ปริมาณวิเคราะห์ของสีย้อมทำเครื่องหมายจากคาร์ดานอลและไนโตรแอนิลีนในดีเซล โดยใช้ไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี

นางสาวปวีณา เอกพรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549

QUANTITATIVE ANALYSIS OF MARKER DYES FROM CARDANOL AND NITRO ANILINE IN DIESEL USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Petrochemistry and Polymer Science
Faculty of Science
Chulalongkorn University
Academic Year 2006

Thesis Title	Quantitative analysis of marker dyes from cardanol and nitro aniline in diesel using high performance liquid chromatography
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Field of Study	Petrochemistry and Polymer Science
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ได้ทำการศึกษาการวิเคราะห์ปริมาณสีย้อมทำเครื่องหมายจากคาร์ดานอลและไนโตรแอนิลีนในดีเซล โดยเทคนิคไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี ใช้เครื่องตรวจวัดแบบการดูดกลืนแสงอัลตราไวโอเลตและแสงขาว จากการเตรียมดีเซลตัวอย่างโดยซิลิกาคอลัมน์ ซึ่งมีเฮกเซนและโทลูอีนเป็นตัวชะ ตามลำดับ แล้วทำการวิเคราะห์ปริมาณสีย้อมด้วยสภาวะที่เหมาะสม คือ คอลัมน์ C_{18} ขนาด 250 × 4.6 มิลลิเมตร วัฏภาคเคลื่อนที่คือ เมทานอลกับ 0.2% แอซิติกแอซิดในน้ำ ด้วยอัตราส่วน 95 ต่อ 5 ที่อัตราการไหล 0.80 มิลลิลิตรต่อนาที ตรวจวัดที่ความยาวคลื่น 390 นาโนเมตร กราฟเทียบมาตรฐานให้ค่าสัมประสิทธิ์สหสัมพันธ์ที่ดี มีค่า 0.9979 ในช่วงความเข้มข้น 1.00-12.0 มิลลิกรัมต่อลิตร จากนั้นทดสอบความถูกต้องและความเชื่อถือได้ของวิธีวิเคราะห์ที่ได้ โดยการประเมินค่าร้อยละของการคืนกลับ และค่าความเที่ยงของวันเดียวกันและต่างวันกัน พบว่าวิธีการวิเคราะห์มีประสิทธิภาพดี และสามารถวิเคราะห์ปริมาณสีย้อมทำเครื่องหมายชนิดนี้ในดีเซลได้ต่ำถึง 2.50 มิลลิกรัมต่อลิตรของดีเซล นอกจากนี้ยังได้ศึกษาถึงปริมาณของสีย้อมในดีเซลในช่วงระยะเวลา 3 เดือน พบว่าปริมาณสีย้อมมีความคงตัว ดังนั้นเทคนิคไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี จึงเหมาะสมสำหรับปริมาณวิเคราะห์ของสีย้อมทำเครื่องหมายจากคาร์ดานอลและไนโตรแอนิลีนในดีเซล ด้วยเวลาวิเคราะห์ที่เร็ว ปริมาณสารตัวอย่างน้อย และสามารถนำผลปริมาณวิเคราะห์ที่ได้ไปยืนยันผลคุณภาพวิเคราะห์จากงานภาคสนามได้อีกด้วย

สาขาวิชา...ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์...ลายมือชื่อนิสิต.....

ปีการศึกษา.....2549.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

477 23670 23: MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
KEYWORDS: MARKER DYES/ CARDANOL/ HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

PAWEENA EKKAPHAN: QUANTITATIVE ANALYSIS OF MARKER DYES FROM CARDANOL AND NITRO ANILINE IN DIESEL USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. THESIS ADVISOR: ASSOC. PROF. AMORN PETSOM, Ph.D., 73 pp.

The quantitative analysis of marker dyes from cardanol and nitro aniline in diesel using high performance liquid chromatography (HPLC) with UV-VIS detector was studied. The sample preparation was performed using silica column chromatography. The column was eluted with hexane and toluene, respectively. The quantitative determination of marker dyes was carried out using C_{18} column (250 × 4.60 mm), methanol/0.2% acetic acid in water with ratio 95:5 as mobile phase, flow rate 0.80 mL/min, wavelength detection at 390 nm. Excellent linear coefficient of 0.9979 was obtained by constructed standard calibration in a range of 1.00-12.0 mg/L. Satisfactory method efficiency was evaluated from recovery and intra- and intermediate precision and the quantitation limit is 2.50 mg/L of marker dyes in diesel fuel. Moreover, the measurement of the amount of this marker dye in diesel stored for 3 months was studied. It was found that marker dye was stable in diesel. Therefore, the developed HPLC method can be used for quantitative determination of marker dyes from cardanol and nitro aniline in diesel with short analysis time, small volumes of sample, and provide quantitative measurement of marker dye to confirm qualitative measurement in the field test.

Field of study..Petrochemistry and Polymer Science.. Student's signature.....
Academic year.....2006..... Advisor's signature.....

ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation and deepest gratitude to my advisor, Assoc. Prof. Dr. Amorn Petsom, for his discerning suggestion, encouragement, and assistance throughout this study. Special thanks are extended to the thesis committee for their invaluable comments.

In addition, I wish to thank the Program of Petrochemistry and Polymer Science, the Department of Chemistry, the Faculty of Science, Chulalongkorn University, for all facilities and grant. Moreover, this work is granted by the Graduate School, Chulalongkorn University, and I am much indebted.

My special thanks also extended to the Institute of Biotechnology and Genetic Engineering (IBGE) for the information and instrumentation used for my overall study.

I am grateful to all members of IBGE for their helpfulness and suggestions. In particular, special thanks go to Supansa, Wasinee, Unchalee, and my colleagues for moral supports, cheerful willingness, heartfelt friendship and encouragement.

Finally, I am eternally grateful to my beloved mother, my grandmother, my aunt, and the rest of my family members for their encouragement and understanding throughout the course of my study.

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LIST OF ABBREVIATIONS AND SYMBOLS

CNSL	cashew nut shell liquid
GC	gas chromatography
HPLC	high performance liquid chromatography
I.D.	internal diameter
LOD	limit of detection
LOQ	limit of quantitation
MDL	method detection limit
MQL	method quantitation limit
MS	mass spectrometry
NMR	Nuclear Magnetic Resonance Spectrometer
PDA	photodiode array
PTFE	Polytetraflouroethylene
RSD	relative standard deviation
SD	standard deviation
UV-VIS	ultraviolet-visible
μL	micro-liter
λ_{max}	Maximum wavelength detection
g	gram
m/z	mass to charge ratio
mg	milligram (s)
mg/L	milligram per liter
mL	milliliter
mm	millimeter
nm	nanometer
ppm	parts per million
R^2	correlation coefficient
R_s	resolution
t_R	retention time

CHAPTER I

INTRODUCTION

1.1 State of problem

At present, large quantities of fuel oils have been consumed for several applications throughout the world. Normally, fuel oils have to be taxed according to the government rates, which are depended on the types and the purposes of the fuel oils. As the different tax rates, the government encountered many problems for instance smuggling of untaxed oils or blending lower priced products such as kerosene, heating oil or diesel fuel into higher priced products and selling the inferior product at a premium price. It is essential to develop method of marking and identifying petroleum products to distinguish them from seemingly identical products exists for these reasons, including to identify various grades of fuels, to distinguish manufacturer's brands, to differentiate similar fuels taxed at different rates and to decrease adulteration, misuse and tax evasion of petroleum products available in the market.

To solve these problems, marker systems have been suggested as methods to identify types of fuel oils and to monitor the tax classification of petroleum products. Several markers systems have been reported and used for a long time. Each marker had the different properties according to the purposes and the matrices. Nowadays, marker dyes were successfully established, for example, quinizarin derivatives , azo compounds, fluorescent markers [Orelup 1988, Friswell *et al.* 1993, Smith 1996, Friswell *et al.* 1999]. The marker dyes must be soluble in hydrocarbon-based nonpolar solvents, and were added to fuel oils at concentrations less than 10 parts per million (ppm) [Orelup 1988]. There are various commercial marker dyes. Red dyes are often various diazo dyes, i.e. Solvent Red 19, Solvent Red 24, and Solvent Red 26. Anthraquinone dyes are used for green and blue shades, i.e. Solvent Green 33, Solvent Blue 35 and Solvent Blue 26. The azo compounds that were aniline derivatives were usually used as marker in fuel oils. Their structures properties have

high electron density functional groups that absorb ultraviolet and visible light. These reasons can easily detected by photometric assay.

Spectrophotometric method has been used for qualitative determination in the field [Orelup 1988, Nowak 1990, Friswell *et al.* 1992, Hallisy 1993, Brenzinger *et al.* 1996, Pual *et al.* 2001]. This method is simple, and low cost. However the drawback of this technique has existed when the constituents of fuel oil have changed. This method also does not provide a good quantitative measurement of marker dye concentration in the field. Quantitative determinations are particularly important in case where qualitative result is suspected, e.g. dilution of a higher-taxed fuel with a lower-taxed fuel. However, laboratory verification is needed to confirm the marker dye concentration. Therefore, a suitable was developed to provide more efficient qualitative and quantitative determination.

There are quantitative analytical methods, i.e. High Performance Liquid Chromatography (HPLC) [Henricsson *et al.* 1996, Timkovich 2000, Pielesz *et al.* 2002, Selvaggini *et al.* 2006], Gas Chromatography (GC) [Zoumalan 1993] or GC-MS techniques [Timkovich 2000] which have been used for marker dyes determination. HPLC technique is the most popular for routine analysis of marker dyes. This effective method can separate and detect a substance at very low concentration (less than 1 ppm) in complex mixtures. Moreover, it is suitable for separating non-volatile species or thermal-unstable compounds. Only very small volumes of sample are needed for analysis.

This research involved quantitative evaluation of marker dyes from cardanol and nitro aniline in diesel using HPLC technique. Novel marker dyes in this research was obtained from Thailand [Suwanprasop *et al.* 2004]. These marker dyes were synthesized by coupling the naturally occurring n-alkylphenol, cardanol, with aniline and its derivatives. Cardanol is obtained from cashew nut shell liquid (CNSL), which is an agricultural byproduct from the manufacturing of cashew nuts and commercially available in the southern part of Thailand. This quantitative method provides accurate concentrations of these novel marker dyes and confirms the qualitative measurement of field test.

1.2 The objective of this research

The objective of this research is to develop a method for quantitative determination of marker dyes derived from cardanol and nitro aniline in diesel fuel using HPLC.

1.3 The scope of this research

The scope of this work is to develop a HPLC method for determination of marker dyes derived from cardanol and nitro aniline in diesel fuel. HPLC separation was carried out by varying the flow rate, mobile phase type and mobile phase percentages in order to obtain the optimum HPLC condition. Sample preparation procedures were developed using column chromatography. Validation of HPLC method will be studied on accuracy, precision, the limits of detection and quantitation, and stability. Moreover, the developed HPLC method will be applied for quantitative analysis of these marker dyes in diesel fuel, and the determined amount of these marker dyes obtained from HPLC and UV-VIS will be compared for verification the results in field test.

CHAPTER II

THEORY

2.1 Azo dyes

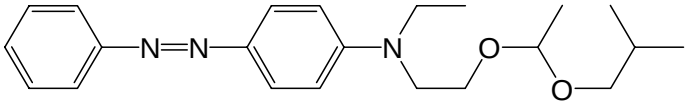
A dye is defined as an organic compound lending visible color when dissolved in the dyed product.

Dyes used for coloring petroleum products such as gasoline, kerosene, and diesel fuel are carried out in order to render them identifiable, and thus make difficult the possible tax evasions, which can result from utilizing said products for purpose that differ from ones for which they are taxed. The coloring materials must satisfy various requirements as the following [Orelup 1988]:

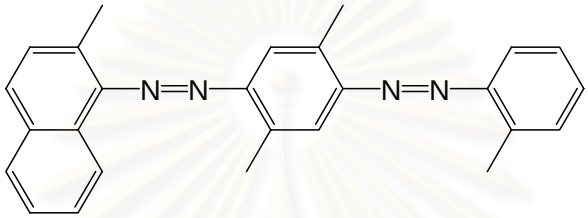
1. to have a high dyeing power;
2. to have a sufficient solubility in organic solvents and fuel oils, in the presence or absence of antiknock agents;
3. to have a high diffusion index;
4. to contain little or no by-product insoluble in fuels;
5. to leave only a minimum deposit of sludge in the engines;
6. to have a proper fastness to light during storage;
7. to be compatible with additives and not cause difficulties during combustion;
8. to be brittle but no powders in the solid form;
9. to be sufficiently fluid to be solubilized in organic solvents, if it is used in the form of concentrated solution;
10. to must primarily be extractable with difficulty from the system in which it is dissolved.

Examples of commercial oil-soluble dyes were shown as;

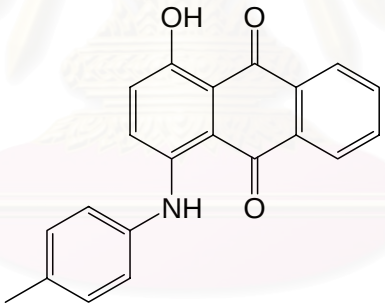
Solvent Yellow 124 (Sudan 455)



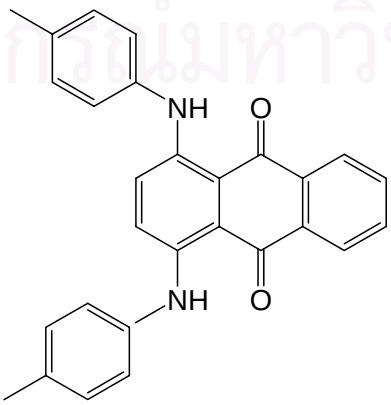
Solvent Red 26 (C.I. 26120)



Solvent Blue 90 (C.I. 60725)



Solvent Green 3 (C.I. 61565)



Azo dyes are the largest and the most important class of dyestuffs, which have been added to petroleum products marking. The general formula of azo compound is Ar-N=N-Ar' which is prepared by coupling a diazotized aromatic amine with an aromatic ring containing a powerfully electron-releasing group, generally –OH, $-NR_2$, -NHR, or $–NH_2$. The diazotized aromatic amine which is dissolved or suspended in cold aqueous mineral acid is treated with sodium nitrite. Diazonium salt is used immediately after preparation because it slowly decomposes even at ice-bath temperatures.

Coupling is an electrophilic aromatic substitution in which the diazonium ion is the attacking reagent. Substitution usually occurs para to activating group. Coupling with phenols, in general, is carried out in a mild alkaline solution, and with amine in a mild acidic solution. It is the most important that the coupling medium be adjusted to the right degree of acidity or alkalinity by the addition of the proper amount of hydroxide or salt like sodium acetate or sodium carbonate [Norman 1987].

2.2 Marker dyes

A marker is defined as a substance which can be used to tag petroleum products for subsequent detection and is colorless in the petroleum products. The marker is dissolved in a liquid to be identified, and then subsequently detected by performing a simple chemical or physical test on the tagged liquid [Orelup 1988].

The important characteristics of certain desirable markers for petroleum include:

1. are entirely foreign to the liquids;
2. can be supplied as highly concentrated solutions in petroleum-compatible solvents;
3. are easily detected by a simple field test;
4. are not obscured by unstable natural components of the liquids;
5. are stable over the anticipated storage life of the tagged liquid (usually three to six months); and
6. have identities which can be confirmed by laboratory methods.

Dyes and markers are need to clearly distinguish chemically or physically similar liquids. Dyes alone are not always adequate to securely and reliably identify liquids. Many dyes are easily removed from products by unauthorized persons. Furthermore, dyes can be occurred by other natural or added substances (particularly dyes present at low concentration, usually one to ten part per million in a mixture of fuels). Because dyes alone have these shortcomings, a combination of a dye and a marker often is used to tag liquid petroleum products.

2.3 Sample pretreatment

The quantitative determination of the added marker dyes in fuel oils is difficult caused by the complicated matrix of petroleum products. The marker dyes are added in fuel oils at minute levels (1-10 ppm), they provide substantially no color to the petroleum product, but undergo a reaction during a detection procedure. The suitable sample pretreatment method is the important step before determines the marker dyes (analytes) following used analytical method. There are two main preparation procedures as the following:

2.3.1 Liquid-liquid extraction

The basic principle of liquid-liquid extraction involves the contracting of a solution with another solvent that is immiscible with the original. The solvent is also soluble with a specific solute contained in the solution. Two phases are formed after the addition of the solvent, due to the differences in densities. The solvent is chosen so that the solute in the solution has more affinity toward the added solvent. Therefore the mass-transfer of the solute from the solution to the solvent occurs.

Azo dyes can change color with a change in pH, like an acid-base indicator, because the chromophoric system in changed by acid-base reaction. The loss of proton changes the electronic structure of these dyes resulting in a change of color. As a result, azo dyes are good markers in fuel oils, which can be detected easily by extraction with simple acid or basic solution. There are two solvent extraction mode used for prepared fuel oil samples before analyzed marker dyes as below:

2.3.1.1 Base extraction [Hallisy 1993, Frederico *et al.* 1999]

Base extraction of the markers from the tagged fuel oils may conveniently be carried out with a dilute, e.g., 1-3% aqueous solution of an alkaline such as NaOH or KOH. Preferably, the extraction solution also includes a water-miscible, petroleum-immiscible or organic solvent, such as methanol. Moreover, the extraction mixture comprises, between various volume of water, between various volume of an water-soluble alkyl amine and co-solvent. The water-soluble alkyl amines include, such as methoxy propyl amine, aminopropyl morpholine, and methoxy ethoxy propylamine and mixtures thereof. Suitable co-solvents include alcohols, such as ethyl alcohol; glycols, such as ethylene glycol, diethylene glycol, propylene glycol, polypropylene glycol; glycerine; esters, such as methyl lactate, ethyl lactate and butyl lactate; sulfolane; dimethyl sulfoxide (DMSO), and dimethyl formamide (DMF). Preferred solvents are the more oxygenated materials, such as glycerine, diethylene glycol and propylene glycol. The base forms a salt with the phenolic-OH, resulting in development of the color and also changing the solubility of the marker so that it is substantially less soluble in petroleum and substantially more soluble in aqueous medium. The markers will be extracted by the aqueous layer and colored by reaction with the extraction mixture.

2.3.1.2 Acid extraction [Friswell *et al.* 1996, Frederico *et al.* 1999]

Acid extraction of the markers carried out with a dilute acidic solution, e.g. a 10% HCl or formic acid solution. It is desirable that acid-extractable markers be available, particularly markers which develop a color sufficiently strong to be clearly differentiated from any background color which might develop from acid reaction with petroleum impurities or develop a color which is sufficiently different from any such background color.

Spectrometric equipment may be used to quantify the amount of markers in aqueous layer that extracted from both extraction modes. The color that is produced is relatively quantitative. The test is not quantitative in the strict sense that exact levels of markers can be tested in tagged fuel oils. Due to the nature of

petroleum products are mixtures of a wide variety of compounds. The level of impurities extractable by extraction solution may vary.

2.3.2 Column chromatography (Adsorption chromatography)

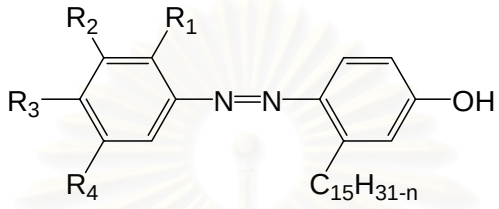
The separation mechanism in adsorption chromatography is based mainly on differences between the adsorption affinities of the sample components for the surface of an active solid. Column chromatography consists of a column of particulate material such as silica or alumina that has a solvent passed through it at atmospheric or low pressure. The separation can be liquid/solid (adsorption) or liquid/liquid (partition). The columns are usually glass or plastic with sinter frits to hold the packing. Most systems rely on gravity to push the solvent through. The sample is dissolved in solvent and applied to the front of the column. The solvent elutes the sample though the column, allowing the components to separate based on adsorption (alumina, hydroxyapatite) or partition (cellulose, diatomaceous earth). The mechanism for silica depends on the hydration. Traditionally, the solvent was non-polar and the surface polar, although today there are a wide range of packing including bonded phase systems. Bonded phase systems usually utilize partition mechanisms rather than adsorption. The solvent is usually changed stepwise, and fractions are collected according to the separation required, with the eluted solvent usually monitored by TLC.

The nature of fuel oils is complicated medium which mixed widely variety of compounds. Therefore, the preteatment of sample is important before analyze the accurate amount of marker dyes. This method separates the added markers from fuel oils by passing through a bed of column packing which selectively retains the marker dye on the packing, e.g. silica [Nowak 1990, Timkovich 2000]. The suitable solvent elutes markers from column. The marker dye is thereby separated from substantially the remainder of the sample. The amount of marker dye will be quantitatively determined by suitable analytical method.

2.4 Literature review

2.4.1 Literature reviews on *marker dyes from cardanol and aniline derivatives*:

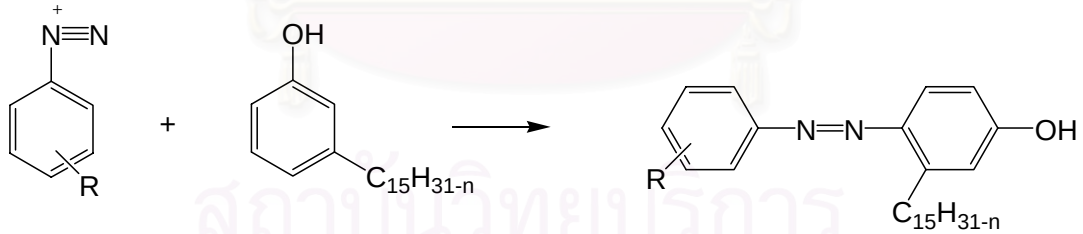
Novel marker dyes in this research is available from Thailand [Suwanprasop *et al.* 2004]. These marker dyes have the general formula:



Where R_{1-4} = $-NO_2$, -Cl, $-OCH_3$, -H, etc.
n = 0, 2, 4, and 6

Figure 2.1: Structure of aniline-cardanal azo.

These marker dyes were synthesized by coupling reaction of cardanol which was obtained from partially purification of decarboxylated cashew nut shell liquid, with diazonium salts of aniline derivatives. Overall reaction was illustrated below:



Aniline derivatives Cardanol Novel nitro aniline-cardanol azo dye

Where R = $-NO_2$, $-Cl_2$, $-OCH_3$, -H, etc.
n = 0, 2, 4, and 6

Figure 2.2: The coupling reaction of cardanol and aniline derivatives.

These synthetic marker dyes provided invisible colors in gasoline and diesel fuel at level of 2-5 ppm. The detection of these marker dyes in fuel oils is performed by extraction the dyed fuel oils with 50% ethylenediamine in solution of 1:1 ethylene

glycol and methanol. The amount of marker dyes measured using an UV/VIS spectrophotometer.

2.4.2 Literature reviews on *analytical procedure of azo dyes in fuel oils*:

Several methods were described in the literatures for analysis of azo dyes in fuel oil by spectrometric and chromatographic techniques. Spectrometric methods have been commonly used for qualitative determination in the field. However, the chromatographic method provided good qualitative and quantitative analysis.

Zoumalan S. [1993] determined the trace concentration of nitrogen-bearing marker dye (0.25-5.0 ppm), in gasoline. The nitrogen-bearing marker dye was selected from the group consisting of 1-(4-morpholino)-3-(alpha naphthylamino)-propane and 1-(4-morpholino)-3-(beta naphthylamino)-propane. A sample of the marked gasoline to be analyzed was prepared by adding a know volume of an internal standard, preferably trioctylamine, to sample. The amount of these dyes was performed by gas chromatograph (GC) with a nitrogen-phosphorus detector (NPD). A regression analysis of the experimental data provided a linear relationship with a 0.9997 correlation coefficient. The concentration of the marker dye was approximately the same as that which was originally present in the gasoline.

Henricsson S. and Westerholm R. [1996] used liquid chromatographic method to determine the concentration of a marker Solvent Yellow 124, *N*-ethyl-*N*-[2-(1-isobutoxyethoxy)ethyl](4-phenylazophenyl)amine, in diesel. The structure of this dye is shown below:

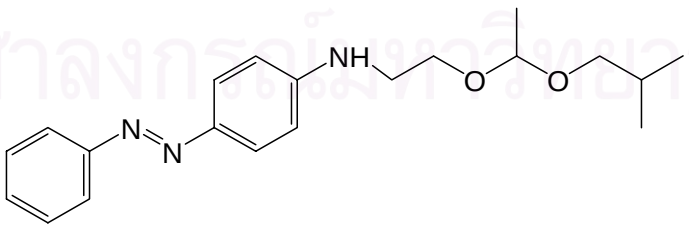


Figure 2.3: Structure of Solvent Yellow 124.

The dyed diesel sample was passed through deactivated silica column, using *n*-hexane and 25% dichloromethane in *n*-hexane as eluents. The eluted dye was then subjected to HPLC analysis. The separations were carried out on a YMC C_8-AQ column (5 μm, 250 x 4.6 mm I.D.). The mobile phase used was acetonitrile-25 mM ammonium acetate buffer (pH 4.8) at ratio 72:28 v/v containing 5 mM diethylamine. The wavelength detection was 420 nm. Chromatography was performed using a mobile-phase flow-rate of 2.0 mL/min. This analytical system had linearity in the range 0.5-100 ng and found to be linear with a 0.996 correlation coefficient. The limit of detection was in the range 60-80 ng, and the limit of quantitation was 0.2 ng. This method was robust and was adapted by routine laboratory; the throughput was 180 samples per day.

Timkovich R. [2000] reported alternative analytical methods for determining the content of regulatory red dye, Solvent Red 26, in diesel. The general structure of this dye is shown below:

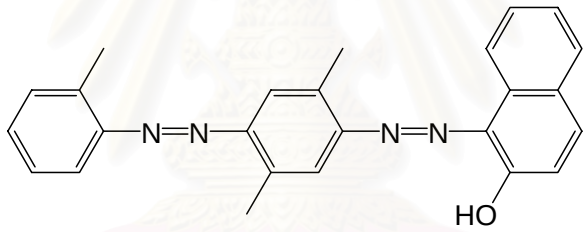


Figure 2.4: Structure of Solvent Red 26.

A preconcentration step was consisting of adsorption chromatography on silica gel, using chloroform and *n*-hexane as eluents. Dye fractions were then determined the concentration by HPLC and GC-MS for increasing the sensitivity of the method. HPLC was performed on Whatman Partil silica gel column (5 μm, 250 x 4.6 mm I.D.) eluted at 1 ml/min using UV-VIS detector set at 519 nm. The mobile phase system was chloroform-hexane (3:7). HPLC and GC-MS greatly extended the lower limit of dye detection. This study achieved the preferred method, HPLC, based upon the combined considerations of column maintenance, analysis time, sensitivity, selectivity, and overall costs.

Pauls T. D. *et al.* [2001] developed a method for identifying an invisibly tagged liquid petroleum hydrocarbon, remarkably using visible dyes consisting essentially of least one anthraquinone and diazo dye, each at level below 1 ppm that they could not be visually detected by human eyes. These dyes are hydrocarbon-soluble and have maximum absorption in the 550-700 nm visible wavelength range. The dye content was determined quantitatively by spectrometric measurement with the SpecTrace^{TM} analyzer. This method provided true quantitative measurements of marker concentrations in the field.

CHAPTER III

EXPERIMENTAL

3.1 Instrument and apparatus

3.1.1 High Performance Liquid Chromatography (HPLC): a ThermoFinnigan HPLC system consists of a vacuum membrane degasser (SCM1000), SpectraSYSTEM® gradient pump (P4000), SpectraSYSTEM® autosampler (AS3000), SpectraSYSTEM® UV-PDA detector (UV6000LP) and Foxy Jr.® fraction collector. ThermoFinnigan, USA.

3.1.2 HPLC column: a Luna 5u C_{18}(2) 100A column, 250 x 4.6 mm I.D., 5 µm, Phenomenex, Australia.

3.1.3 A SecurityGuard™ Cartridge Holder with C_{18} Cartridge (4.0 x 3.0 mm), Phenomenex, Australia.

3.1.4 A Glass Micro Filtration Apparatus: A 1000 ml flask, 250 ml glass funnel, 47 mm fritted glass support base, and anodized aluminum clamp for HPLC mobile phase filtration, KONTES Ultra-ware™, USA.

3.1.5 A rotary vacuum evaporator, EYELA, Japan.

3.1.6 Ultrasonic cleaner, D.S.C. Group, Thailand.

3.1.7 HPLC Autosampler vials 1.8 mL with caps, ThermoFinnigan, USA.

3.1.8 Filter membrane 47 mm, 0.45 µm, type Nylon and PTFE, Satorius, Geramany.

3.1.9 A vacuum pump and compressor, GAST Manufacturing, USA.

3.1.10 Vortex mixer, Scientific Industries.

3.1.11 Micro-pipettes and tips.

3.1.12 Volumetric pipettes 5, 10 mL.

3.1.13 Volumetric flaks 5, 10, 25 mL.

3.1.14 Round-bottom flask 50 mL.

3.1.15 Beakers 50, 150, 250, 600, and 1000 mL.

3.1.16 Glass syringes 5 mL.

3.1.17 Glass column with PTFE stopcock.

3.1.18 Conical glass funnel.

All glass apparatus was washed thoroughly in detergent, rinsed with double distilled water and then rinsed with elution solvent before used.

3.2 Chemicals

3.2.1 The marker dye

4-(4-nitro-phenylazo)-cardanol, 4-(2-chloro-4-nitro-phenylazo)-cardanol, 4-(2-chloro-5-nitro-phenylazo)-cardanol, and 4-(2-methoxy-4-nitro-phenylazo)-cardanol were commercial dyes available from Thailand.

3.2.2 Organic solvents and reagents

Toluene, hexane, propylene glycol, potassium hydroxide (Carlo Erba, Milan, Italy), and ethyl acetate (Merck, Darmstadt, Germany) were analytical grade (AR grade). Methanol, acetonitrile (Merck, Darmstadt, Germany), and toluene (Carlo Erba, Milan, Italy) were HPLC grade. Acetic acid was analytical grade supplied by Merck, Darmstadt, Germany.

The HPLC grade organic solvents was filtered with PTFE membrane and degassed before used.

3.2.3 Silica gel 60 (230-400 mesh), Merck, Darmstadt, Germany.

3.2.4 Commercial diesel.

3.3 The study of the chromatographic characteristics of crude marker dyes from cardanol and nitro aniline derivatives

Crude marker dyes: 4-(4-nitro-phenylazo)-cardanol, 4-(2-chloro-4-nitro-phenylazo)-cardanol, 4-(2-chloro-5-nitro-phenylazo)-cardanol, and 4-(2-methoxy-4-nitro-phenylazo)-cardanol diluted with toluene and cardanol were studied their chromatographic characteristics using HPLC condition listed in Table 4.2. The results of study are reported in Section 4.1.

3.4 The preliminary purification methods of marker dye

The marker dyes used in this study were mixture of cardanol derivatives. They were purified before used as standard material.

The preliminary purification procedure was performed by using column chromatography. The crude marker dye, 4-(4-nitro-phenylazo)-cardanol (2.50 g), was curried out on a silica gel column. The column was eluted with 100% hexane, 100% toluene, and 50% toluene in ethyl acetate, respectively. The toluene fractions were combined in a round-bottom flask and the solvent was removed on a rotary evaporator to dryness at 60°C. The fractions containing same products were combined and tested for their chromatographic properties by HPLC and UV-VIS detection at 390 nm. Characterization was further performed by mass spectrometer and NMR spectrometer. The results are shown in Section 4.2.

3.5 Preparation of standard solutions

3.5.1 1000 mg/L Stock standard solutions

The 1,000 mg/L stock standard solutions of 4-(4-nitro-phenylazo)-cardanol was prepared by dissolving 0.0250 g of each standard and diluting them to the mark with toluene in 25.00 mL volumetric flasks.

3.5.2 100 mg/L Standard solutions

The 100 mg/L standard solutions of 4-(4-nitro-phenylazo)-cardanol was prepared by pipetting 1.00 mL of each 1,000 mg/L stock solution into 10.00 mL volumetric flasks and then diluting to the mark with toluene.

3.5.3 Standard solutions for calibration curves

The concentrations of standard solutions: 1.00, 2.00, 4.00, 6.00, 8.00, 10.0, and 12.0 mg/L were prepared by pipetting 10.0, 20.0, 40.0, 60.0, 80.0, 100, and 120 µL, respectively of each 100 mg/L standard solution into 2 mL vial and then diluting to 1,000 µL with toluene by micropipette. These standard solutions were studied calibration curves.

3.5.4 Standard solutions for studying linearity ranges

The concentrations of standard solutions: 1.00, 2.00, 4.00, 6.00, 8.00, 10.0, 12.0, 15.0, 18.0, 20.0, and 25.0 mg/L. The concentration levels 1 to 7 were prepared by procedure in 3.4.3. The concentrations: 15.0, 18.0, 20.0, and 25.0 mg/L were prepared by pipetting 150, 180, 200, and 250 µL, respectively of each 100 mg/L standard solution into 2 mL vial and then diluting to 1,000 µL with toluene by micropipette. These standard solutions were studied linearity ranges.

3.6 The study of High Performance Liquid Chromatographic conditions

The HPLC conditions were developed by varying the flow rate, mobile phase type, mobile phase percentages, and wavelength detection in order to obtain the optimum HPLC conditions. 10 µL of the standard solutions, spiked standard solutions and sample solutions were injected into HPLC system under optimum HPLC conditions listed in Table 4.2. A chromatogram of standard solutions is in APPENDIX D.

3.7 The study of selectivity evaluation of the HPLC conditions

The selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other component in the sample. In this study, the selectivity of HPLC was determined by the peak retention time and resolution of analyte at the optimum chromatographic conditions in Table 4.2. The results are shown in Table 4.3.

3.8 The study of standard calibration curve

The procedure to study the calibration curves of standard 4-(4-nitro-phenylazo)-cardanol can be described as following:

3.8.1 Series of 1.00, 2.00, 4.00, 6.00, 8.00, 10.0 and 12.0 mg/L standard solution were analyzed respectively by HPLC under the optimum conditions (Table 4.2).

3.8.2 The relationships between concentration and peak area were plotted. The intercepts, slopes, and correlation coefficients are reported in Section 4.5.

3.9 The study of linearity range

The procedure for studying the linearity ranges of standard 4-(4-nitro-phenylazo)-cardanol can be described as following:

3.9.1 Series of 1.00, 2.00, 4.00, 6.00, 8.00, 10.0 and 12.0 mg/L standard solution were analyzed respectively by HPLC under the optimum conditions (Table 4.2).

3.9.2 The relationships between concentration and peak area were plotted. The intercepts, slopes, and correlation coefficients are reported in Section 4.6.

3.10 The study of detection limits and quantitation limits (LOD and LOQ)

The detection limit and quantitation limit of the instrument were defined as the amount of analytes in standard solution that yield as peaks at signal-to-noise ratio equal to 3 and 10, respectively. The procedure can be described as follows:

3.10.1 The 10.0 mg/L standard solutions were prepared from respective 1,000 ppm stock solution.

3.10.2 The 1.00 mg/L standard solutions and concentration below 1.00 mg/L were prepared by diluting 10.0 mg/L of standard solution from step 3.10.1.

3.10.3 The standard solutions from step 3.10.2 were injected into HPLC system under the optimum conditions (Table 4.2). The peak signals of compound were measured from the chromatogram.

3.10.4 The detection limit and quantitation limit of compound was determined from the concentration that gave the peak signal as high as 3 and 10 times of the baseline signal, respectively.

3.10.5 The detection limit and quantitation limit of compound are illustrated in Section 4.7.

3.11 The sample pretreatment procedure

The amount of 4-(4-nitro-phenylazo)-cardanol in diesel were determined by HPLC. The marked diesel samples were prepared by pipetting the appropriate amount of 100 mg/L marker dye stock solution into 5 mL volumetric flask and then diluting them to the mark with diesel. The appropriate amount of marker dyes in diesel samples were typically in range 1.00-10.0 ppm.

The sample pretreatment procedure of marked diesel (1.00-10.0 mg/L) was performed using column chromatography over pasture pipette packed with silica gel. The column was eluted with 100% hexane (15 mL), 100% toluene (30 mL), and 50% toluene in ethyl acetate (10 mL), respectively. The yellowish fractions were combined in a round-bottom flask and the solvent was removed on a rotary evaporator to dryness at 60°C. The dried residue was re-dissolved in 5.00 ML toluene and then filtered through 0.45 µm PTFE membrane before HPLC analysis.

3.12 The study of matrix calibration curve

The matrix calibration curves were studied in unmarked diesel matrices. The procedure for the study can be described as followed:

3.12.1 Blank solution was prepared by using unmarked diesel sample passed through silica column according to procedure in Section 3.11.

3.12.2 The standard solutions at 1.00, 2.00, 4.00, 6.00, 8.00, 10.0, and 12.0 mg/L were prepared by pipetting 10.0, 20.0, 40.0, 60.0, 80.0, 100, and 120 µL, respectively of each 100 mg/L standard solution into 2 mL vials and then diluting to 1,000 µL with the blank solution in 3.12.1 as diluting solvent instead of toluene by micropipette.

3.12.3 Standard solutions in unmarked diesel matrix from 3.12.2 were injected respectively into HPLC system under the optimum conditions (Table 4.2).

3.12.4 The matrix calibration curves were constructed by plotting concentration versus peak area. The intercepts, slopes, and correlation coefficients are illustrated in Section 4.8.

3.13 The study of matrix effect

The matrix effect in sample was studied using diesel matrix. The statistical analysis of the calibration curve of standard solution in toluene (Section 3.8) was performed against the matrix solution curve (Section 3.12) using two tailed paired *t*-test at 95% confidence level. The *t*-value is reported in Table 4.4.

3.14 The study of method quantitation limits (MQL)

The method quantitation limit was defined as the amount of analyte in spiked standard solution that yields a peak at signal-to-noise ratio equal to 10. The procedure can be described as follows:

3.14.1 The spiked samples were prepared by spiking known amount of standard solution into diesel sample and preconcentrated by procedure in Section 3.11.

3.14.2 Blank sample was prepared in the same way as spiked samples but not to be added to the standard solution.

3.14.3 The blank and spiked sample from step 3.14.1 and 3.14.2 were injected into HPLC system under the optimum conditions (Table 4.2). Peaks of compound were measured from the chromatograms.

3.14.4 The MQL of compound was found from the concentration that gave the peak signal as high as 10 times of the baseline signal. The result is described in Section 4.10.

3.15 The study of method detection limits (MDL)

The method detection limit is considered to be the lowest concentration of analyte in sample that can be detected but not necessarily quantified, under the stated conditions of the test. In this research, the method detection limits were obtained by calculation from the method quantitation limit by using the signal-to-noise ratio equal to 3 in Section 3.14. The result is described in Section 4.10.

3.16 The study of accuracy

The accuracy of a method is the closeness of the measured values to the true value (concentration) of the sample. In this study, the accuracy was performed by spiking marker dyes into diesel, approximately at MQL, 2-fold, and 4-fold of MQL and passing through silica column procedure (Section 3.11). Each spiked level was carried out for five batches. Each sample was performed for five runs using optimum HPLC conditions (Table 4.2).

The accuracy of this method was reported in terms of recovery (%) of determined amount of analytes spiked in diesel sample. Results are described in Section 4.11.

3.17 The study of precision

Precision is the amount of scatter in the results obtained from multiple analyses of homogeneous sample(s). In this study, precision was divided into intra-assay precision or repeatability obtained by repeatedly analyzing the same sample in one day and intermediate precision which was obtained when the assay was performed on different days. The study was investigated by using the spiked standard solutions with known amounts in unmarked diesel, approximately at MQL, 2-fold and 4-fold of MQL, passing through the silica column. The eluted of marker dye was repeated 5 times within each batch for intra-assay precision study. Then, the eluted of marker dye was repeated on 5 different days for an intermediate precision study. Precision was expressed by percentage of relative standard deviation (%RSD) of concentration. Results are discussed in Section 4.12.

3.18 Sample stability

The stability of the analyte was determined by using diesel matrix spiked with 4-(4-nitro-phenylazo)-cardanol followed by normal sample preparation procedure in Section 3.11. These preconcentrated solutions were divided into 7 vials, stored under the same conditions, and then analyzed on different days within a 3-months period. The concentrations were back calculated using linear equations of freshly prepared

standard solutions and the recoveries were reported as percentage of the original spiked concentration. The recovery results are summarized in Table 4.7 and illustrated as control charts in Figure 4.9-4.11.

3.19 UV-VIS method

UV-VIS method is usually used for quantitative analysis of marker dyes in the field test. Because almost all marker dyes can be absorb VIS range. In this work, quantitative analysis of marker dyes using UV-VIS spectrophotometer [Suwanprasop *et al.* 2004] was studied to compare results from HPLC method.

Since marker dye in this work was phenyl azo compounds that have weakly basic character. This type of marker can be detected in diesel fuel by extraction into an appropriate alkaline aqueous solution that reacts with a marker dye, and produces color in an aqueous phase.

In this work, the extraction system was 5 mL of 1% potassium hydroxide in a solution of 1:1 propylene glycol and methanol added into 25 mL marked diesel at seven concentration levels, 1.00, 2.00, 4.00, 6.00, 8.00, 10.0, and 12.0 mg/L, shaken for 30 min. The mixture was left at room temperature until two phases were observed. The lower phase, which developed reddish-violet color, was drawn off for detected recording the maximum adsorption at 510 nm using UV-VIS spectrophotometer. The relationships between concentration and absorbance were plotted. The intercepts, slopes, and correlation coefficients are reported in Section 4.14.

3.20 Method verification

For confirming quantitative analysis of marker dye in the field test using UV-VIS method with HPLC method, the statistical analysis was studied. In this study, the procedure for sample preparation and quantitative analysis was carried by spiking marker dyes into diesel fuel, approximately concentration at 2.50, 5.00, and 10.0 mg/L. For HPLC analysis, the samples were prepared as described in Section 3.11 and then the amount of marker dye was detected by HPLC method. On the other hand, the

samples were prepared base on Section 3.19 for UV-VIS analysis and detected by UV/VIS spectrophotometer.

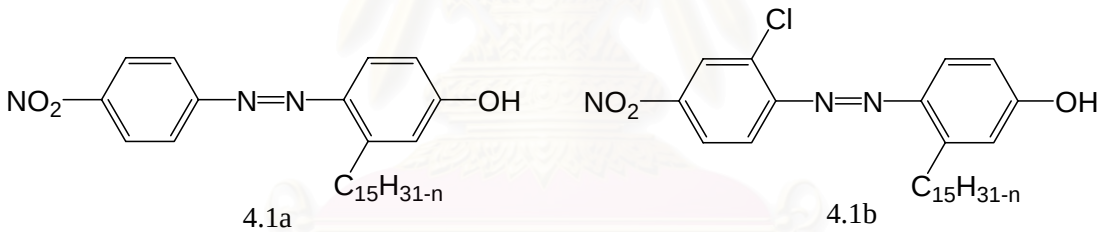
The statistical analysis of marker dye amount in diesel fuel between using HPLC method and UV-VIS method was performed using two tailed paired *t*-test at 95% confidence level. The *t*-value is reported in Table 4.8.

CHAPTER IV

RESULTS AND DISCUSSION

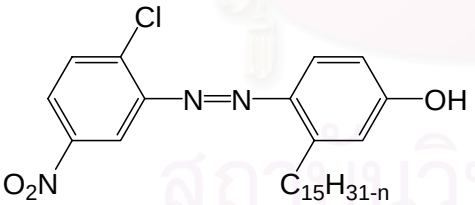
4.1 The chromatographic characteristics of crude marker dyes from cardanol and nitro aniline derivatives

Marker dyes used in this work were available from Thailand [Suwanprasop *et al.* 2004] including 4-(4-nitro-phenylazo)-cardanol (Figure 4.1a), 4-(2-chloro-4-nitro-phenylazo)-cardanol (Figure 4.1b), 4-(2-chloro-5-nitro-phenylazo)-cardanol (Figure 4.1c), and 4-(2-methoxy-4-nitro-phenylazo)-cardanol (Figure 4.1d). We studied chromatographic characteristics of these compounds and cardanol by optimum HPLC condition listed in Table 4.2. Their chromatograms are shown in APPENDIX D.

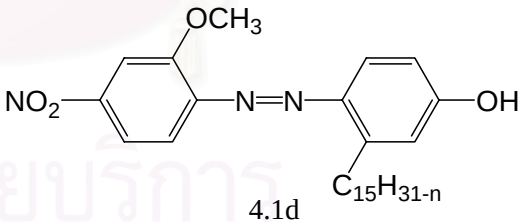


4.1a

4.1b



4.1c



4.1d

where n = 0, 2, 4, 6

n = 0 $(C_{14}H_{28})CH_3$

n = 2 $(CH_2)_7CH=CH(CH)_2CH_3$

n = 4 $(CH_2)_7CH=CHCH_2CH=CH(CH_2)_2CH_3$

n = 6 $(CH_2)_7CH=CHCH_2CH=CHCH_2CH=CH_2$

Figure 4.1: The structures of nitro-aniline cardanol azo. [Suwanprasop *et al.* 2004].

The chromatogram of cardanol detected at 278 nm is shown in APPENDIX C. It was observed that cardanol could be separated into respective three major peaks pattern of their derivatives with retention time 8.83, 10.57, and 12.99 min, respectively. A comparison of chromatograms of each marker dye and cardanol at 278 nm indicates that these crude marker dyes remained unreacted cardanol derivatives in reaction mixture.

Moreover, the chromatogram of each marker dye at 390 nm showed all the same that comprised of three major peaks pattern but respective peak separated with different retention time depending on substitution group on aniline. Retention time of each marker dye by HPLC conditions listed in Table 4.2 is reported in APPENDIX B.

According to the chromatographic data, we should further qualify marker dyes namely 4-(4-nitro-phenylazo)-cardanol to be used throughout this work because it is used as a commercial marker dye in fuel oils.

Furthermore, the chromatographic characteristics of mixed marker dyes included 4-(4-nitro-phenylazo)-cardanol/4-(2-chloro-4-nitro-phenylazo)-cardanol and 4-(4-nitro-phenylazo)-cardanol/4-(2-methoxy-4-nitro-phenylazo)-cardanol with ratio 1:1 using HPLC conditions listed in Table 4.2 were studied. The chromatogram of mixed marker dyes in APPENDIX D showed chromatographic pattern of each compound in mixture. It was represented that we could detect our mixed marker dyes in case of marked fuel imitation.

4.2 The preliminary purification of marker dyes

The marker dyes used in this research were a mixture of cardanol derivatives namely 4-(4-nitro-phenylazo)-cardanol. These marker dyes were synthesized by coupling reaction of cardanol which was obtained from partially purification of decarboxylated cashew nut shell liquid, with diazonium salts of nitro-aniline. They were purified before being used as standard material. Separation and purification were achieved by silica column chromatography (Section 3.4), obtaining reddish-brown viscous liquid with 68% yield. The structure of 4-(4-nitro-phenylazo)-cardanol is:

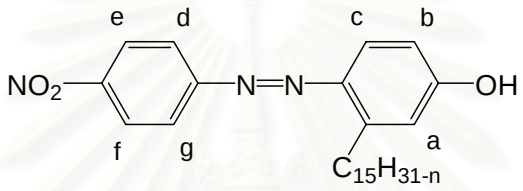


Figure 4.2: The structure of 4-(4-nitro-phenylazo)-cardanol [Suwanprasop *et al.* 2004].

A comparison of the chromatograms of marker dyes and cardanol before purification using HPLC conditions in Table 4.2 are shown in APPENDIX D. Furthermore, the chromatogram of marker dyes and cardanol after purification are shown in Figures 4.2 and 4.3, respectively.

According to the cardanol containing three major components of unsaturated side chain monoene (36%), diene (20%), and triene (41%) [Sood *et al*. 1986], the chromatogram of cardanol and marker dyes comprised of three major peaks showed all the same peak area ratio. It could be estimated that the elution of marker dyes was triene, diene, and monoene side chain in its molecule, respectively. It was indicated that the compounds after purification for using in this work are the marker dyes from coupling reaction of cardanol with diazonium salts of nitro-aniline with a small amount of unreacted cardanol in the reaction mixture.

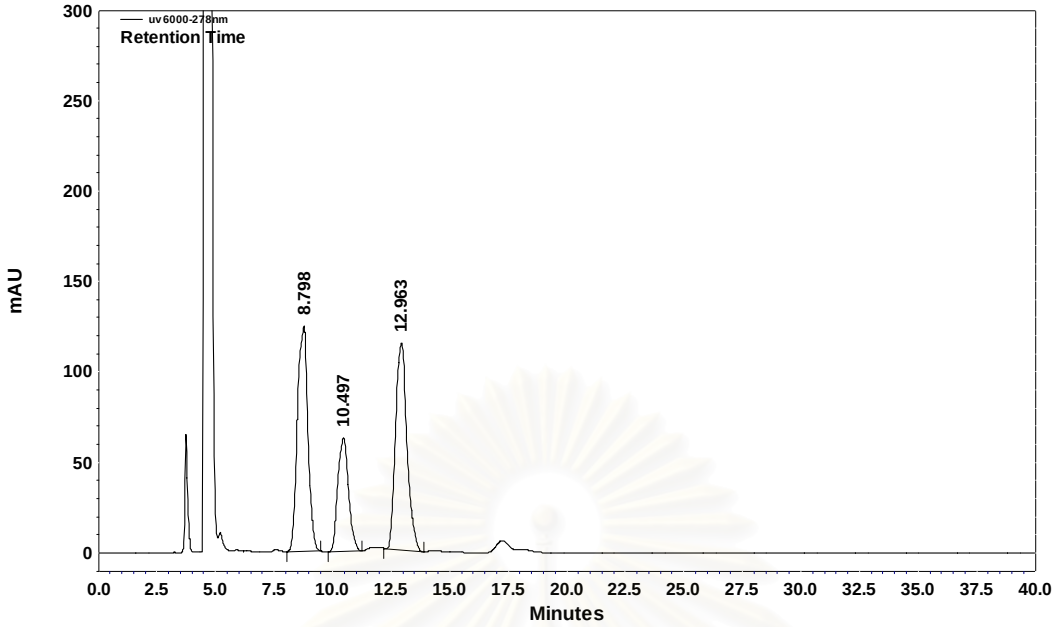


Figure 4.3: A chromatogram of three major peaks of cardanol after purification using HPLC conditions as shown in Table 4.2 detected at 278 nm.

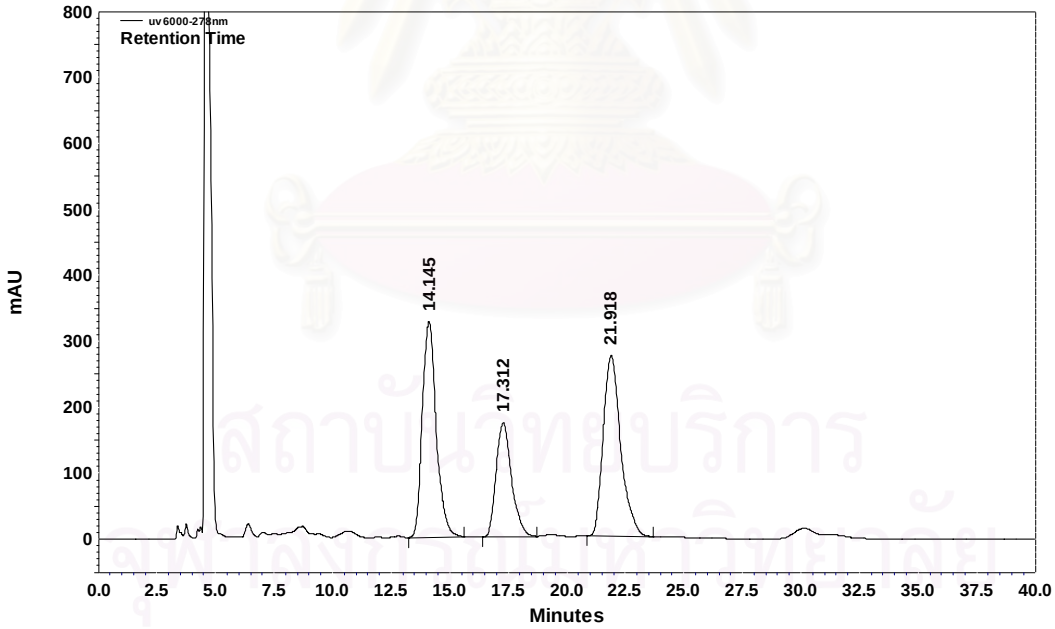


Figure 4.4: A chromatogram of three major peaks of 4-(4-nitro-phenylazo)-cardanol after purification using HPLC conditions as shown in Table 4.2 detected at 278 nm.

After purification, the marker dyes were tested for their chromatographic characteristics using HPLC condition in Table 4.2 (Figure 4.5) detected wavelength at 390 nm. It was observed that purified marker dye could be separated into respective three major peaks pattern of cardanol derivatives in its molecule. Our HPLC condition could detect all peaks with sufficient resolution. The chromatogram of marker dyes from this section was further used as HPLC pattern for this work.

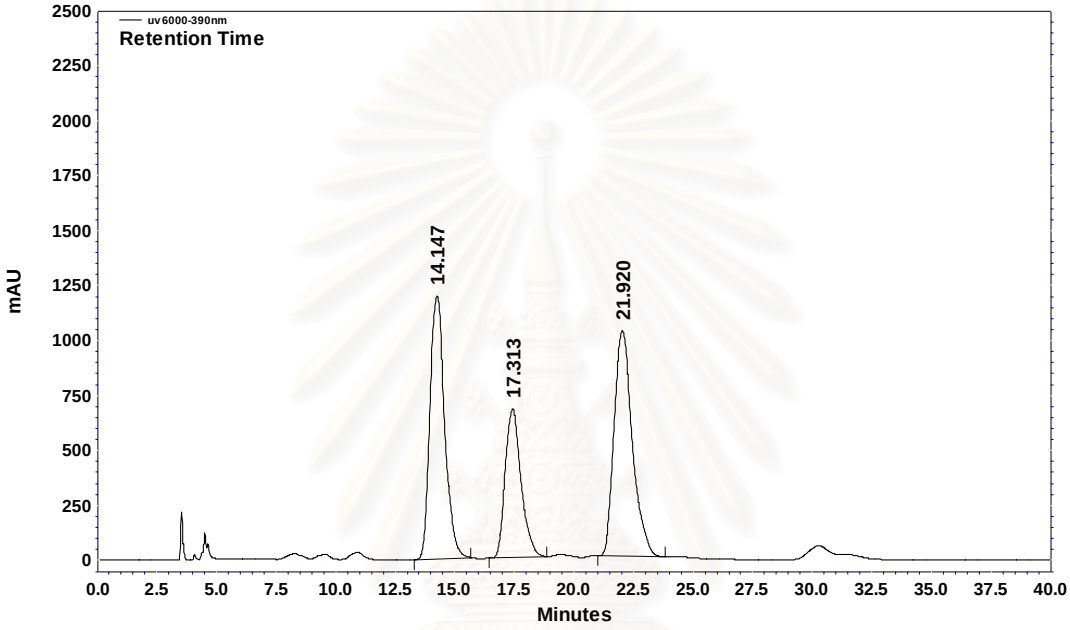


Figure 4.5: A chromatogram of three major peaks of 4-(4-nitro-phenylazo)-cardanol after purification using HPLC conditions as shown in Table 4.2 detected at 390 nm.

The characterizations of these compounds were performed by UV-VIS and NMR.

UV-VIS spectrum of this compounds in APPENDIX A is the spectrum of 4-(4-nitro-phenylazo)-cardanol representing all cardanol derivatives in its molecules because of their similarity in structure. Therefore, UV-VIS data was used to differentiate this compound from other species.

The ^{1}H-NMR spectrum ($CDCl_3$) of 4-(4-nitro-phenylazo)-cardanol after purification in APPENDIX B show the presence of a para-substituted azo benzene

ring at δ_H 8.36 (2H, *d*, position e and f) and 7.95 (2H, *d*, position d and g). The ^{1}H-NMR spectra also demonstrated the existence of a coupled cardanol in the marker dye, whose δ_H at the position a, b, and c were at 6.81 (s), 6.76 (d), and 7.75 (d), respectively. In addition, the δ_H of the methylene proton adjacent to the aromatic ring of a coupled cardanol was shifted downfield from 2.80 (in unreacted cardanol) to 3.12 (in the marker dye product); this downfield shift amy be due to the presence of azo substituted cardanol. The ^{1}H-NMR spectrum of 4-(4-nitro-phenylazo)-cardanol also implied that there was the amount of unreacted cardanol in the reaction mixture, as suggested by the amount of unreacted cardanol at δ_H 2.80. The assignments are concluded in Table 4.1.

Table 4.1: The ^{1}H-NMR spectral data of 4-(4-nitro-phenylazo)-cardanol (Figure 4.2).

Position	δ_H, Multiplicity
a	6.81, *d*
b	6.76, *d*
c	7.75, *d*
d	7.95, *d*
e	8.36, *d*
f	8.36, *d*
g	7.95, *d*

The ^{13}C-NMR spectrum ($CDCl_3$) of 4-(4-nitro-phenylazo)-cardanol (APPENDIX B) showed prominently at δ_C 123.2 and 124.8, attributable to carbon position d and g, and e and f, respectively. The ^{13}C-NMR spectrum of this compound also revealed the presence of oxycarbon in an aromatic ring (C-OH) at δ_C 156.3, methylene and methyl groups at δ_C 14.1-35.8, and double bonds in an aromatic ring and side chain at δ_C 112.5-136.8.

4.3 HPLC method optimization

HPLC optimization for separation of marker dye was performed on reverse-phase C_8 or C_{18} column. Since reverse-phase C_{18} column was available in routine laboratory, the C_{18} column has been definitely preferred. The separation was tested on Phenomenex® Luna C_{18} 4.6 x 250 mm I.D., 5 µm column using water/acetonitrile as mobile phase but could not achieve good separation because the solvent strength of mobile phase was insufficient. Adjustment of gradient elution or using different condition did not improve the separation further but instead resulted in co-elution of other compounds. Therefore, a new tactic of changing the selectivity or varying the band spacing was tested. Result of changing organic mobile phase from acetonitrile to methanol was likely to give an even better separation. Adding small amount of acid in aqueous could achieve good separation. The function of acid in this separation increased retention and decreased tailing peak. Therefore, C_{18} column and methanol/water adding small volumes of acid were selected for determine of marker dyes in this study.

The HPLC method was developed and the optimum condition is reported in Table 4.2. The chromatograms of standard solution are shown in APPENDIX D.

Table 4.2: High Performance Liquid Chromatographic conditions.

HPLC Parameter	HPLC Conditions
Analytical column	4.6 x 250 mm I.D., 5 µm Phenomenex® Luna C_{18}
Mobile phase	Isocratic Methanol/0.2%Acetic acid (95:5)
Flow rate	0.80 mL/min for 40 min
Injection volume	10 µL
Detector	PDA-UV detector
Wavelength	390 nm

4.4 Results of selectivity evaluation

Because it is very important for the analytical method to have no (or minimal) interference from other species contained in the sample matrix, method selectivity must be proved. The selectivity of HPLC method was evaluated by peak retention time (t_R) matched with the value of standards and resolution values of critical pairs (R_S). Resolution values of critical pairs (R_S) can be calculated from Equation 4.1 as below:

$$R_S = 2 \times \left(\frac{t_{R,B} - t_{R,A}}{W_{b,A} + W_{b,B}} \right) \qquad (4.1)$$

When $t_{R,B}$ is retention time of component B which is more strongly retained, $t_{R,A}$ is retention time of component A which is less strongly held, $W_{b,A}$ is width of peak for component A at the base line, and $W_{b,B}$ is width of peak for component B at the base line. If R_S value equal to or greater than 1.5, this indicates good separation.

Table 4.3 summarized the selectivity data. The chromatographic pattern of 4-(4-nitro-phenylazo)-cardanol shows three major peaks having same ratio peak area. As shown in Figure 4.2, resolutions of all critical pairs are at least 4.28 (baseline resolution) which is acceptable for quantitative analysis.

Table 4.3: Retention time and resolution of 4-(4-nitro-phenylazo)-cardanol by HPLC conditions listed in Table 4.2 (n = 9).

Peak	Retention time (min)	Resolution
1	14.04 ± 0.02	-
2	17.20 ± 0.04	4.28
3	21.83 ± 0.05	8.29

4.5 The standard calibration curve

The aim of this research is to determine the level of 4-(4-nitro-phenylazo)-cardanol. To determine the amount presents in the diesel samples, a linear calibration curve of pure standards were constructed for analyte at seven concentration levels from 1.00-12.0 mg/L using the optimum HPLC conditions listed in Table 4.2. The chromatogram of each pure standard concentration levels is shown in APPENDIX D. A plot and calibration curve of total area of three major peaks versus concentration was shown as below:

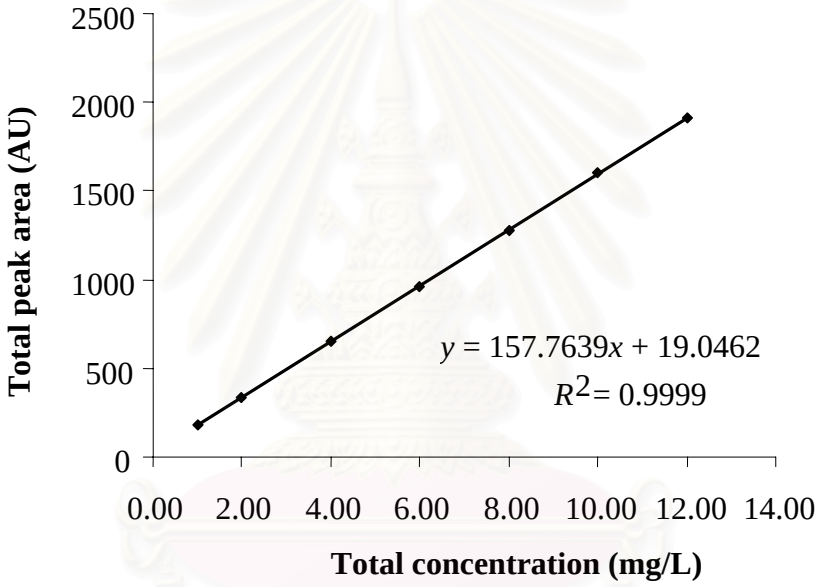


Figure 4.6: The calibration curve of 4-(4-nitro-phenylazo)-cardanol using HPLC conditions listed in Table 4.2.

From a plot in Figure 4.6 showed correlation coefficient (R^2) value approaching 1.00 indicating very high linear relationship within this concentration range.

4.6 The linearity range

From previous section, we are confident that the instrument response is linearly proportional to the analyte concentration from 1.00-12.0 mg/L. To be very useful, an analytical method should have a large magnitude of linear range. To further tested the linearity or dynamic range of the analytical method, concentration levels were extended to near the lowest concentration at which quantitative measurements can be made up to the concentration at which instrument response departs from linearity or become saturated. We extended our study from 1.00 to 25.0 mg/L using linear line to predict best fit curves over this range. A plot is linear (see Figure 4.7).

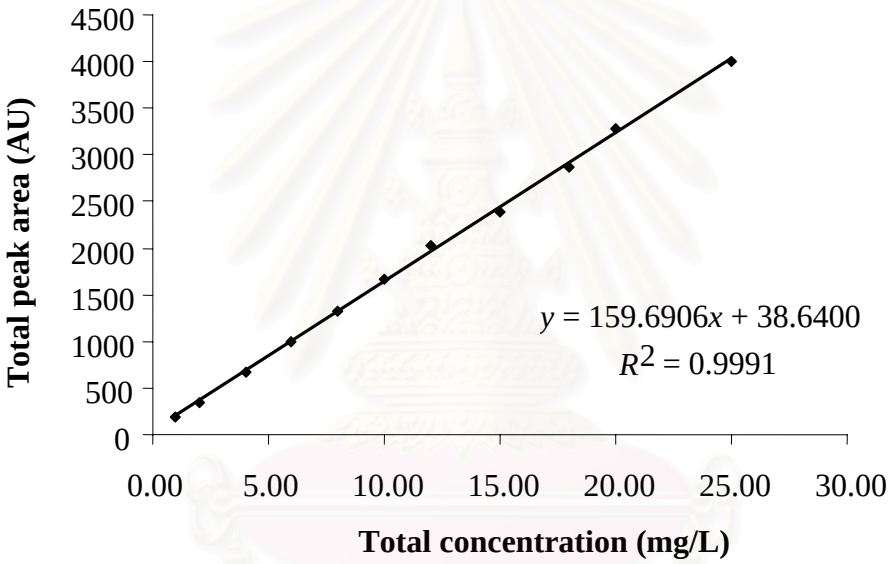


Figure 4.7: The relationship between concentration of 4-(4-nitro-phenylazo)-cardanol and peak area by HPLC conditions listed in Table 4.2.

Excellent linear coefficients (>0.9990) was obtained for all major peaks covering a large concentration range in good agreement with our previous observations. Because excellent precision was observed over several concentration levels, we are confident that the analytical procedure can be accurately determined the amount of 4-(4-nitro-phenylazo)-cardanol up to 25.0 mg/L.

4.7 The detection limits and quantitation limits

The detection limits (LOD) and quantitation limits (LOQ) are defined as the lowest amount of an analyte in standard solutions that can be reliable detected and quantitated that yielding an instrumental signal significantly different from the blank or background signal equal to 3 and 10, respectively. It is very important to establish the lower end of the practical operating range of a method to be certainly that the result is accurate.

LOD and LOQ values of marker dye in this work are 0.25 and 0.80 mg/L, respectively, with calculated from the minimum peak area/height. This research does not involve analysis of low trace levels of 4-(4-nitro-phenylazo)-cardanol. Therefore, LOD and LOQ in this study are sufficient for quantitative analysis of 4-(4-nitro-phenylazo)-cardanol in commercial diesel samples for routine test.

4.8 Results of matrix calibration curve

Since the linearity of the response to pure standards may be different from real samples, it is crucial to construct the matrix calibration curves. Matrix calibration curves ranged from 1.00-12.0 mg/L were constructed (see Figure 4.8). The chromatograms of each matrix standard concentration levels and blank solution are shown in APPENDIX D.

The linear plot was obtained with good correlation coefficient data (0.9979), indicating linear detector responses both in pure solution and in diesel matrix. The results imply that the diesel matrix does not interfere with signal and that linear instrument responses are observed from 1.00-12.0 mg/L.

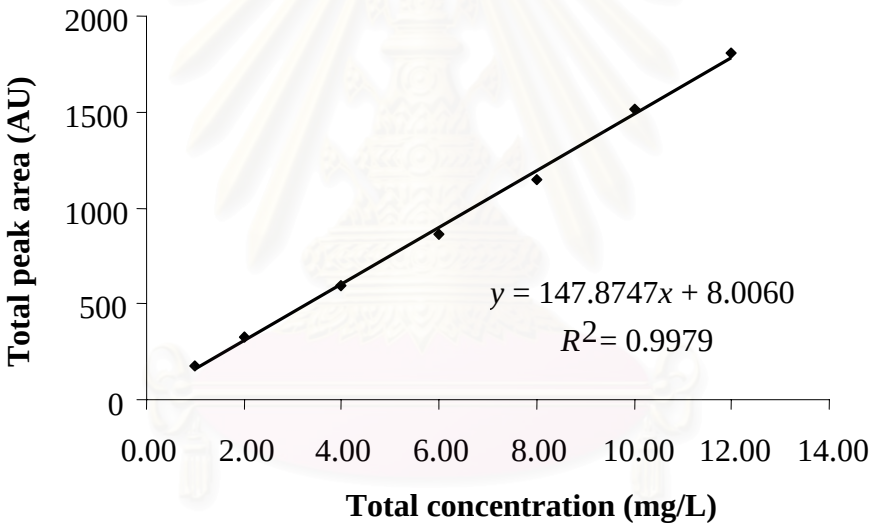


Figure 4.8: The calibration curve of 4-(4-nitro-phenylazo)-cardanol in diesel matrix by HPLC conditions listed in Table 4.2.

4.9 Results of the matrix effect study

Since matrix components may either elute at the same time as the analyte of interest. The effect of diesel matrix on the separation of all major peaks was determined as described in Section 3.13. A suitable statistical tool for comparing results of two methods (two calibration curves) is the paired *t*-test at 95% confidence interval of mean. Taking the null hypothesis that is no significant difference in the peak area given by pure standard solution (Section 4.4) and standard solution in diesel matrix (Section 4.7). The *t*-value is given in Table 4.4 compared with the critical value (P = 0.05) which is 2.45 (n = 7). The *t*-calculated value is more than *t*-critical value. Hence, the null hypothesis is rejected for this marker dye and diesel matrix should be affected for the analysis of this marker dye. Therefore, calibration curve of 4-(4-nitro-phenylazo)-cardanol in diesel matrix instead of calibration curve of this marker dye in toluene was used throughout this work.

Table 4.4: The *t*-calculated value of two tailed paired *t*-test between standard solution and standard in diesel matrix at 95% confidence level (n = 7).

Total concentration (mg/L)	Total peak are (AU)		Paired *t*-test	*t*-critical
	Standard solution	Standard in diesel matrix		
1.00	181.12	171.55		
2.00	334.00	323.13		
4.00	648.94	591.86		
6.00	961.13	865.71	3.98	2.45
8.00	1277.75	1146.11		
10.0	1600.83	1512.72		
12.0	1913.40	1803.58		

4.10 Method detection limits (MDL) and method quantitation limits (MQL)

Method quantitation limit (MQL), is defined as the lowest amount of analytes that can be quantitated at signal-to-noise ratio equal to 10 after passing through sample preparation steps. MQL is determined from corresponded sample size, dilution factor, and instrument sensitivity (LOQ). Similarly, method detection limit (MDL) is defined resemblingly the MQL, but it is the lowest amount of analyte that the method can be detected at the signal-to-noise ratio of 3. In this work, the MDL value was obtained by the calculation from the corresponded to MQL at the signal-to-noise ratio of 3.

The MDL and MQL values of this analytical process were obtained to be 0.75 and 2.50 mg/L, respectively. These MDL and MQL data indicate that our overall process comprising sample preparation procedure and an HPLC method being good sensitivity protocol for quantitative analysis of cardanol azo dye in diesel.

Since the spiked solutions were diluted during sample preparation process and affected by diesel matrix, then the MDL and MQL value would be higher than the previously established LOD and LOQ values (0.25 and 0.80 mg/L).

4.11 Method accuracy

Accuracy of an analytical method can be indicated by closeness of the measurements to its true or accepted value. In this research, accuracy of the method was investigated by back-calcualtions to determine the spiked concentration of marker dyes at MQL, 2-fold MQL, and 4-fold MQL levels as described in Section 3.11. The chromatogram of each spiked sample is shown in APPENDIX D. The accuracy of the method is evaluated by using the recovery of the compound as illustrated in Table 4.5. The recovery of an analyte is calculated using Equation 4.2.

$$\% \text{ Recovery} = \left(\frac{Q_{\text{determined}} - Q_{\text{sample}}}{Q_{\text{spiked}}} \right) \times 100 \qquad (4.2)$$

Where Q_{spiked}, Q_{sample}, and $Q_{\text{determined}}$ are the amount of spiked standard, the determined amount of analyte in the sample before spiking standard, and the determined amount of analyte in the sample after spiking standard, respectively. In this case, Q_{sample} is equal to zero.

Results in Table 4.5 show high accuracy of the method with the recoveries for spiked standard at MQL, 2-fold, and 4-fold of MQL ranging from 81.83 to 85.49 % with RSD < 2.50%. The values of recovery and RSD for marker dye in this study were obtained to be in the acceptable range according to the AOAC Peer-Verified methods, yielding recovery (80-110%) and RSD (7.3-11%) at ppm levels [AOAC 1993].

Table 4.5: The amount of 4-(4-nitro-phenylazo)-cardanol spiked in diesel analyzed by HPLC conditions listed in Table 4.2 (n = 5).

Level	Spiked (mg/L)	Mean recovery (% ± SD)	RSD (%)
MQL	2.50	81.84 ± 1.81	2.21
2-fold MQL	5.00	85.49 ± 1.32	1.52
4-fold MQL	10.0	81.83 ± 0.67	0.82

4.12 Method precision

Precisely, detector responses may be different between pure solutions and sample matrixes either from matrix interferences or different distribution of compounds in real matrixes. Therefore, it is necessary to carry out method precision studies in real matrixes to evaluate the reliability of our procedure. Method precision was studied at three levels, MQL, 2-fold MQL, and 4-fold MQL. The method precision was studied by repeated analyses on the same day (intra-assay precision) and on different days (intermediate precision).

The intra-assay precision was demonstrated as percent relative standard deviation (%RSD) which implies reproducibility of the method. The values of the mean and %RSD data of the experiment were obtained from five separate overall runs each day, while five consecutive days for the intermediate precision. The results were summarized in Table 4.6. %RSD values ranging from 0.40-3.79% indicate that excellent intra-assay precision based on the AOAC Peer-Verified methods can be achieved within the same day (%RSD <6% at ppm levels). The overall %RSD ranged 1.49-3.25% also indicate acceptable intermediate precision based on AOAC Peer-Verified methods [AOAC 1993]. Therefore, this method can be used with confident that it will provide reliable data on different analyses.

Table 4.6: %Recovery and %RSD of 4-(4-nitro-phenylazo)-cardanol spiked in diesel analyzed by HPLC conditions listed in Table 4.2 (n = 5).

Precision		Mean Recovery (% ± SD)			RSD (%)		
		MQL	2-fold MQL	4-fold MQL	MQL	2-fold MQL	4-fold MQL
Intra-assay	Day 1	82.77 ± 1.80	87.15 ± 1.13	82.92 ± 0.33	2.18	1.30	0.40
	Day 2	84.23 ± 0.88	93.53 ± 1.47	87.08 ± 0.65	1.05	1.57	0.74
	Day 3	82.20 ± 2.13	92.82 ± 0.76	90.43 ± 0.75	2.59	0.82	0.83
	Day 4	84.31 ± 2.68	91.17 ± 2.66	87.96 ± 0.50	3.18	2.92	0.57
	Day 5	85.27 ± 3.23	91.64 ± 3.48	89.04 ± 1.68	3.79	3.80	1.89
Intermediate	Overall	83.76 ± 1.25	91.26 ± 2.48	87.49 ± 2.84	1.49	2.72	3.25

4.13 Sample stability

The stability of analytes should be determined to see how long a sample can be stored without degradation. The sample stability was determined by column chromatography of spiked standard solutions in unmarked diesel matrix as procedure in Section 3.11. A batch of sample was prepared and stored in the same condition up to 3 months. Samples were pull and analyzed at 7 different intervals using the optimum HPLC condition in Table 4.2. The results are summarized in form of %recovery in Table 4.7.

The replicate measurements at fixed time intervals, the mean and acceptable range of replicate measurements are plotted using control chart. Control charts are used to monitor the variability and to provide a graphical display of statistical control. In the control chart shown Figures 4.9-4.11, replicate measurements are plotted as function of time. A common approach is to use the average or expected value as the center line, and use a multiple of standard deviation to set the control limits. In this work, the 2SD values set the upper and the lower control limits or the values within which the measurements must fall. Approximately 95% of the data should lie within the range X ± 2SD. When X value is recovery of determined amount of original spiked sample after passed separation procedure and HPLC system. These are tracked to see if there is trend or a systematic deviation from the center line. The recovery calculated from matrix linear equation is plotted on the control chart. If the value falls outside the control limit, the sample must to be analyzed before that time.

The control charts of analyte at all 3 concentration levels, MQL, 2-fold MQL, and 4-fold MQL throughout 3 months were significantly deviated from the control limits as illustrated in Table 4.7. Therefore, it was concluded that the stability of marker dye in diesel fuel was found to be stable and amount of marker dye in diesel fuel stored over a period of at least 3 months could be measured using HPLC procedure with accurate quantitative determination.

Table 4.7: %Recovery of 4-(4-nitro-phenylazo)-cardanol spiked in diesel and storage up to 3 months analyzed by HPLC conditions listed in Table 4.2.

Concentration level (mg/L)	%Recovery calculated compared with matrix linear equation of each day							X – 2SD	X + 2SD	Day of storage
	0	15	30	45	60	75	90			
2.50	84.27	83.21	81.12	80.43	80.1	79.42	78.87	78.22	85.46	90
5.00	85.72	85.68	85.67	86.56	85.46	85.41	85.04	84.18	89.46	90
10.0	82.48	81.82	81.81	81.77	81.71	81.54	81.07	80.49	83.17	90

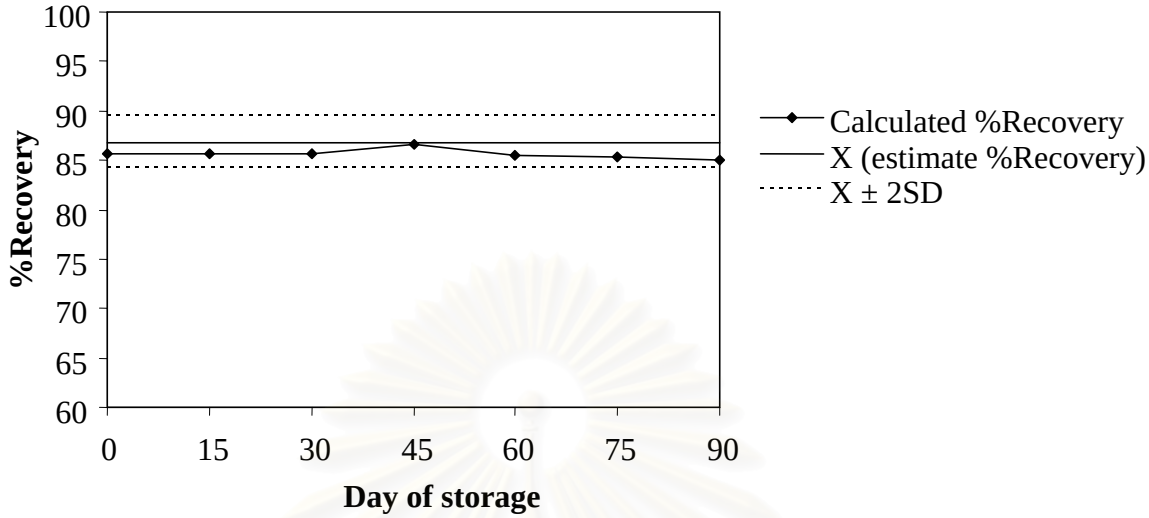


Figure 4.9: A control chart of 4-(4-nitro-phenylazo)-cardanol spiked in diesel at 2.5 mg/L and storage up to 3 months analyzed by HPLC conditions in Table 4.2.

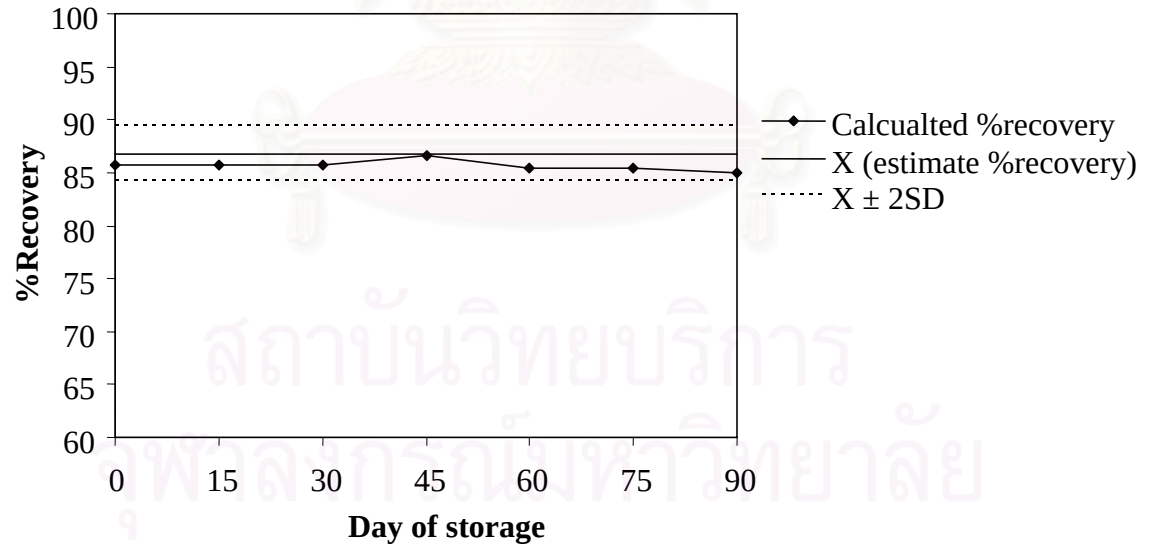


Figure 4.10: A control chart of 4-(4-nitro-phenylazo)-cardanol spiked in diesel at 5.00 mg/L and storage up to 3 months analyzed by HPLC conditions in Table 4.2.

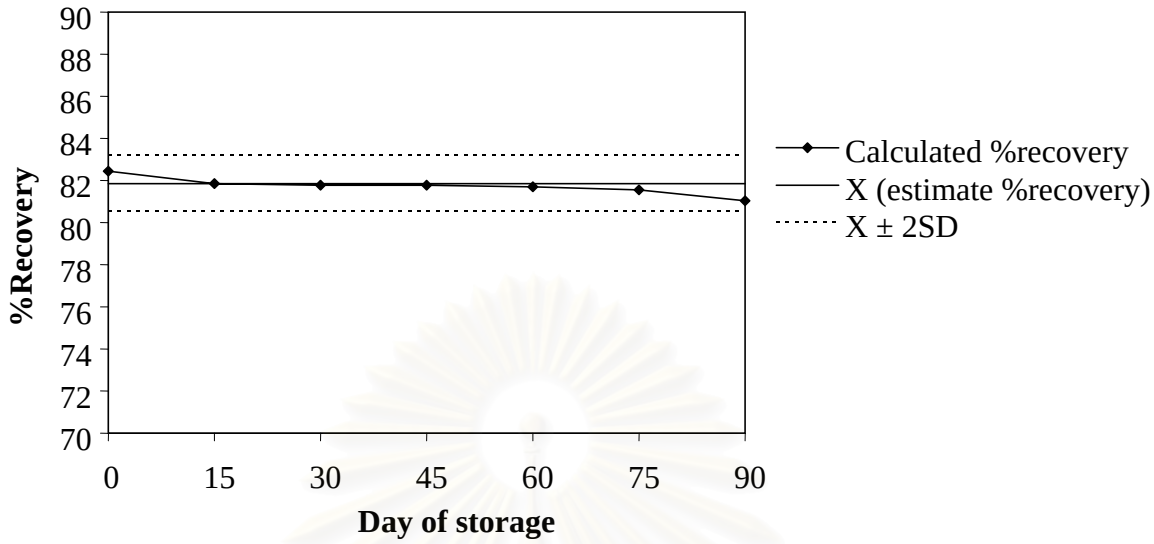


Figure 4.11: A control chart of 4-(4-nitro-phenylazo)-cardanol spiked in diesel at 10.0 mg/L and storage up to 3 months analyzed by HPLC conditions in Table 4.2.

4.14 Results of UV-VIS study

The standard solutions were prepared by adding marker dye at concentration from 1.00-12.0 mg/L into diesel fuel. This dyed diesel fuel was then extracted with a 1% potassium hydroxide in a solution of 1:1 propylene glycol and methanol. The calibration curve was established by plotting absorbance at wavelength 510 nm against the concentration of the marker dye in diesel fuel as shown in Figure 4.12. The UV-VIS spectrum of marker dye in diesel was shown in APPENDIX A.

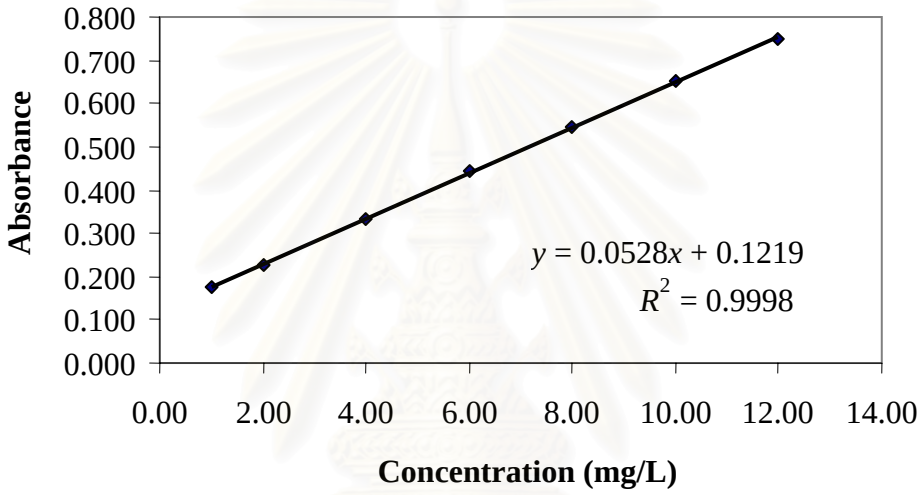


Figure 4.12: The calibration curve of 4-(4-nitro-phenylazo)-cardanol in diesel fuel using UV-VIS detection at wavelength 510 nm.

From a plot in Figure 4.12 showed correlation coefficient (R^2) value approaching 1.00 indicating very high linear relationship within this concentration range. Therefore, this relationship could be used for quantitative analysis of marker dye in diesel in field test.

4.15 Results of method verification

To compare quantitative analysis of marker dye in the field test using a UV-VIS and HPLC methods, the known amounts of marker dye at levels of 2.50, 5.00, and 10.0 mg/L in diesel fuel were determined by UV-VIS and HPLC methods. For UV-VIS analysis, the linear equation $y = 0.0528x + 0.1219$ with $R^2 = 0.9998$ was used to evaluate amount of compounds after sample preparation as described in Section 3.19. For HPLC analysis, the samples were prepared according to Section 3.11. The matrix calibration curve (Section 4.8), giving linear equation $y = 147.8747x + 8.0060$ with $R^2 = 0.9979$ was used.

Table 4.8 lists a comparison of the concentrations of marker dye in diesel fuel determined by UV-VIS and HPLC methods.

The all determined levels of marker dye using UV-VIS method were more than concentrations of marker dye using HPLC method. Because the absorbance data were obtained from all components in diesel matrix that could be absorb in the same wavelength range as the analyte. HPLC method is more selective method for determined marker dye without matrix effect but concentration values were less than values by UV-VIS method caused partial sample amounts loss whereas sample preparation.

Using paired *t*-test analysis at 95% confidence interval of mean, the statistical less than the critical *t*-test value indicates that UV-VIS and HPLC methods give no significant difference in the determined amount of marker dye in diesel fuel. Moreover, the HPLC method is more accurate and specific than UV-VIS method. Therefore, the HPLC data could be used to confirm quantitative analysis of marker dye in diesel fuel in the field test by UV-VIS analysis.

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Table 4.8: The determined concentrations of 4-(4-nitro-phenylazo)-cardanol in diesel fuel and paired t-test at 95% confidence level by UV-VIS and HPLC method.

Spiked (mg/L)	Determined concentration (mg/L ± SD)		Paired *t*-test	
	UV-VIS	HPLC	statistical	critical
2.50	2.42 ± 0.13	2.07 ± 0.04		
5.00	4.79 ± 0.21	4.39 ± 0.02	1.74	4.3
10.0	10.10 ± 0.19	8.21 ± 0.08		

CHAPTER V

CONCLUSIONS AND SUGGESTION FOR FUTURE STUDY

In this study, high performance liquid chromatography (HPLC) was developed for separation and analysis of marker dyes derived from nitro aniline and cardanol in diesel fuel.

The work covered the preliminary purification of marker dyes namely 4-(4-nitro-phenylazo)-cardanol before using as standard material. Simple and reliable preparation methods for these marker dyes were proposed. Successful separations and purifications were performed by column chromatography. Reddish-brown viscous liquid were obtained with 68% yield. After purification, the compounds were tested for their chromatographic characteristics and further confirmed by UV-VIS and NMR to match their profiles. In comparisons with the characteristic data of marker dye and cardanol, it was represented that the compounds after purification for use in this work are the marker dyes from coupling reaction of cardanol with diazonium salts of nitro-aniline with a small amount of unreacted cardanol in the reaction mixture.

The quantitative determination of marker dyes was carried out using reverse-phase C_{18} column (250 × 4.60 mm), methanol/0.2% acetic acid in water with ratio 95:5 as mobile phase, flow rate 0.80 mL/min, wavelength detection at 390 nm. The selectivity of HPLC method was evaluated by resolution values of critical pairs (R_s) and matching peak retention time (t_R) with standards. The method is capable to separate marker dyes to respective three major peaks pattern of cardanol derivatives in its molecule. The method showed good analytical characteristics, having linear relationship of pure standard solution (R^2>0.9990) up to 25.0 mg/L and R^2>0.9970 with matrix effect. The method detection limit and method quantitation limit were higher than the detection limit and the quantitation limit because of sample preparation procedure.

The method accuracy and precision were performed for the determined amounts of marker dye at three levels, MQL (2.50 mg/L), 2-fold MQL (5.00 mg/L), and 4-fold MQL (10.0 mg/L), spiked in diesel matrix. Percent recovery values ranging from 81.83 to 85.49 % with RSD < 2.50% demonstrated excellent method accuracy that also met AOAC Peer-Verified methods. In addition, the method precision were tested for both intra-assay precision and intermediate precision showing excellent precision with %RSD ranged 1.49-3.25% indicating high precision under AOAC Peer-Verified methods. By comparison of sample blank with spiked sample of diesel matrix, it was found that there was no matrix that disturbed the analysis. Therefore, the developed method can be used to analyze this marker dye accurately and precisely. Moreover, the high stability of marker dye in diesel fuel was found. Therefore, the amount of marker dye in diesel fuel stored over a period of at least 3 months could be determined accurately.

The concentrations of marker dye in diesel fuel were determined by UV/VIS and HPLC. Using statistical paired *t*-test analysis at 95% confidence interval of the mean, no significant difference was obtained for the concentrations of marker dye in diesel fuel using UV-VIS and HPLC methods. The HPLC method gave less concentrations of marker dye in diesel fuel than those determined using the UV-VIS method with more selective method. Therefore, more accurate HPLC data could be used to confirm quantitative analysis of marker dye in diesel fuel in the field test by UV-VIS analysis.

For future work, the determined amount of marker dyes should be performed by the HPLC method with more sensitive and selective fluorescence detector. Because marker dye structure generally possess delocalized electrons formally present in conjugated double bond that exhibits fluorescence property. Furthermore, the developed HPLC method could be used for analysis of mixed marker dyes in fuel oils that showed the unique chromatographic pattern.

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APPENDICES

APPENDIX A

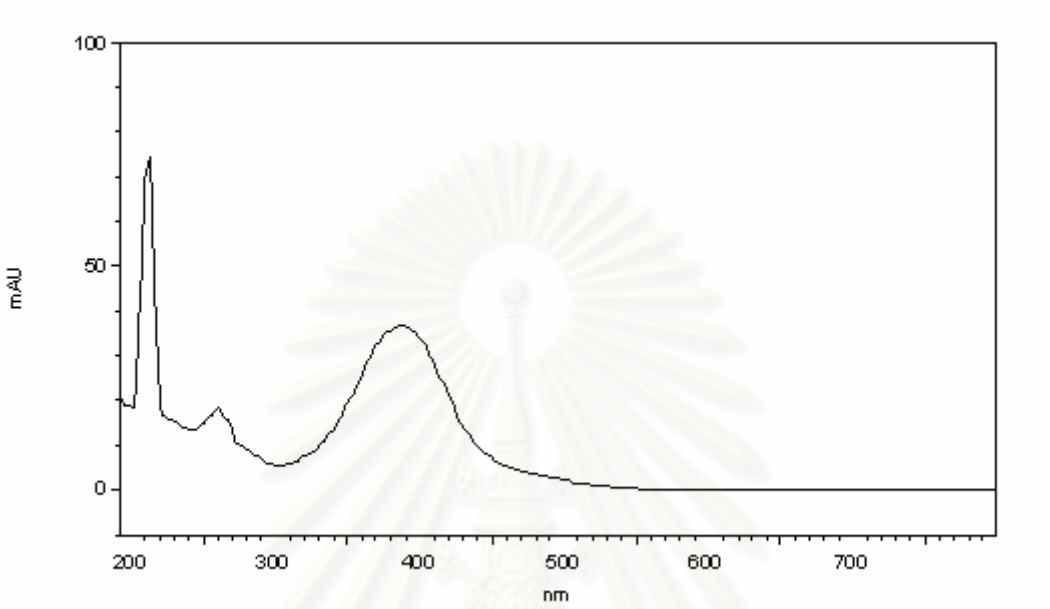

Figure A-1: UV-VIS spectrum of 4-(4-nitro-phenylazo)-cardanol at maximum wavelength 390 nm.

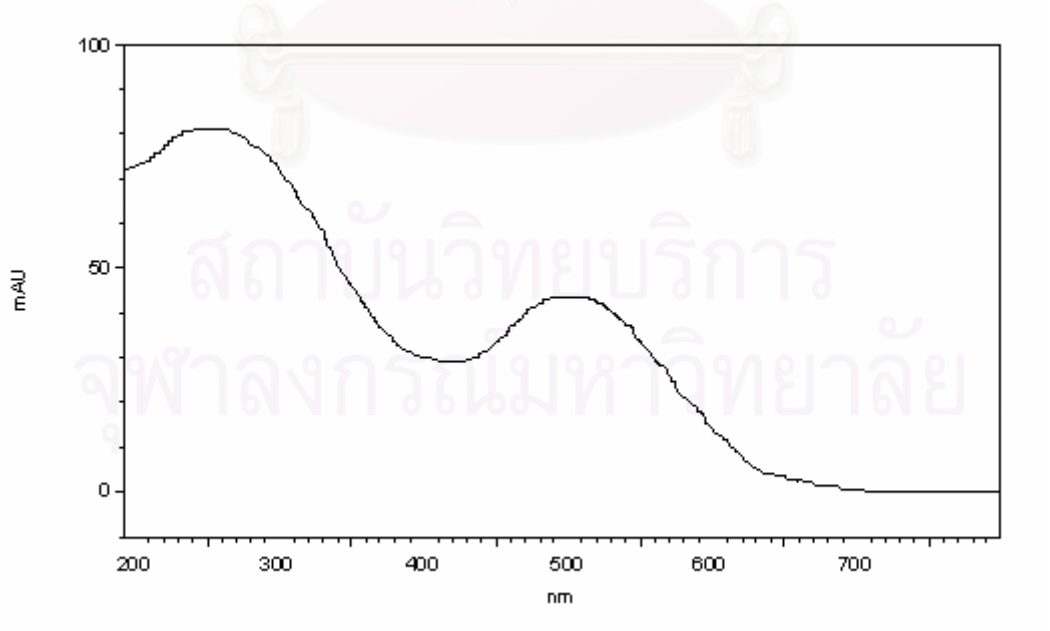

Figure A-2: The UV-VIS absorption of 4-(4-nitro-phenylazo)-cardanol in diesel at maxiumn wavelength 510 nm.

APPENDIX B

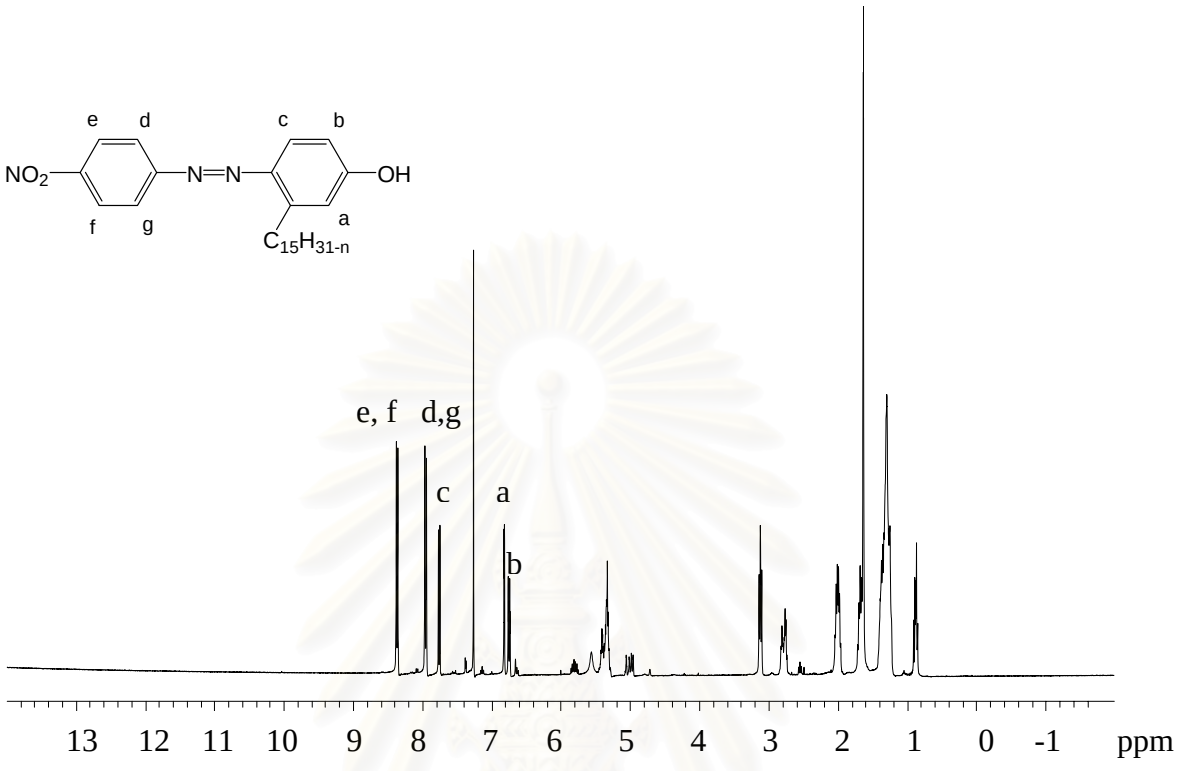

Figure B-1: ^{1}H-NMR spectrum of 4-(4-nitro-phenylazo)-cardanol.

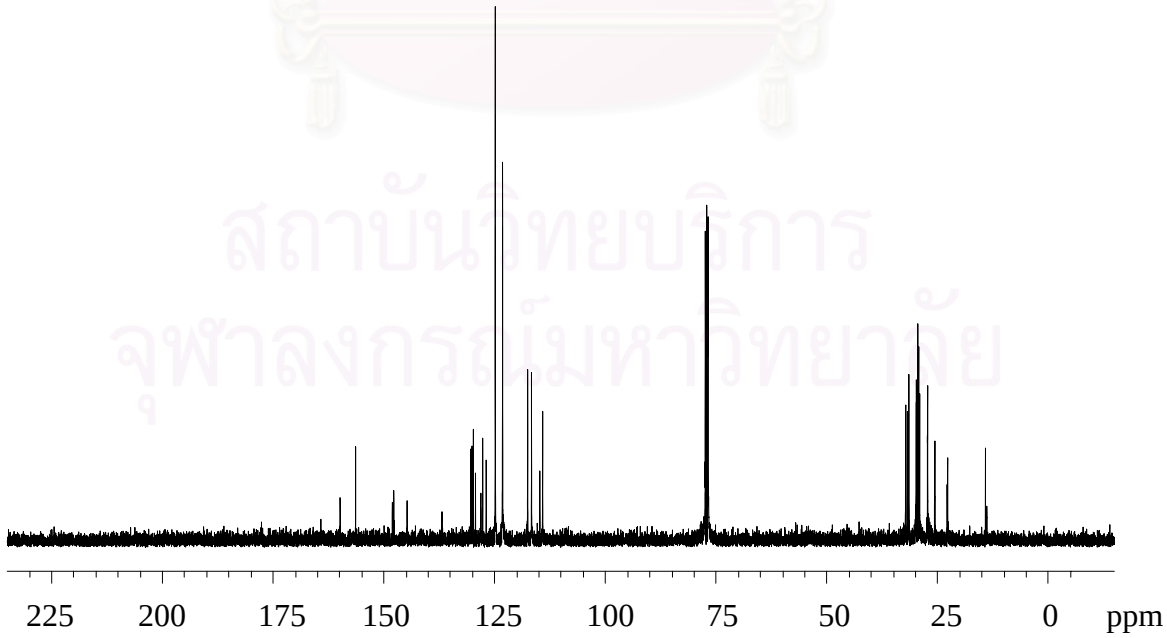

Figure B-2: ^{13}C-NMR spectrum of 4-(4-nitro-phenylazo)-cardanol.

APPENDIX C

Table B-1: Retention time of marker dyes from cardanol and nitro aniline derivatives by HPLC condition listed in Table 4.2 at 390 nm (n = 3).

Marker dye (-R group)	Retention time (min) of three major peaks		
	Peak 1	Peak 2	Peak 3
$4NO_2$	14.05 ± 0.17	17.19 ± 0.21	21.79 ± 0.29
$2Cl\text{-}4NO_2$	19.44 ± 0.11	24.11 ± 0.08	30.98 ± 0.04
$2Cl\text{-}5NO_2$	17.48 ± 0.21	21.65 ± 0.18	27.97 ± 0.07
$2OCH_3\text{-}4NO_2$	12.18 ± 0.16	14.75 ± 0.21	18.63 ± 0.12

APPENDIX D

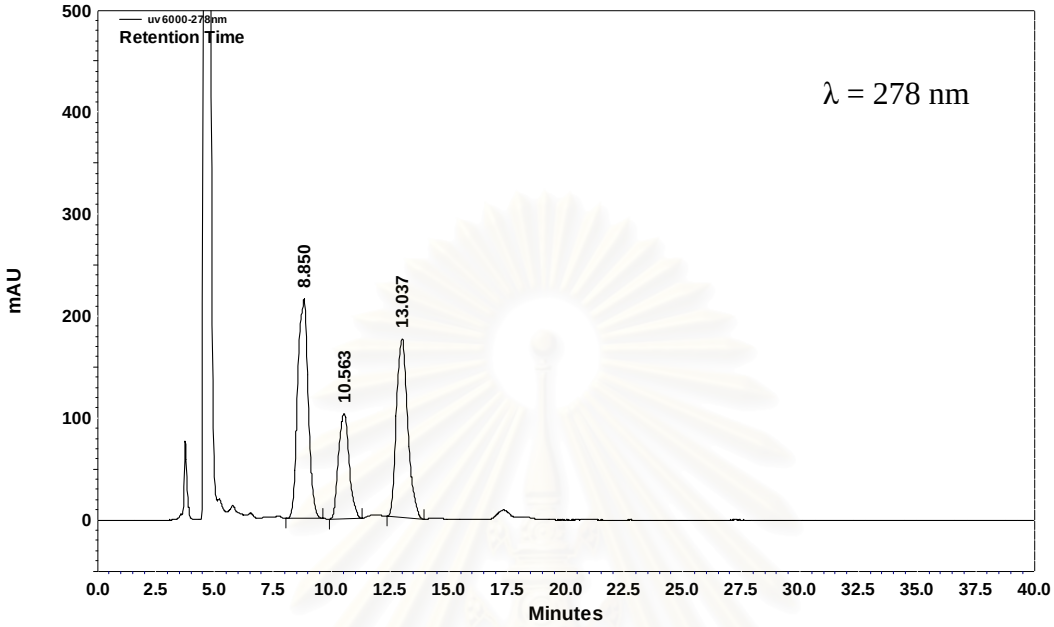

Figure D-1: A chromatogram of three major peaks of cardanol before purification using HPLC conditions as shown in Table 4.2.

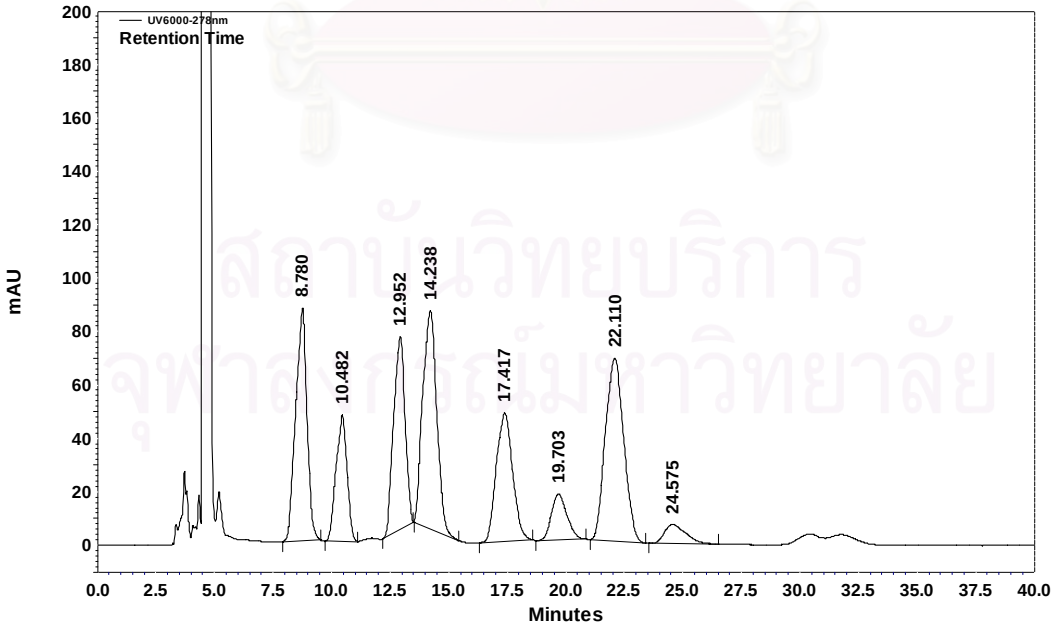

Figure D-2: A chromatogram of 4-(4-nitro-phenylazo)-cardanol before purification using HPLC conditions as shown in Table 4.2.

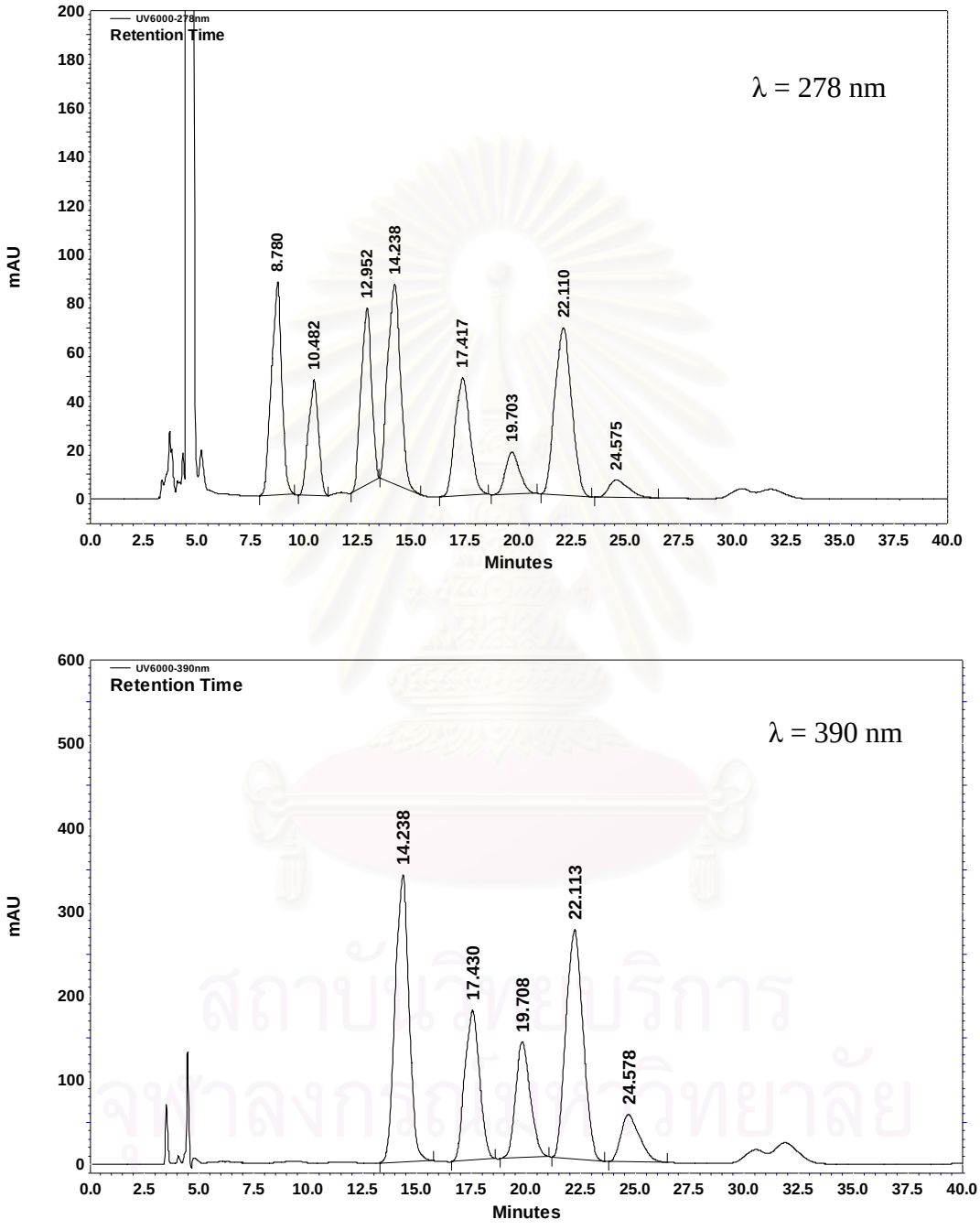

Figure D-3: Chromatograms of 4-(4-nitro-phenylazo)-cardanol using HPLC conditions as shown in Table 4.2.

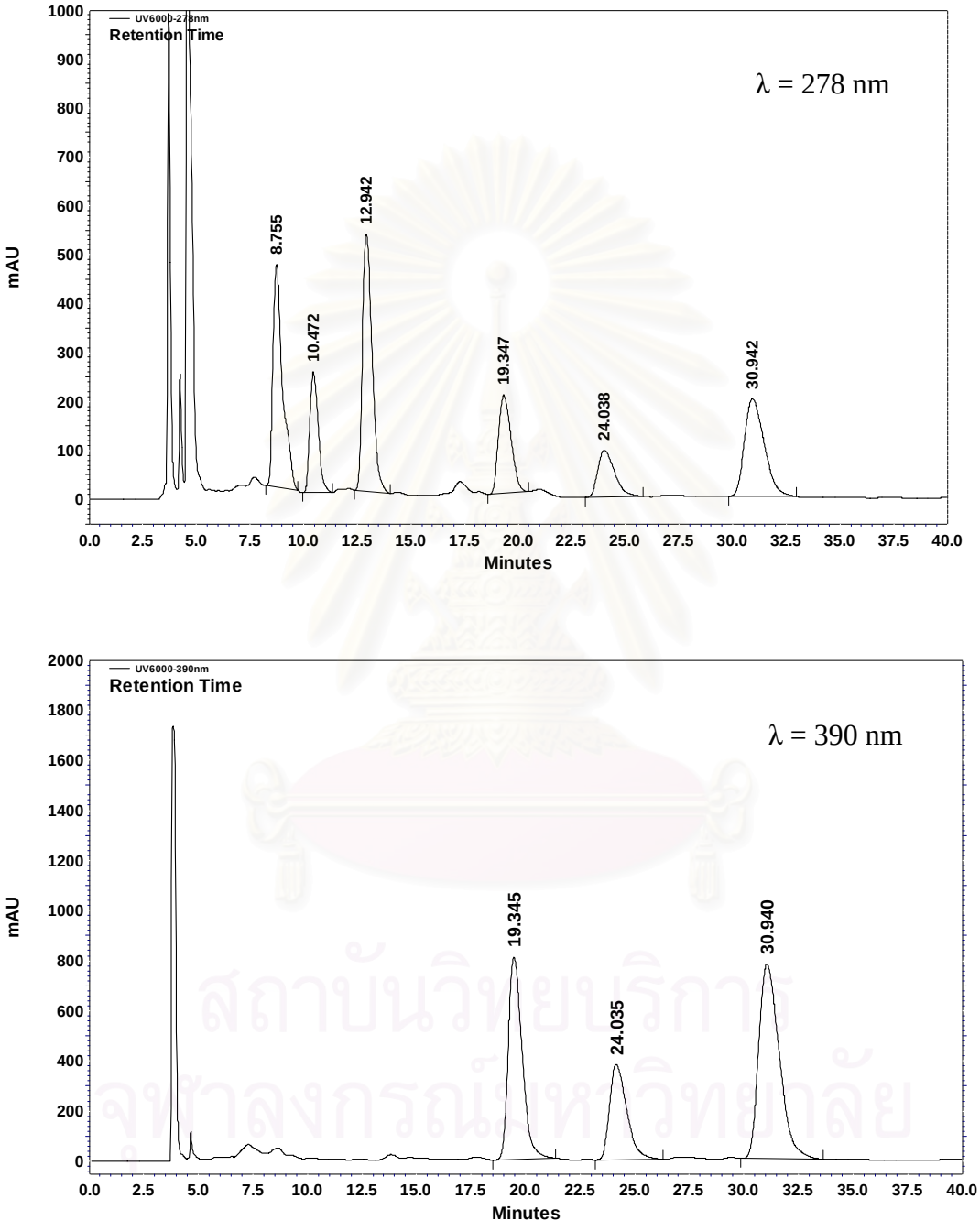

Figure D-4: Chromatograms of 4-(2-chloro-4-nitro-phenylazo)-cardanol using HPLC conditions as shown in Table 4.2.

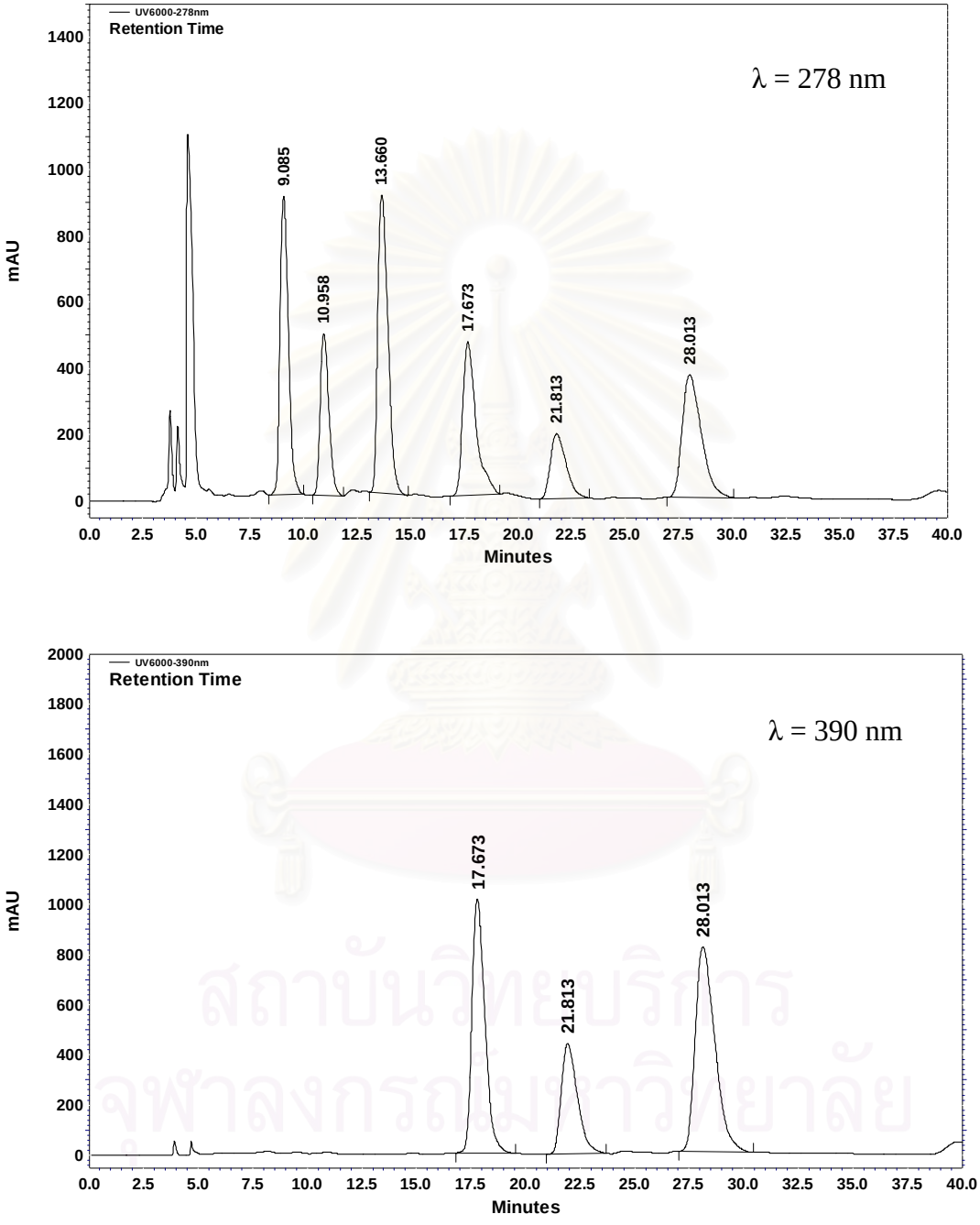

Figure D-5: Chromatograms of 4-(2-chloro-5-nitro-phenylazo)-cardanol using HPLC conditions as shown in Table 4.2.

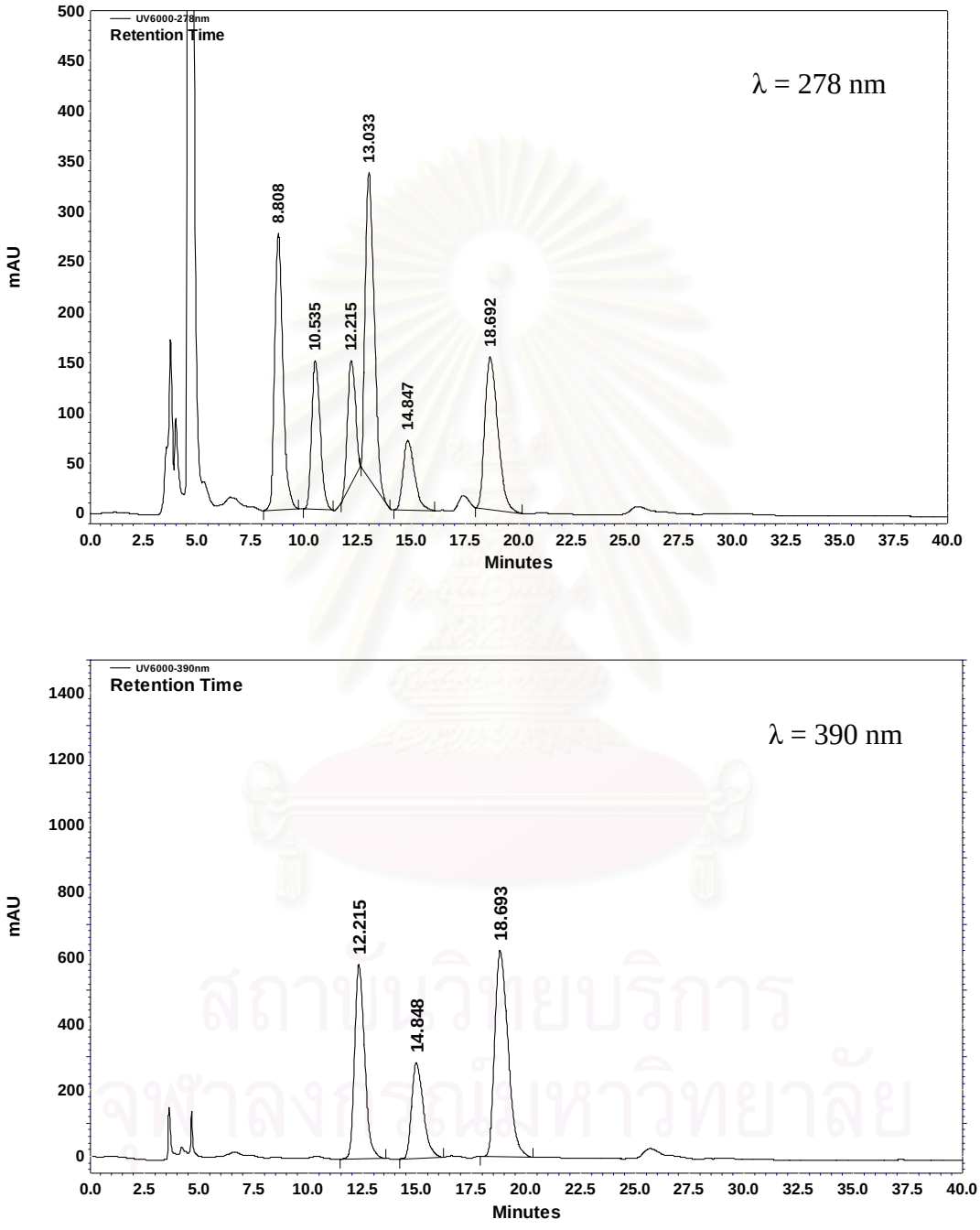

Figure D-6: Chromatograms of 4-(2-methoxy-4-nitro-phenylazo)-cardanol using HPLC condition as shown in Table 4.2 detected.

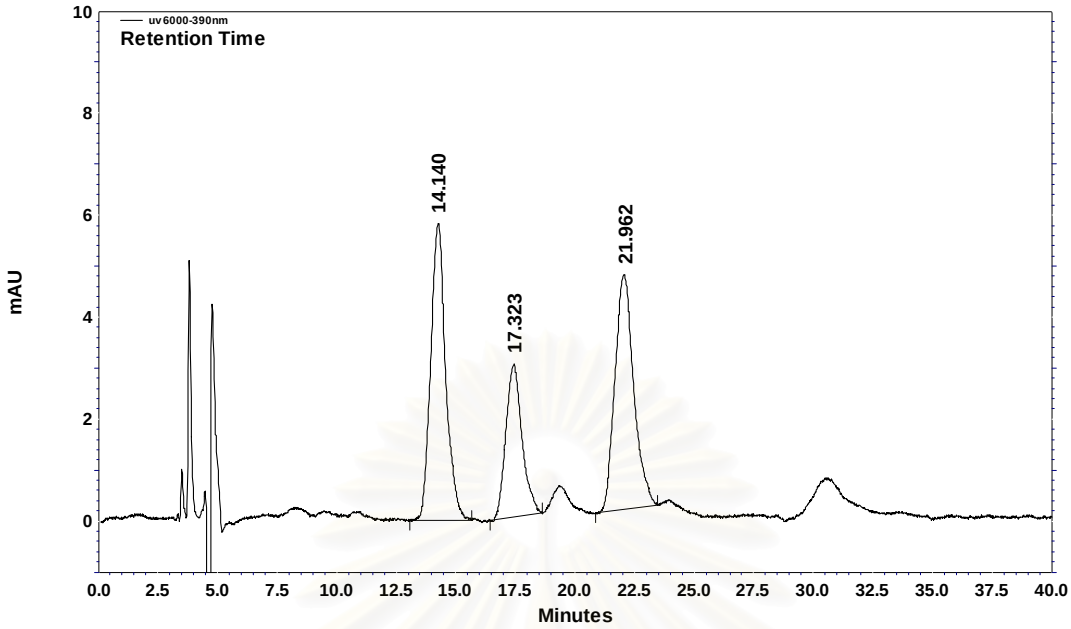

Figure D-7: A Chromatogram of mixed marker dyes 4-(4-nitro-phenylazo)-cardanol/4-(2-chloro-4-nitro-phenylazo)-cardanol (1:1) using HPLC conditions as shown in Table 4.2.

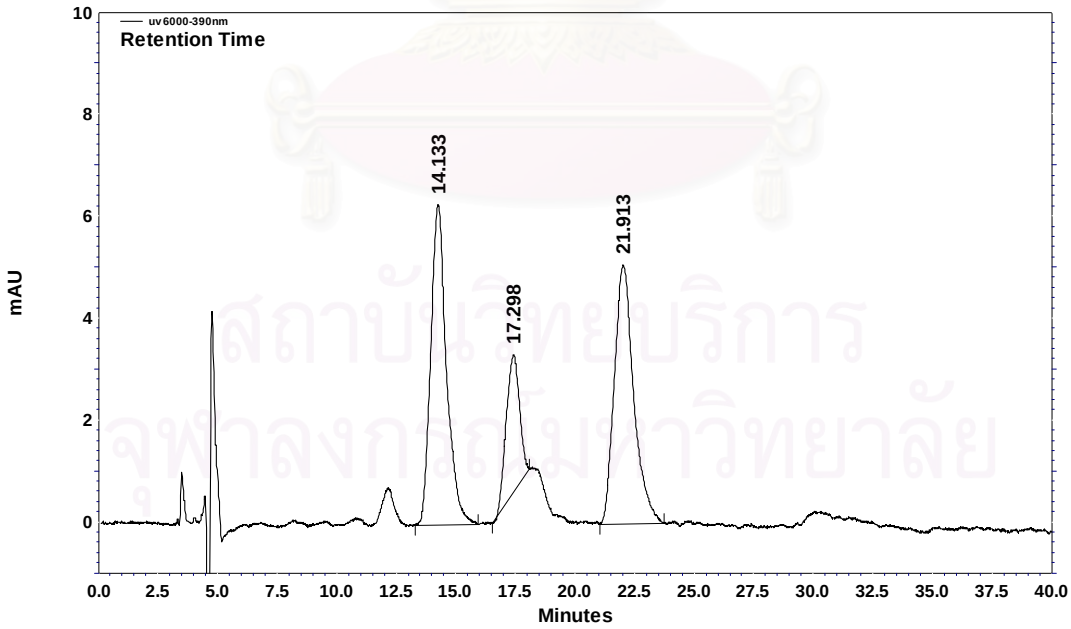

Figure D-8: A Chromatogram of mixed marker dyes 4-(4-nitro-phenylazo)-cardanol/4- (2-methoxy-4-nitro-phenylazo)-cardanol (1:1) using HPLC conditions as shown in Table 4.2.

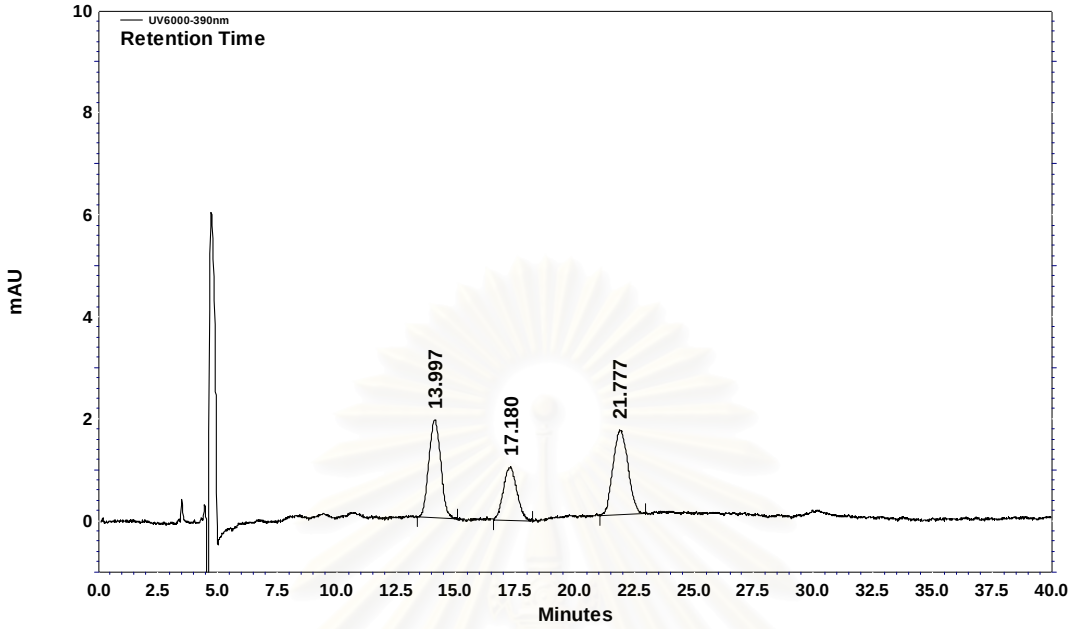

Figure D-9: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 1.00 mg/L for calibration curve.

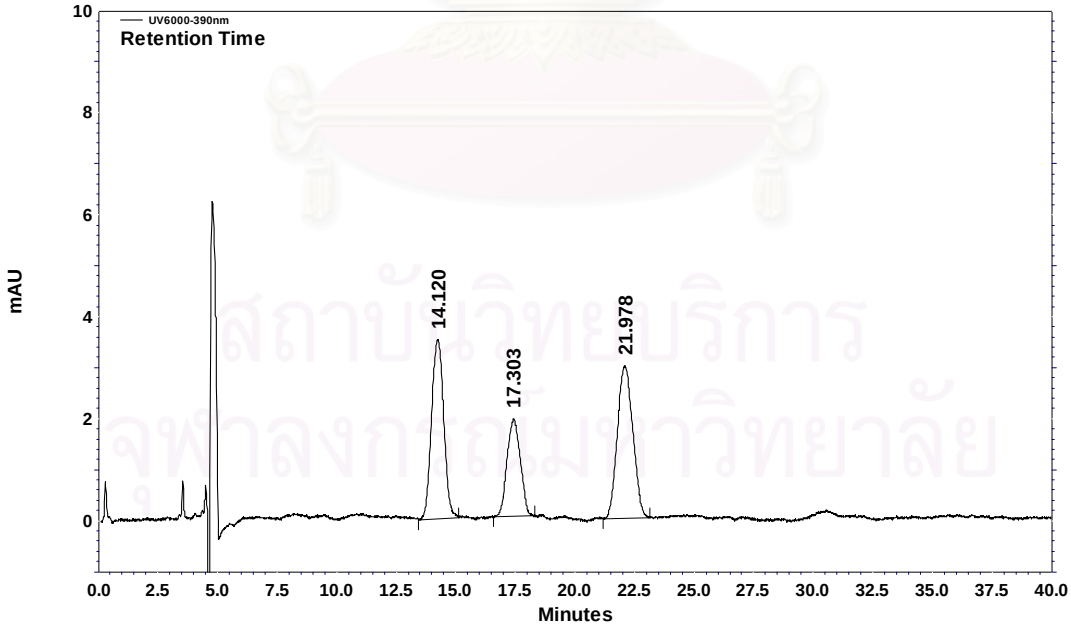

Figure D-10: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 2.00 mg/L for calibration curve.

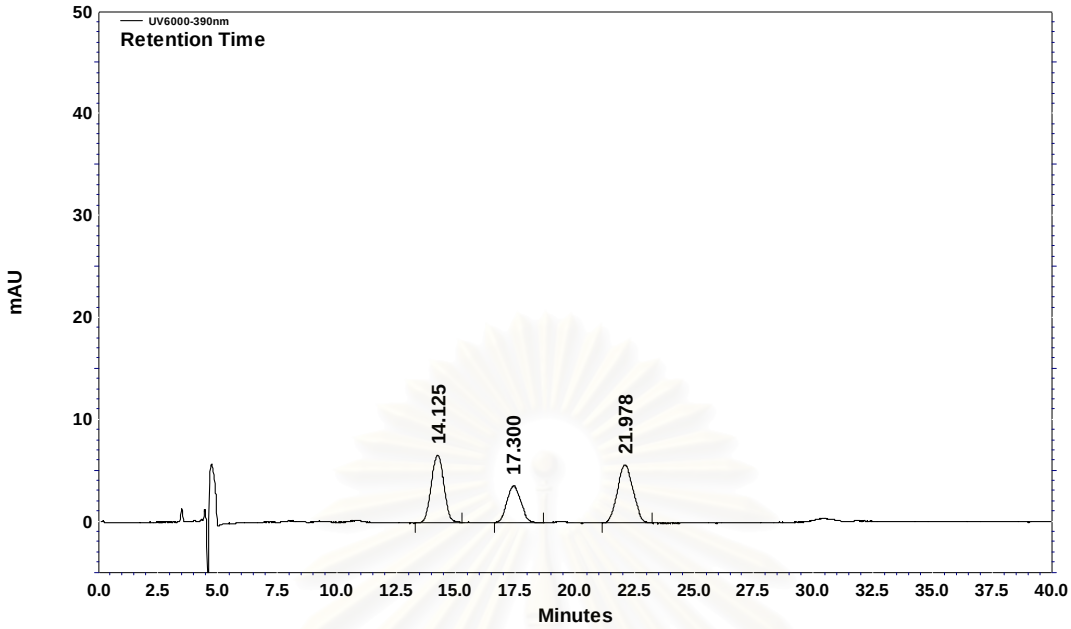

Figure D-11: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 4.00 mg/L for calibration curve.

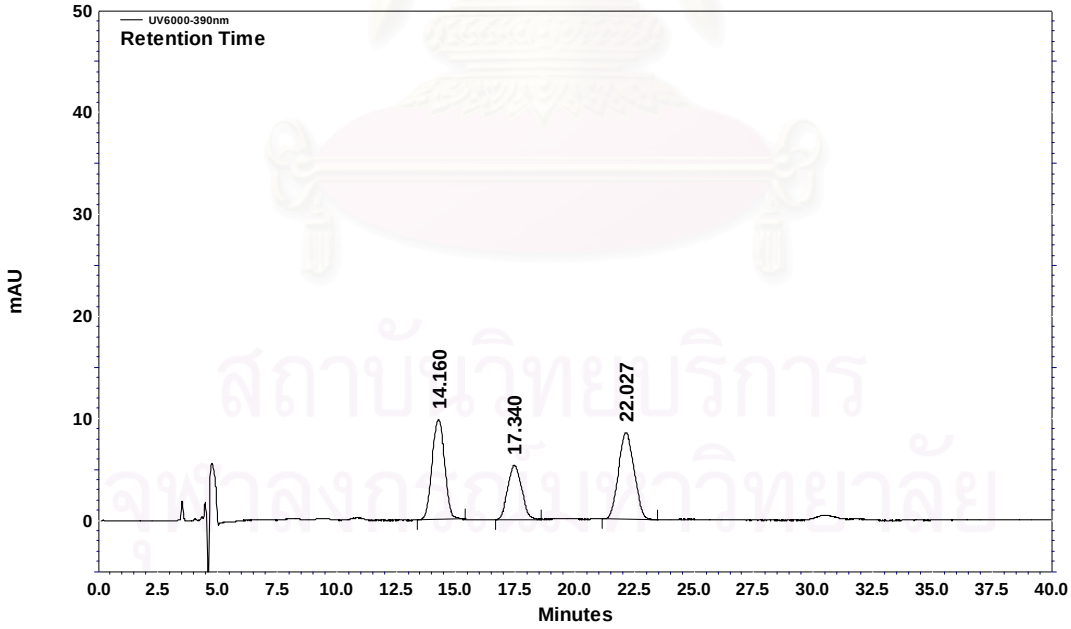

Figure D-12: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 6.00 mg/L for calibration curve.

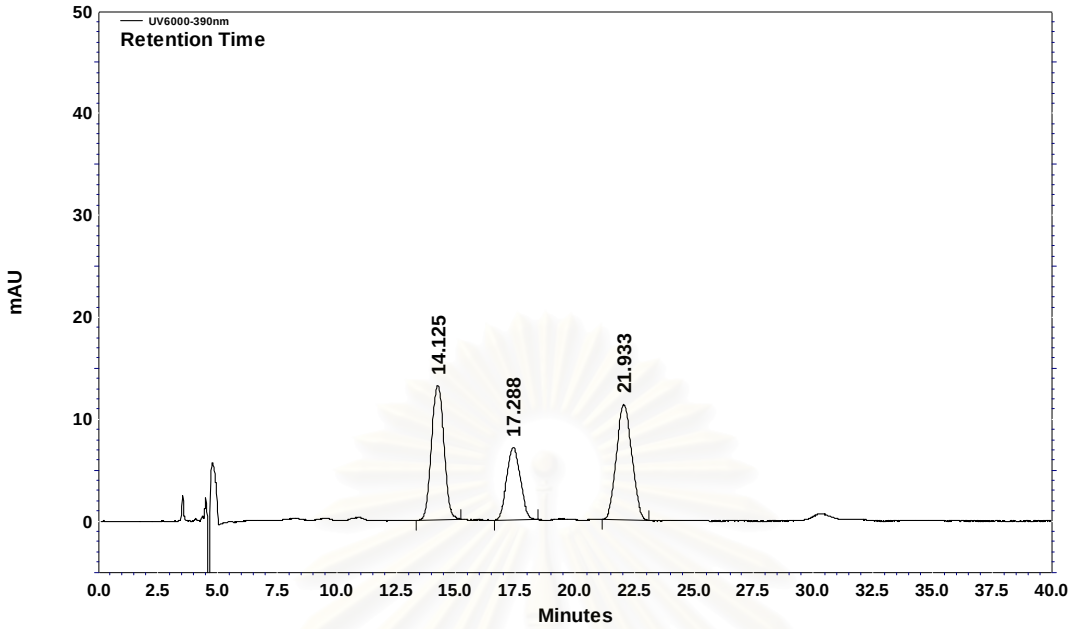

Figure D-13: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 8.00 mg/L for calibration curve.

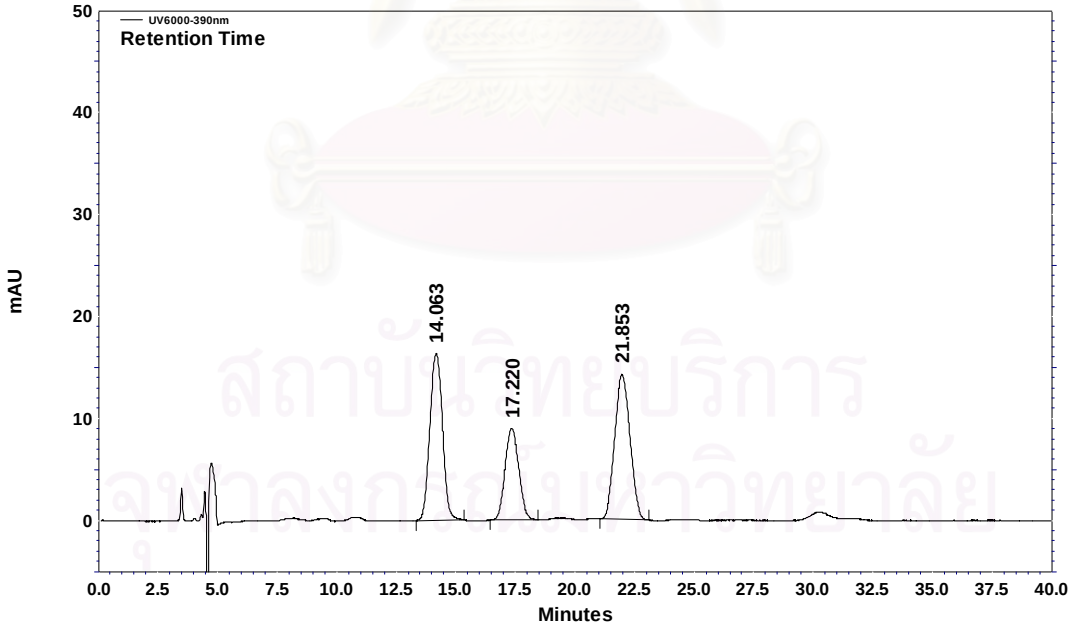

Figure D-14: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 10.0 mg/L for calibration curve.

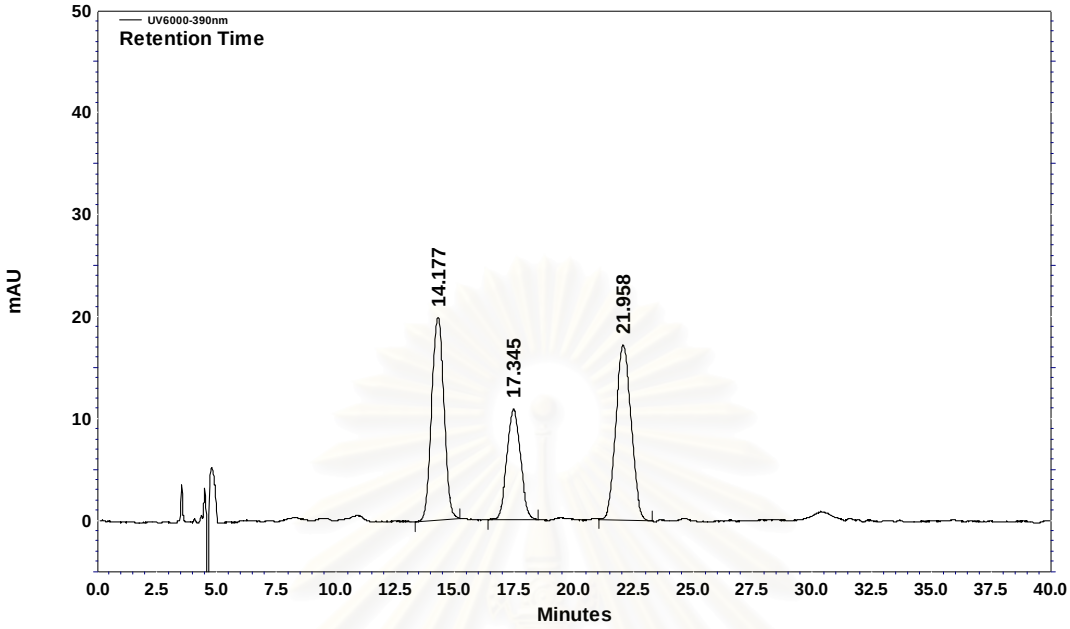

Figure D-15: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 12.0 mg/L for calibration curve.

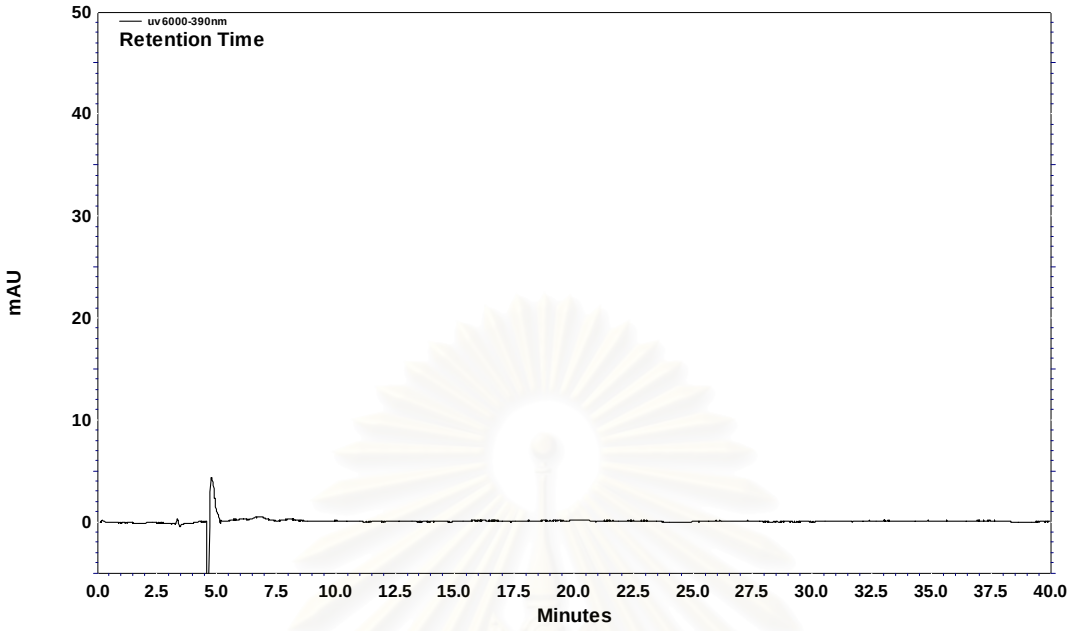

Figure D-16: A chromatogram of diesel matrix blank solution.

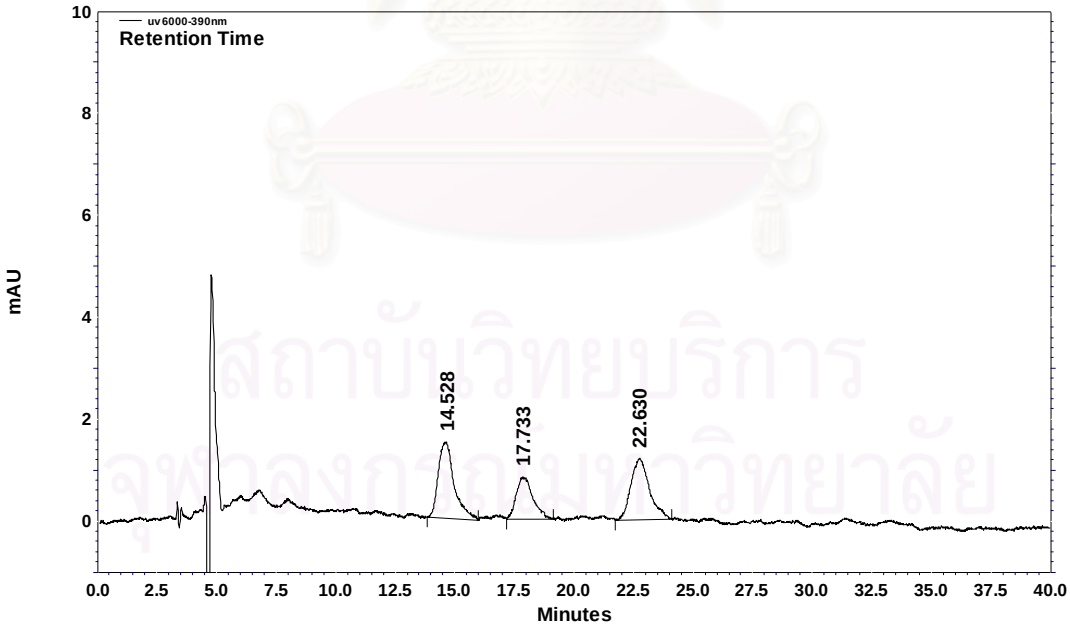

Figure D-17: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 1.00 mg/L for matrix calibration curve.

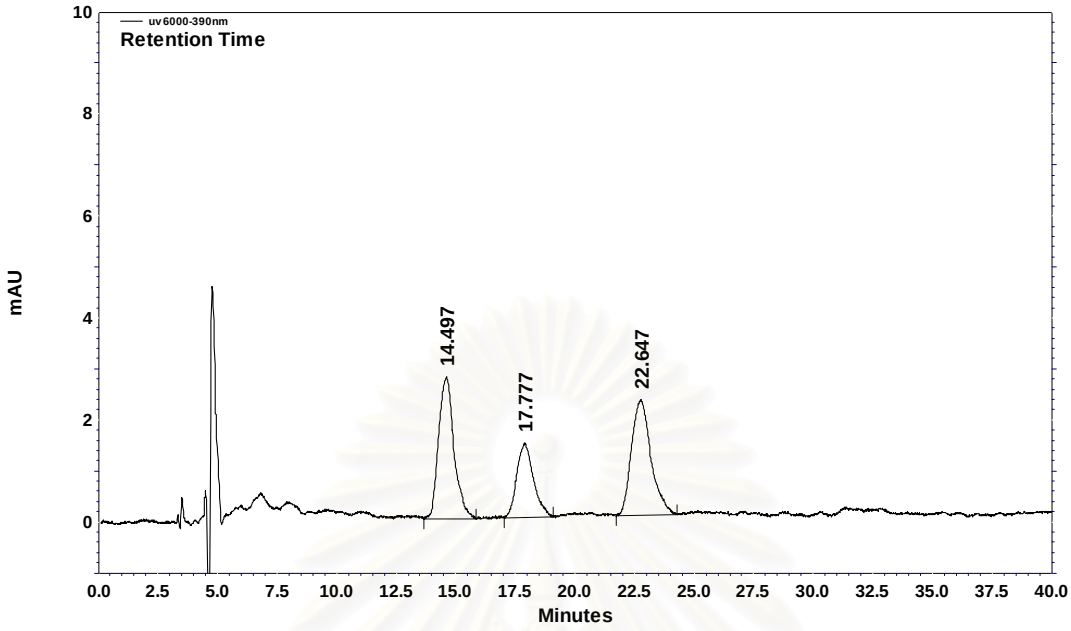

Figure D-18: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 2.00 mg/L for matrix calibration curve.

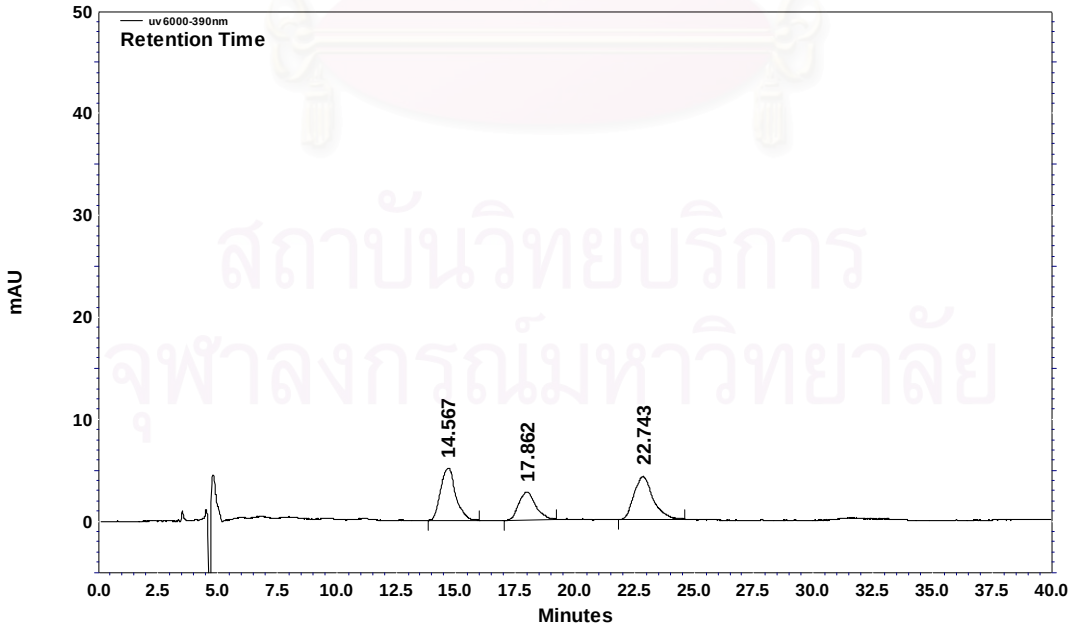

Figure D-19: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 4.00 mg/L for matrix calibration curve.

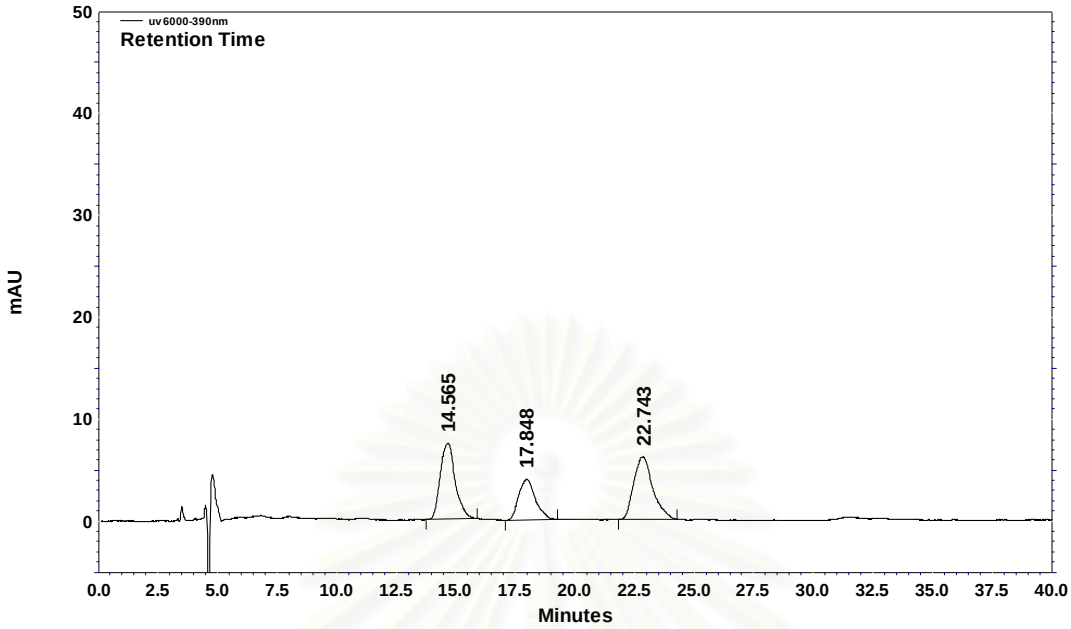

Figure D-20: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 6.00 mg/L for matrix calibration curve.

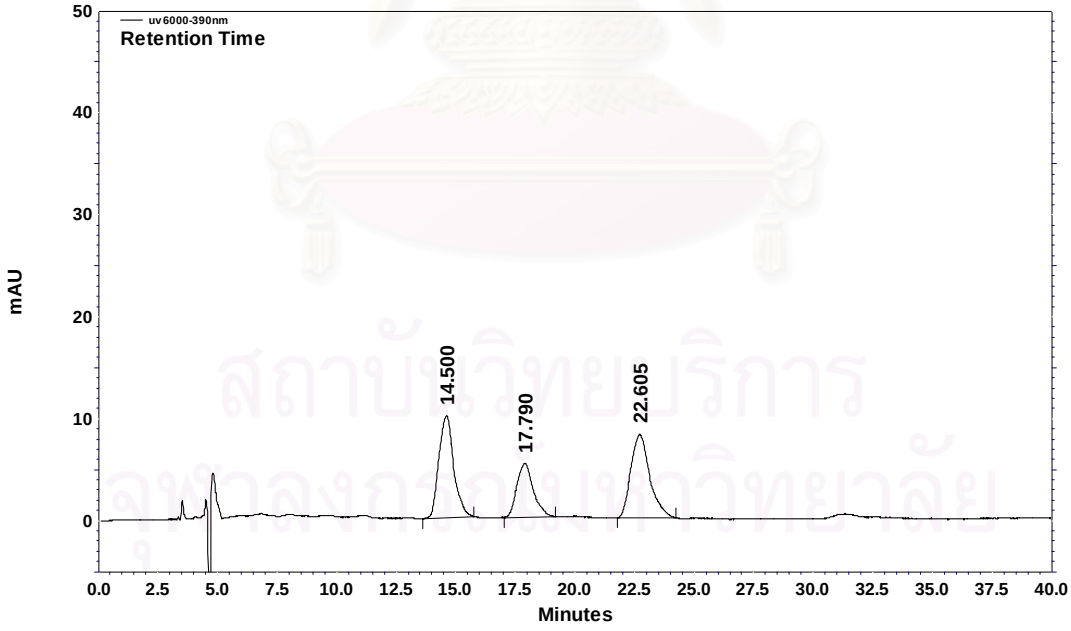

Figure D-21: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 8.00 mg/L for matrix calibration curve.

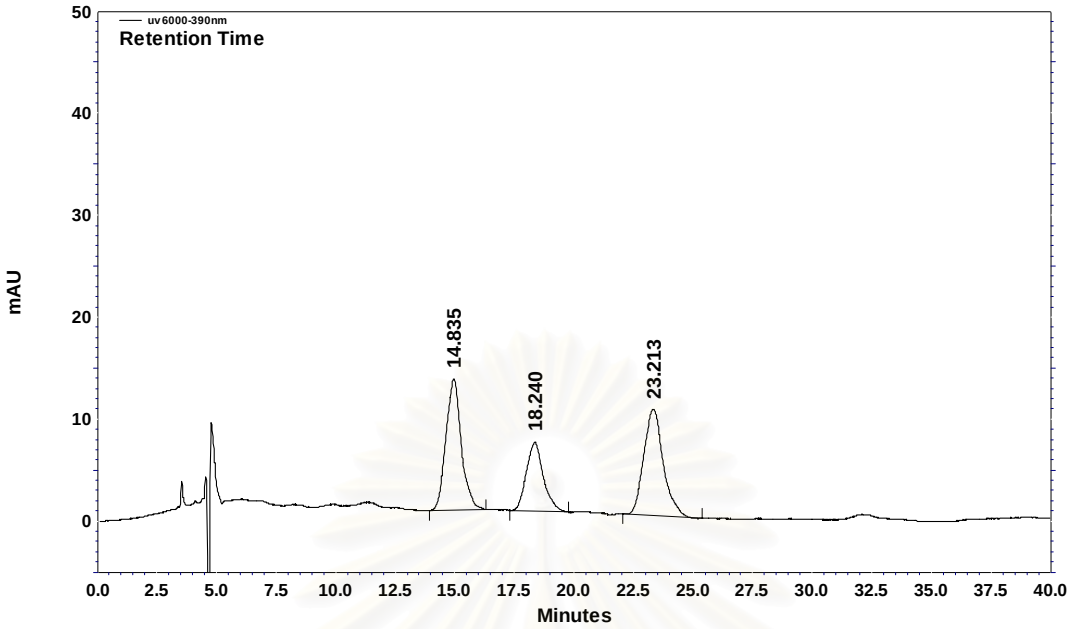

Figure D-22: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 10.0 mg/L for matrix calibration curve.

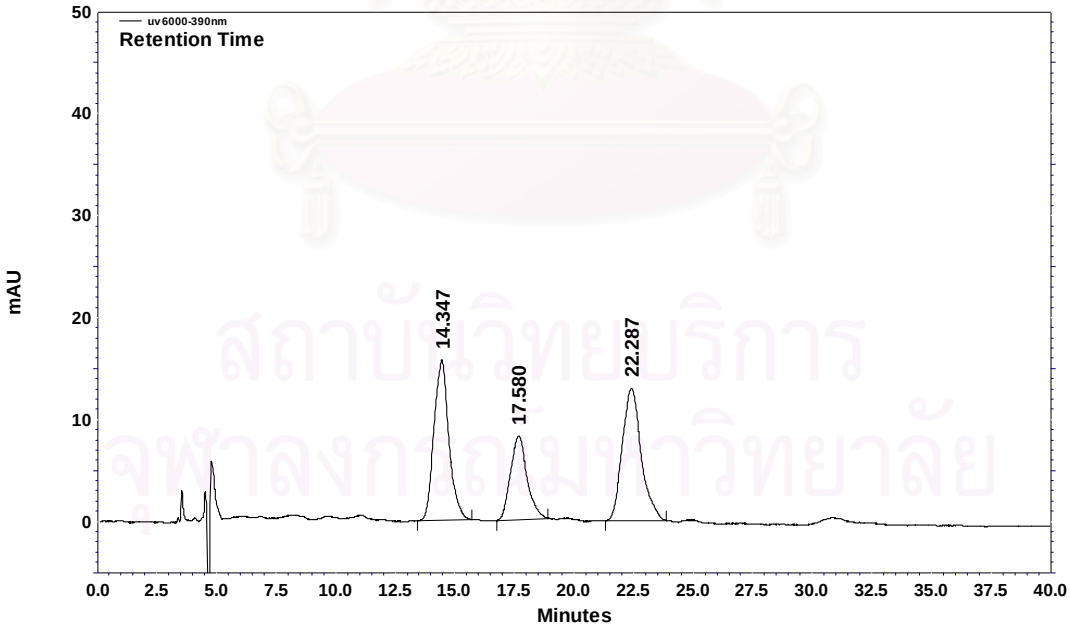

Figure D-23: A chromatogram of 4-(4-nitro-phenylazo)-cardanol 12.0 mg/L for matrix calibration curve.

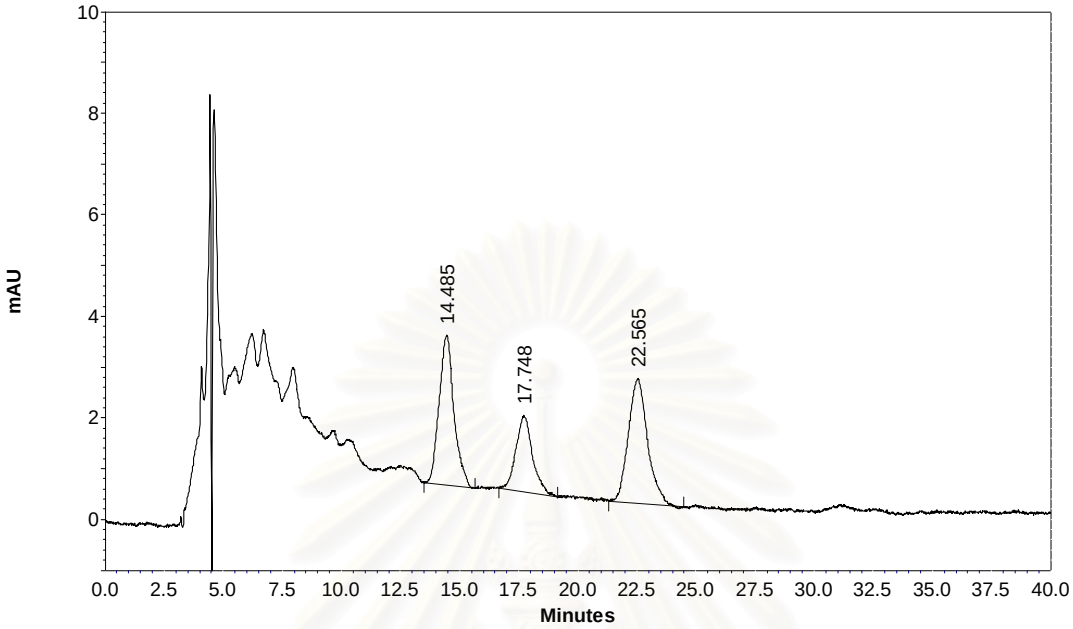

Figure D-24: Chromatogram of spiked diesel fuel at MQL level.

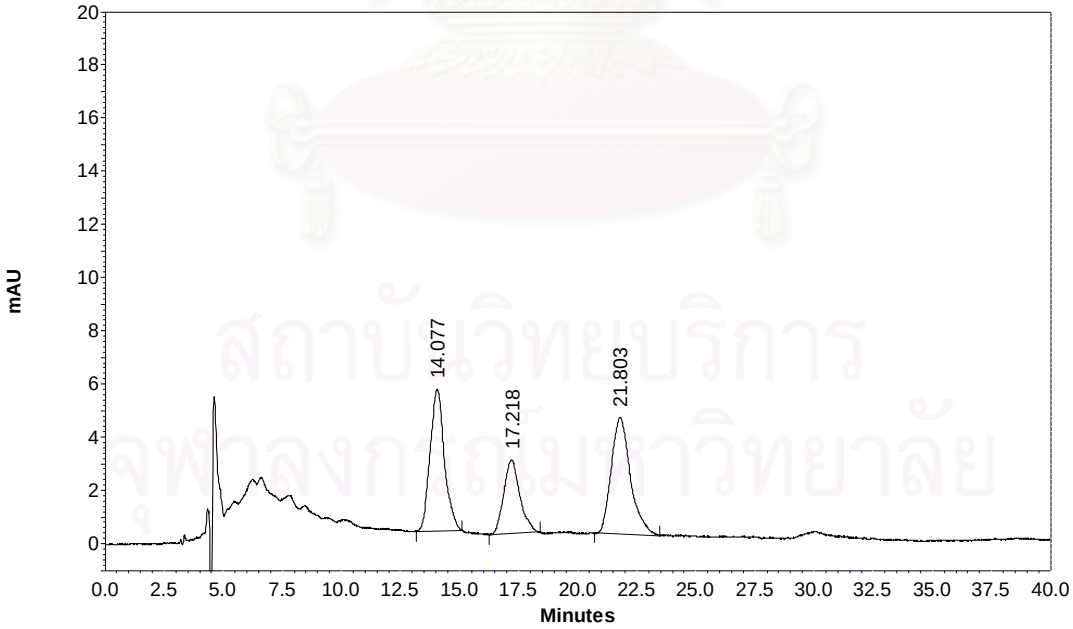

Figure D-25: Chromatogram of spiked diesel fuel at 2-fold MQL level.

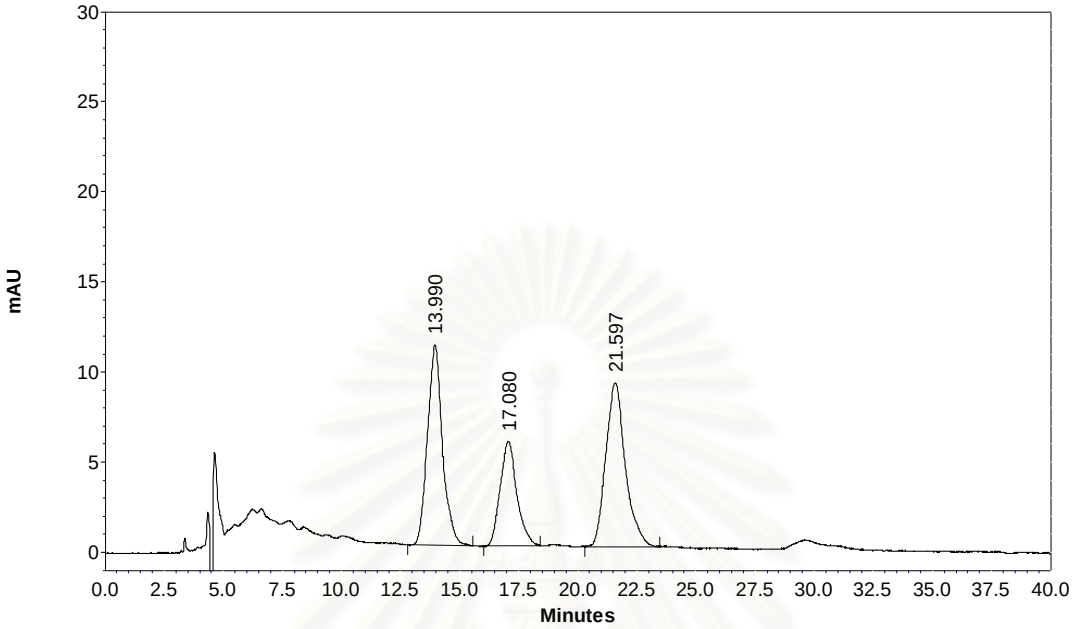

Figure D-26: Chromatogram of spiked diesel fuel at 4-fold MQL level.

VITA

Miss Paweena Ekkaphan was born on Sunday 14th February, 1982, in Supanburi, Thailand. In 2004, she graduated with a Bachelor's degree of Science in Chemistry, from Chulalongkorn University. After that, she has been studied for a Master's degree of Science in Program of Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University, and completed the program in 2007.