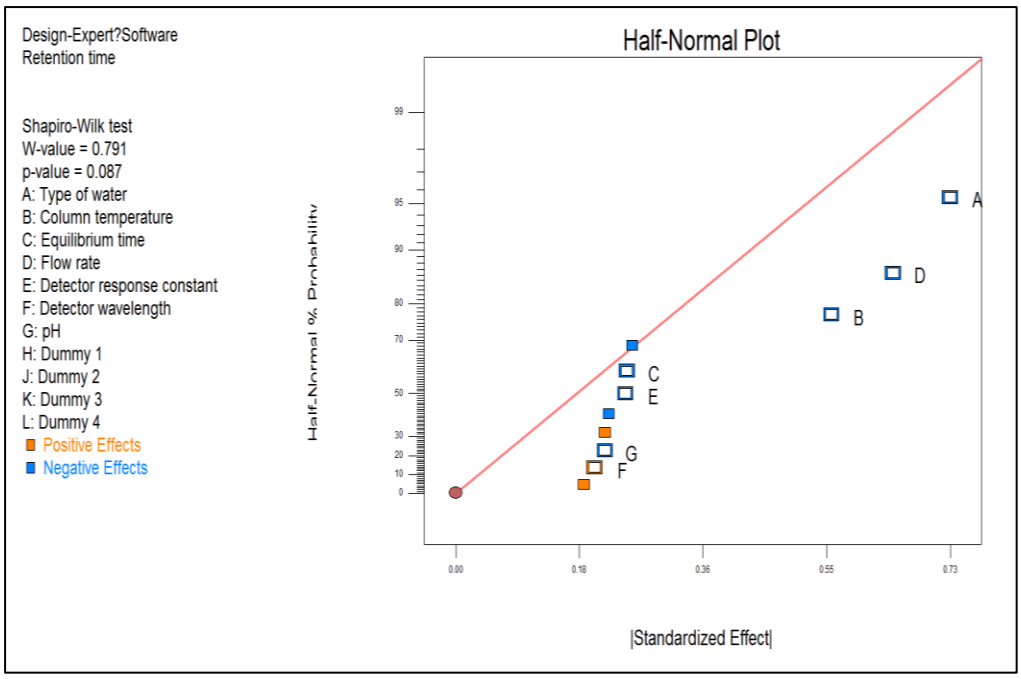


Figure 4.10: Half-normal plot of cyanuric acid retention time in method I: water as mobile phase for all seven factors, excluding the dummy factors

Table 4.9: ANOVA results of retention time for all factorial models of cyanuric acid in method I: water as mobile phase

Source	Sum of Square	df	Mean Square	F value	p-value Prob>F
Model	4.41	7	0.63	4.15	0.0938
A-Type of water	1.59	1	1.59	10.49	0.0317
B-Column temperature	0.92	1	0.92	6.05	0.0697
C-Equilibrium time	0.19	1	0.19	1.26	0.3249
D-Flow rate	1.25	1	1.25	8.21	0.0457
E-Detector response constant	0.19	1	0.19	1.24	0.3283
F-Detector wavelength	0.13	1	0.13	0.83	0.4145
G-pH	0.14	1	0.14	0.95	0.3842
Residual	0.61	4	0.15		
Corrected Total	5.02	11			

Based on the ANOVA results, all of the seven factors and the overall model showed that their relative p-values are larger than 0.0500, specified that all factors and overall model are insignificant. Therefore, the selected half-normal plot and ANOVA results have been assembled by eliminating the factors that indicating large p-values until the model was testified to be significant.

The new half-normal plot for several selected significant factors, type of water used (Factor A), column temperature (Factor B) and flow rate (Factor D) have been generated and attached as Figure 4.11. Selected factorial model of ANOVA test has also been shown (Table 4.10).

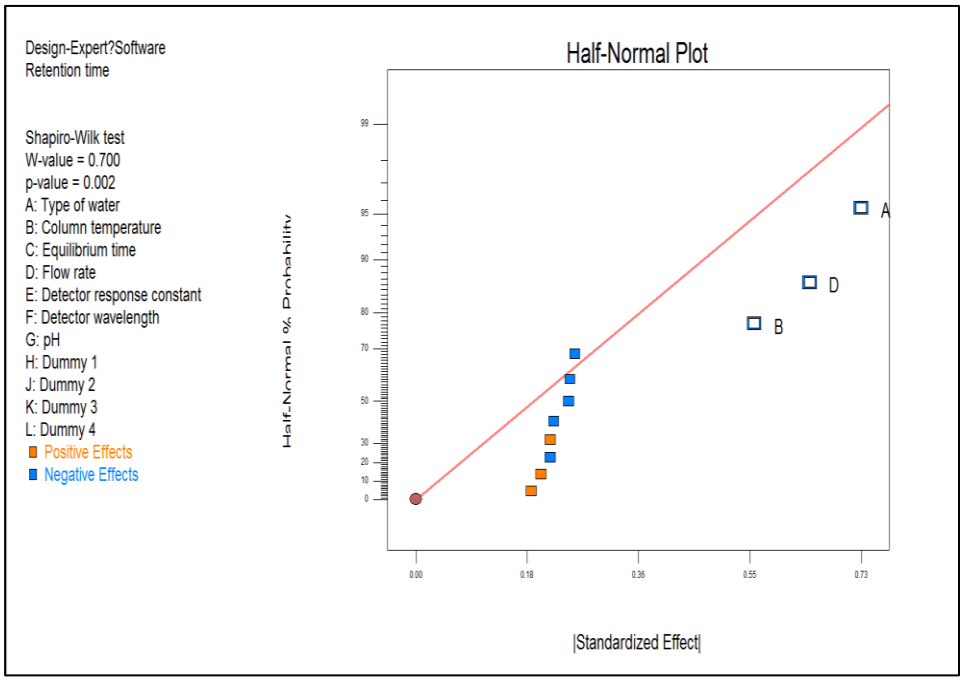


Figure 4.11: Half-normal plot of cyanuric acid retention time in method I: water as mobile phase for three selected factors

Table 4.10: ANOVA results of cyanuric acid retention time for selected factorial models in method I: water as mobile phase

Source	Sum of Square	df	Mean Square	F value	p-value Prob>F
Model	3.76	3	1.25	7.97	0.0087
A-Type of water	1.59	1	1.59	10.14	0.0129
B-Column temperature	0.92	1	0.92	5.85	0.0419
D-Flow rate	1.25	1	1.25	7.93	0.0226
Residual	1.26	8	0.16		
Corrected Total	5.02	11			

As factors such as type of water used (Factor A), column temperature (Factor B) and flow rate (Factor D) have been selected, the p-values of these factors and overall model have been reduced to less than 0.0500. This indicates that factor A, B and D are the significant factors.

Normal plot of residuals for retention time (Figure 4.12) has been evaluated to ensure that the results of retention time response fulfilled the normality assumption. It can be observed that the data points are distributed along the fitting line, explained that there is bias nor outlier exist in the data.

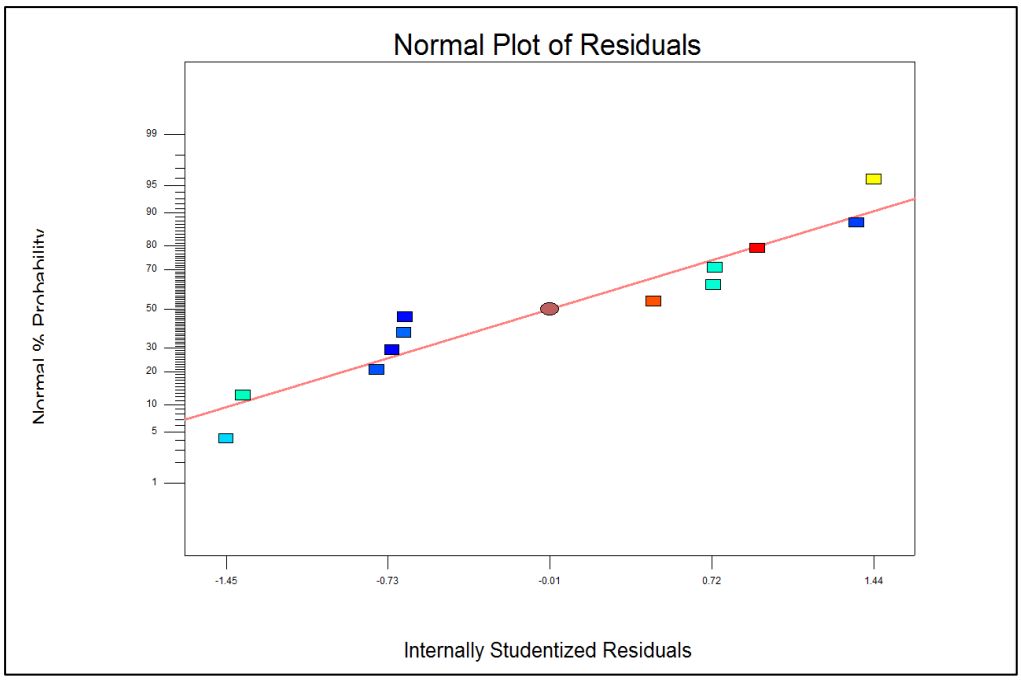


Figure 4.12: Normal plot of residuals of cyanuric acid retention time in method I: water as mobile phase

It has been validated that three significant factors were contributed to the variation of retention time results, including the factors: type of water, column temperature and flow rate. Equations related to these three factors have been offered.

For experiments that employed Millipore water as mobile phase, the retention time result is predicted to be:

Retention time = 9.54033 - 0.11073* Column temperature - 3.22333* Flow rate

While for experiments that utilized mineral water as the mobile phase, the peak height is written as:

Retention time = 8.81167 - 0.11073 * Column temperature - 3.22333* Flow rate

4.6.1.4 Discussion on Significant Factors Determined

Table 4.11 listed the significant factors for each of the studied responses to serve as the summary of the results obtained.

Table 4.11: Summarised significant factors for the responses of cyanuric acid in method I: water as mobile phase

HPLC responses	Significant factors
Peak area	• Type of water • Column temperature
Retention time	• Type of water • Column temperature • Flow rate

It has been simplified that the major three factors that significantly affect the HPLC responses of cyanuric acid determined in this mrthod, employing water as the mobile phase are the type of water used as mobile phase, column temperature and flow rate. Each of the significant factors will be discussed on the reason of its significant influence on the responses.

Two types of water being used to examine the HPLC responses on determination of cyanuric acid, Millipore water used as Level -1 mobile phase while mineral water used as Level +1 mobile phase. The great difference between these two mobile phases is their relative ionic strength. Millipore water is classified as a type of ultrapure water that produced under a series of water purification and monitoring technologies of tap water through Milli-Q Integral system. Tap water is purified to reach a resistivity of 18.2MΩ· cm for ultrapure water production. Resistivity is known as the reciprocal of conductivity and used to monitor the ion purity of water. The higher the resistivity applied to the water purification system, the lower concentration of ion dissolved in water and the higher the water's ability to resist electric current. With resistivity of 18.2MΩ·cm, Millipore water is considered as ultrapure (Type 1) water that eliminates all UV-absorbing ionic contaminant whereas mineral water is rich in minerals and nutrients (sodium ion, chloride ion, nitrites, nitrates and so on) that is essential to human (EMD Millipore Corporation, 2014). When analyzing ionic compound, ionic strength of mobile phase influenced the responses. The affection is even more obvious as the ionic strength between Millipore water and mineral water is extremely different (Nueu, U.D., 2002). The ionic compounds exist in mineral water tend to prevent the cyanuric acid from retaining on the column, faster elution of cyanuric acid out of the column and thus the responses of CYA detected reduced. The reverse phenomena can be observed in Millipore water condition, where the cyanuric acid tends to retain more strongly to the column, increase the response results.

Another essential parameter that can significantly impact on the liquid chromatography responses is column temperature. By raising the temperature, the viscosity of the eluent decreased and enhanced the diffusivity and mass transfer of solutes, results in lower backpressure of the separation systems, which allows the fast analysis. In other words, the energy of the column system being increased as the temperature raised and thus leads to faster reaction or activity of the mobile phase, analyte ion and also the retention activity (Yang, Y., 2006). It can be speculated that with elevated column temperature, the overall retention of HPLC is reduced. The reverse reaction is predicted to be observed for the runs that has a lower column temperature.

On top of that, flow rate is another significant factor that alters the HPLC responses severely, especially for the retention time response. Generally, increasing the flow rate is proven to be the easiest way to decrease the analysis time (Thermo Electron Corporation, 2004). According to Liu, et al. (2007), flow rate act on the backpressure in the way of increasing the back pressure by increasing the flow rate. Apart from that, by increasing the flow rate, the resolution of the peak will be decreasing, lower the sensitivity.

4.6.2 Method II: Sodium 1-heptanesulphonate-acetonitrile as Mobile Phase

4.6.2.1 Chromatographic Responses Results of Cyanuric Acid

HPLC responses of cyanuric acid determined in mobile phase sodium 1-heptanesulphonate-acetonitrile (83:17, v/v) have been gathered and tabulated in Table 4.12. The factors that would influence the liquid chromatography responses significantly have been and discussed.

Table 4.12: Responses results of cyanuric acid in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

Run	Peak area, mAU*min	Retention time, min
1	1059.37	3.596
2	963.025	2.242
3	898.321	2.231
4	806.797	2.355
5	935.899	2.238
6	919.503	2.294
7	825.435	2.342
8	1440.95	3.317
9	1462.33	3.398
10	997.005	3.581
11	1124.92	3.607
12	1385.67	3.328

4.6.2.2 Analysis on Peak Area Response of Cyanuric Acid

Half-normal plot of peak area (Figure 4.13) has been provided to screen the standardise effects of all seven factors (percentage of sodium 1-heptanesulphonate, column temperature, equilibrium time, flow rate, detector response constant and detector wavelength) on the peak area response. Four dummy factors have been excluded. ANOVA results have also been tabulated as Table 4.13 by "Design Expert" software.

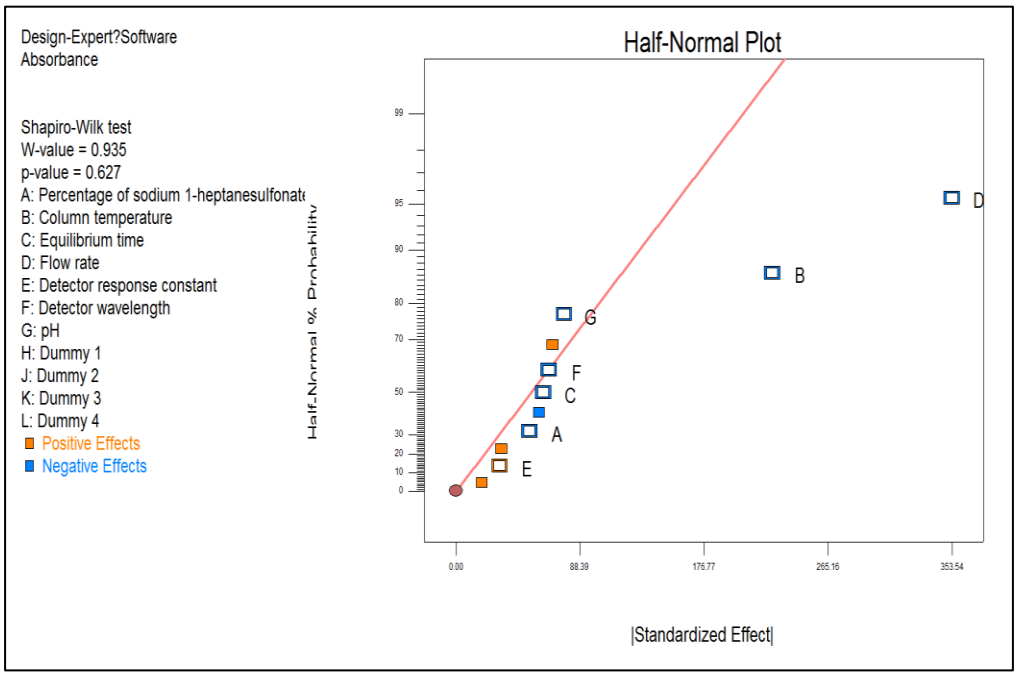


Figure 4.13: Half-normal plot of cyanuric acid peak area for all seven factors, excluding the dummy factors in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

Table 4.13: ANOVA results of peak area for all factorial models of cyanuric acid in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

Source	Sum of Square	df	Mean Square	F value	p-value Prob>F
Model	5.813E+005	7	83041.47	11.48	0.0164
A-Percentage of sodium 1-heptanesulphonate	8238.80	1	8238.80	1.14	0.3461
B-Column temperature	1.526E+005	1	1.526E+005	21.09	0.0101
C-Equilibrium time	11621.27	1	11621.27	1.61	0.2738
D-Flow rate	3.750E+005	1	3.750E+005	51.82	0.0020
E-Detector response constant	2883.90	1	2883.90	0.40	0.5621
F-Detector wavelength	13152.02	1	13152.02	1.82	0.2489
G-pH	17825.91	1	17825.91	2.46	0.1916
Residual	28943.12	4	7235.78		
Corrected Total	6.102E+005	11			

As monitored from the tabulated ANOVA results (Table 4.13), the p-value of the overall model, column temperature (factor B) and flow rate (factor D) are less than 0.0500, showing that the model and these factors are ascertained to be significant. A new selected significant factors half-normal plot and ANOVA results have been included as Figure 4.14 and Table 4.14 respectively so that a clearer overview can be established.

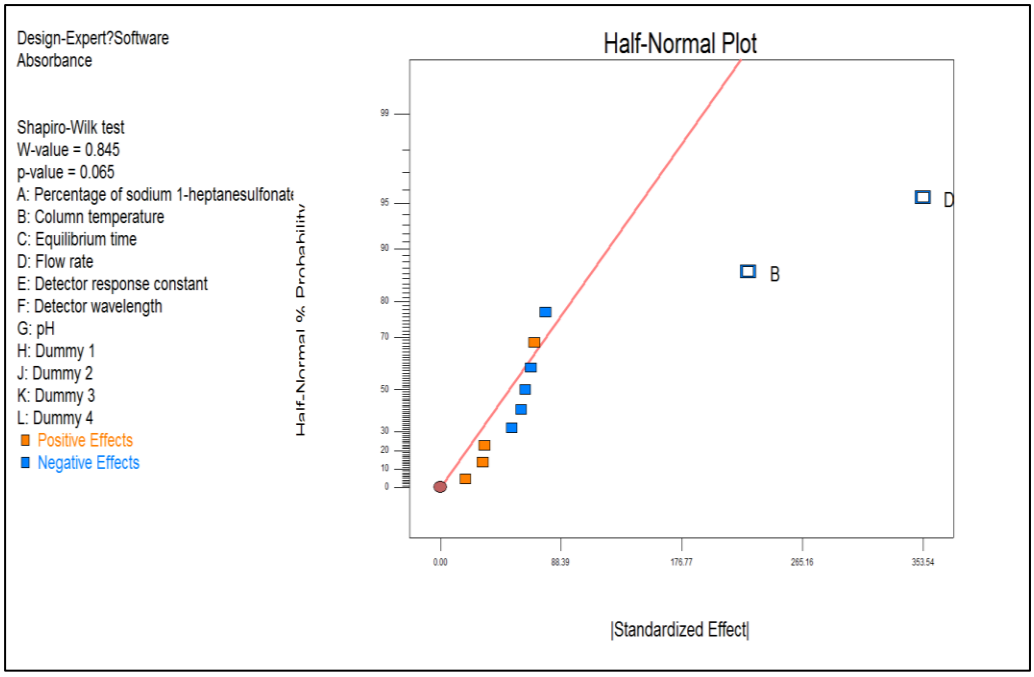


Figure 4.14: Half-normal plot of cyanuric acid peak area in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase for two selected factors

Table 4.14: ANOVA results of cyanuric acid peak area for selected factorial models in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

Source	Sum of Square	df	Mean Square	F value	p-value Prob>F
Model	5.276E+005	2	5.276E+005	28.72	0.0001
B-Column temperature	1.526E+005	1	1.526E+005	16.61	0.0028
D-Flow rate	3.750E+005	1	3.750E+005	40.83	0.0001
Residual	82665.01	9	82665.01		
Corrected Total	6.102E+005	11			

Half-normal plot has ordinary described as one of the effective graphical approaches to interpret the data obtained as insignificant effects are often found in a straight line, whereas significant factors generally deviate from the straight line (Dejaegher and Heyden, 2007). As observed from the half-normal probability plot of selected factors (Figure 4.17), it is quite apparent that factor

B (column temperature) and D (flow rate) deviate from the straight line as the other points lining on the line. This finding implied that these two factors are the significant factors and justified by their p-values which are smaller than 0.0500.

The normality assumption has been fulfilled as points of factors were distributed along the fitting line as shown in the normal plot of residuals (Figure 4.15), disclosed that there is no bias or outlier existed and the results are all normally distributed.

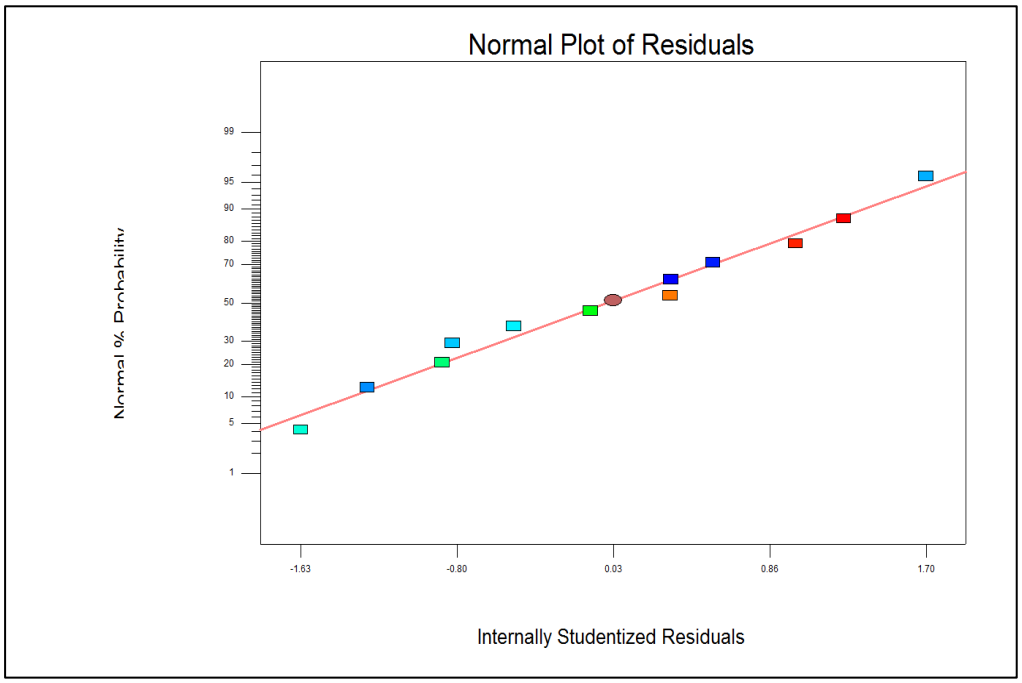


Figure 4.15: Normal plot of residuals of cyanuric acid peak area in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

Equations related to the two significant factors, column temperature and flow rate have been learned, so that the peak area results can be forecasted by just

substituting the known values of column temperature and flow rate for future experiments.

Peak area = 3512.57208 - 22.55275 * Column temperature - 767.72083* Flow rate

4.6.2.3 Analysis on Retention Time Response of Cyanuric Acid

Half-normal plot of retention time response has been shown as Figure 4.16, screening the standardise effects on the retention time of cyanuric acid detected. ANOVA results for all seven factors have been evaluated and tabulated as Table 4.15. The effects of dummy factors have been neglected.

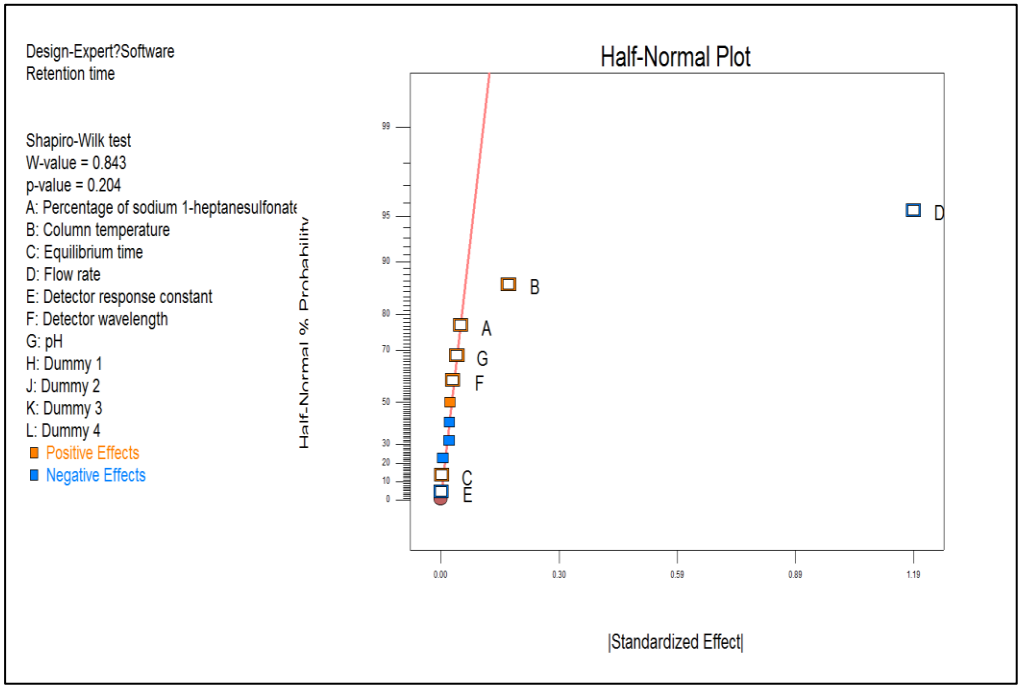


Figure 4.16: Half-normal plot of cyanuric acid retention time in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase for all seven factors, excluding the dummy factors

Table 4.15: ANOVA results of retention time for all factorial models of cyanuric acid in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

Source	Sum of Square	df	Mean Square	F value	p-value Prob>F
Model	4.33	7	0.62	277.72	< 0.0001
A-Percentage of sodium 1-heptanesulphonate	7.450E-003	1	7.450E-003	3.34	0.1415
B-Column temperature	0.087	1	0.087	38.98	0.0034
C-Equilibrium time	1.875E-005	1	1.875E-005	8.413E-003	0.9313
D-Flow rate	4.23	1	4.23	1898.28	< 0.0001
E-Detector response constant	4.083E-006	1	4.083E-006	1.832E-003	0.9679
F-Detector wavelength	2.730E-003	1	2.730E-003	1.23	0.3305
G-pH	4.921E-003	1	4.921E-003	2.21	0.2115
Residual	8.914E-003	4	2.229E-003		
Corrected Total	4.34	11			

Based on the ANOVA results, the p-value of column temperature and flow rate is smaller than 0.0500, specified that these two factors are the significant factors that affect the retention time response remarkably. A half-normal plot and ANOVA results for the selected factors have been assembled by eliminating the insignificant factors and attached as Figure 4.17 and Table 4.16 respectively.

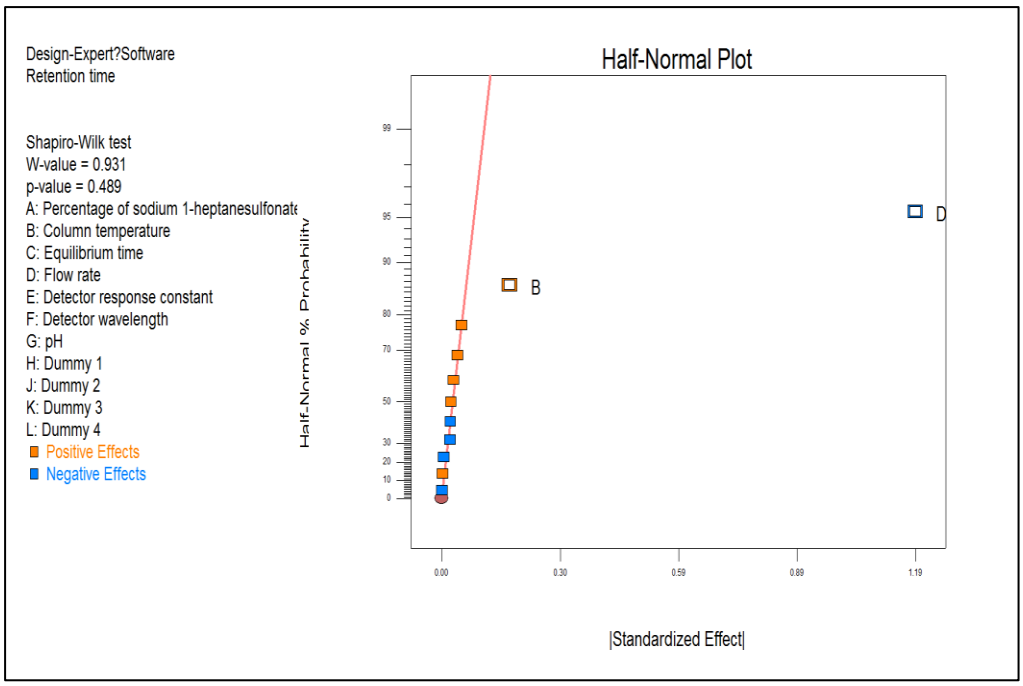


Figure 4.17: Half-normal plot of cyanuric acid retention time in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase for two selected factors

Table 4.16: ANOVA results of cyanuric acid retention time for selected factorial models in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

Source	Sum of Square	df	Mean Square	F value	p-value Prob>F
Model	4.32	2	2.16	808.22	< 0.0001
B-Column temperature	0.087	1	0.087	32.52	0.0003
D-Flow rate	4.23	1	4.23	1583.91	< 0.0001
Residual	0.024	9	2.671E-003		
Corrected Total	4.34	11			

Normal plot of residuals for retention time (Figure 4.18) has been estimated to ensure that the results for retention time response fulfilled the normality assumption, explained by the data points that distributed along the fitting line.

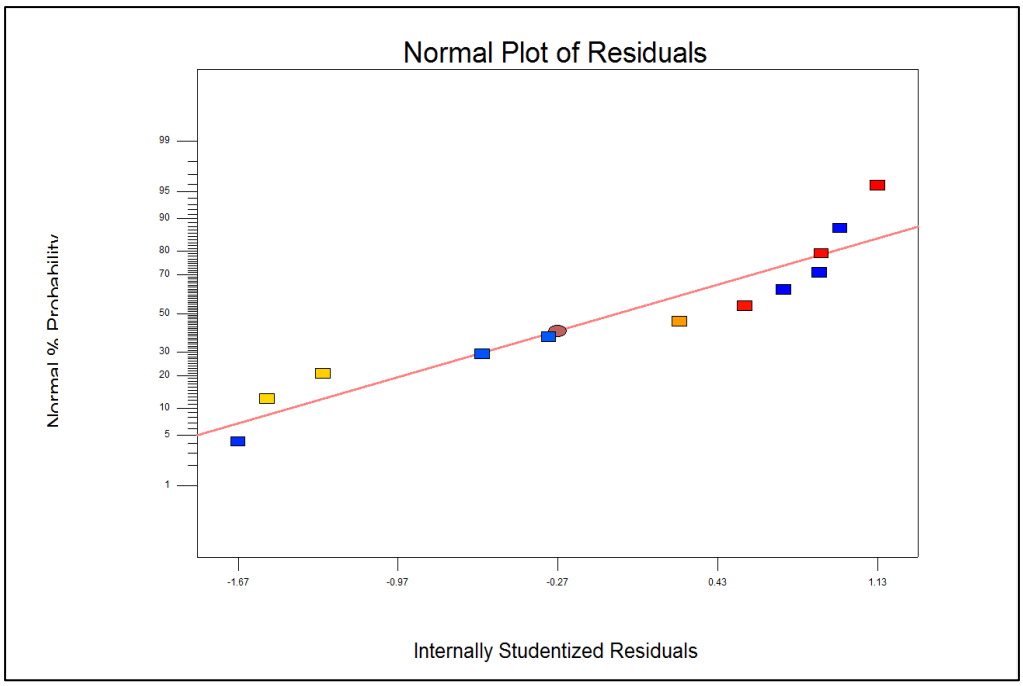


Figure 4.18: Normal plot of residuals of cyanuric acid retention time in method I: sodium 1-heptanesulphonate-acetonitrile as mobile phase

It has been validated that two significant factors contributed to the variation of results obtained in the CYA retention time responses, including the factors: column temperature and flow rate. Equations related to these factors have been introduced.

Retention time = 8.30442 +0.017017 * Column temperature -5.93750 * Flow rate

4.6.2.4 Discussion on Significant Factors Determined

Table 4.17 which included all significant factors for each of the studied responses has been attached to summarise the results obtained.

Table 4.17: Summarised significant factors for the responses of cyanuric acid in method II: sodium 1-heptanesulphonate-acetonitrile as mobile phase

HPLC responses	Significant factors
Peak area	• Column temperature • Flow rate
Retention time	• Column temperature • Flow rate

There are two major factors that significantly affect the HPLC responses of cyanuric acid determined in the mobile phase, sodium 1-heptanesulphonate-acetonitrile (83:17, v/v), including column temperature and flow rate.

The essential parameter that significantly impact on the liquid chromatography responses is column temperature. As shown in Table 4.17, column temperature has been discovered to be the significant factor that manages to influence responses of HPLC notably. By raising the temperature, the selectivity has been decreased and overall retention in RP-HPLC has reduced. Fundamentally, increasing temperature will also increase the solubility of the solute in mobile phase, decrease the solvent viscosity and reduce the retention. The effect is even more serious as ion-pair reagent is involved in this determination method. By altering the temperature, the ion-pair reagent is

being destabilizing and even disrupting the structure or characteristic of ion-pair reagent (Chen, Mant and Hodges, 2003).

Apart from column temperature, flow rate is another significant factor that alters the HPLC responses severely, especially for the retention time response. Same old theory can be implemented in this case, increasing the flow rate will tends to increase the plate height, increase the resolution of response and decrease the analysis time. An interesting phenomenon has been observed, in which flow rate also influences the peak area response crucially. It can be explained that HPLC detectors are operated in a way that output signal is shown as the number of analyte detected at a given time. In short, peak area is defined to be the integration of signal over time. Thus, by altering the flow rate, the peak area will also be altered (Nueu, U.D., 2002).

4.6.3 Comparison between Two Determination Methods

A clear comparison between the robustness of two sets of determination methods has been tabulated as Table 4.18.

Table 4.18: Differences between robustness results of two sets of determination methods

Mobile phase	**Method I: Water**	**Method II: Sodium 1-heptanesulphonate-acetonitrile (83:17, v/v)**
Significant factors	Type of water, column temperature, flow rate	Column temperature, flow rate
p-values of overall model	Peak area = 0.3009 Retention time = 0.0938	Peak area = 0.0164 Retention time = <0.0001

From Table 4.18, the determination method that applies water as the mobile phase is found to be more robust than sodium 1-heptanesulphonate-acetonitrile. By referring the p-values of overall model, the overall model of method utilising water as mobile phase is insignificant. It can be speculated that by associating all factors, the effect of each parameter has been counterbalanced by each other, the whole system does not show significant effect of factors. Thus, specific parameters have to be selected manually to outline the factors that more significantly affect the responses. On the contrast, significant factors have been detected in method employing sodium 1-heptanesulphonate-acetonitrile in the overall screening process. This indicates that there are undoubtedly some significant factors that capable of influencing the responses result more crucially compared with other factors. Hence, determination method employing sodium 1-heptanesulphonate-acetonitrile as mobile phase has concluded to be less robust.

4.7 Future Research Work

As the effect of cyanuric acid on living health has become more adverse and gaining more and more worldwide attention, the determination and validation methods of cyanuric acid are manifested to be imperative. Future research works should include the following so that a more complete analysis of cyanuric acid is being carried out:

I. Response Surface Method (RSM) should be performed after the Plackett-Burman design if and only if the significant factor is two or

more than two. This is to ensure the rigidity of the investigated significant factors as RSM is a software utilised just to analyse the relation between the significant factors.

II. Analysis of sample based cyanuric acid should be involved so to monitor the true conditions and responses of HPLC in the present of sample as the parameters being altered.

III. Determination of melamine-cyanurate complex should also be covered in the project as this insoluble complex is the complex that leads to adverse health problem.

IV. Other CYA detection method should also be validated so that a more comprehensive overview and analysis can be acquired.

Chapter 5

CONCLUSION

In this project, cyanuric acid has been detected by two different determination methods. In method I, mobile phase water has been used. Sodium 1-heptanesulphonate-acetonitrile (83:17, v/v) mobile phase has been utilised for method II. These determination methods have been validated on linearity, sensitivity, precision and robustness test based on International Conference on Harmonization ICH Q2 (R1) guidelines.

The UV spectrums of cyanuric acid in both determination methods have been obtained. Maximum absorption peak observed has been chosen to be the chromatographic detection wavelength. For determination method I that applied distilled water as the mobile phase, the detection wavelength has been set at 210 nm. For method II that employed sodium 1-heptanesulphonate-acetonitrile as mobile phase, the detection wavelength has been set at 213 nm. The chromatograms of cyanuric acid have also been generated. As a result, the retention time was observed at 2.875 min and 2.806 min for method I and II, respectively. Linearity of peak area was tested in the range 1 – 20 µg/mL. The regression coefficients of cyanuric acid were excellent, in which 0.9999 for method I and 0.9981 for method II. The detection limit (LOD) and quantification limit (LOQ) were determined by analysing the calibration line by LINEST function in EXCEL. For cyanuric acid detected in method I, the LOD and LOQ were 0.02 and 0.06 µg/mL respectively. As for LOD and LOQ of cyanuric acid in method II, the results were 0.08 and 0.25 µg/mL

respectively. Precision is validated on the retention time response of intra-day (precision) and inter-day (intermediate precision) injections. Both precision and intermediate precision of retention time were below 1.3% RSD for method I and below 2.7% RSD for method II, respectively.

Plackett–Burman design screens two base level ("+" and "-") of each factor. It was used to carry out the robustness test in this project, in which 11 (4n-1) factors were assessed with 12 (4n) experiments and the results were evaluated by "Design Expert" software. Combination methods of graphical (half-normal plot, normal plot) and statistical (ANOVA) have been implemented to analyse the chromatographic responses, peak area and retention time results. For cyanuric acid determined in method I, seven factors were utilised, including types of mobile phase, mobile phase pH, column temperature (^{o}C), flow rate (mL/min), detection wavelength (nm), equilibrium time (min) and detector response constant (min). The other factors were represented by dummy. The significant factors were discovered to be the type of water as mobile phase, column temperature and flow rate. As for method II, the seven factors being analysed are percentage of sodium 1-heptanesulphonate, mobile phase pH, column temperature (^{o}C), flow rate (mL/min), detection wavelength (nm), equilibrium time (min) and detector response constant (min). The significant factors determined in this method were column temperature and flow rate. It is outlined that determination method I: water as mobile phase is more robust than method II, more appropriate for cyanuric acid detection as it manages to avoid problems of inter-laboratory and inter-instrumental studies.

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